Acta Biomaterialia 58 (2017) 323–336

Contents lists available at ScienceDirect

Acta Biomaterialia

journal homepage: www.elsevier.com/locate/actabiomat

Full length article

Engineered myocardium model to study the roles of HIF-1α and HIF1A-AS1 in paracrine-only signaling under pathological level oxidative stress

Aylin Acun [a], Pinar Zorlutuna [a,b,*]

[a] University of Notre Dame, Bioengineering Graduate Program, United States
[b] University of Notre Dame, Aerospace and Mechanical Engineering Department and Bioengineering Graduate Program, United States

ARTICLE INFO

Article history:
Received 22 February 2017
Received in revised form 11 May 2017
Accepted 15 June 2017
Available online 16 June 2017

Keywords:
Oxidative stress
Tissue engineering
Hypoxia inducible factor
Cardiomyocyte
Endothelial cell

ABSTRACT

Studying heart tissue is critical for understanding and developing treatments for cardiovascular diseases. In this work, we fabricated precisely controlled and biomimetic engineered model tissues to study how cell-cell and cell-matrix interactions influence myocardial cell survival upon exposure to pathological level oxidative stress. Specifically, the interactions of endothelial cells (ECs) and cardiomyocytes (CMs), and the role of hypoxia inducible factor-1α (HIF-1α), with its novel alternative regulator, HIF-1α antisense RNA1 (HIF1A-AS1), in these interactions were investigated. We encapsulated CMs in photo-crosslinkable, biomimetic hydrogels with or without ECs, then exposed to oxidative stress followed by normoxia. With precisely controlled microenvironment provided by the model tissues, cell-cell interactions were restricted to be solely through the secreted factors. CM survival after oxidative stress was significantly improved, in the presence of ECs, when cells were in the model tissues that were functionalized with cell attachment motifs. Importantly, the cardioprotective effect of ECs was reduced when HIF-1α expression was knocked down suggesting that HIF-1α is involved in cardioprotection from oxidative damage, provided through secreted factors conferred by the ECs. Using model tissues, we showed that cell survival increased with increased cell-cell communication and enhanced cell-matrix interactions. In addition, whole genome transcriptome analysis showed, for the first time to our knowledge, a possible role for HIF1A-AS1 in oxidative regulation of HIF-1α. We showed that although HIF1A-AS1 knockdown helps CM survival, its effect is overridden by CM-EC bidirectional interactions as we showed that the conditioned media taken from the CM-EC co-cultures improved CM survival, regardless of HIF1A-AS1 expression.

Statement of Significance

Cardiovascular diseases, most of which are associated with oxidative stress, is the most common cause of death worldwide. Thus, understanding the molecular events as well as the role of intercellular communication under oxidative stress is upmost importance in its prevention. In this study we used 3D engineered tissue models to investigate the role of HIF-1α and its regulation in EC-mediated cardioprotection. We showed that EC-mediated protection is only possible when there is a bidirectional crosstalk between ECs and CMs even without physical cell-cell contact. In addition, this protective effect is at least partially related to cell-ECM interactions and HIF-1α, which is regulated by HIF1A-AS1 under oxidative stress.

1. Introduction

Myocardial infarction (MI), or heart attack, is one of the most common causes of death worldwide [1]. The immediate action taken after a heart attack is to reoxygenate the infarct tissue by restoring blood flow. However, the sudden flow of oxygen-rich blood to the ischemic area induces oxidative stress and often results in further cell death causing reperfusion injury (RI) [1–3], which can lead to the death of the patient [4]. Several mechanisms and metabolic pathways are suggested to be involved in this phenomenon [5] however, a detailed understanding of the progression and prevention of RI is yet to be discovered.

A possible means of treatment during and after RI is to use bioactive factors that would protect the cells from the damage

* Corresponding author at: 143 Multidisciplinary Research Building, University of Notre Dame, Notre Dame, IN 46556, United States.
E-mail address: Pinar.Zorlutuna.1@nd.edu (P. Zorlutuna).

http://dx.doi.org/10.1016/j.actbio.2017.06.023
1742-7061/

caused by the oxidative stress that occurs once the blood flow is restored. Heart tissue has endogenous protective processes in the early stages of MI as well as its aftermath. A candidate mediator of cardioprotection is endothelial cell (EC)-cardiomyocyte (CM) communication via paracrine factors such as nitric oxide (NO), endothelin 1 (EDN1), angiopoietin-II, and prostaglandin I_2 [6]. One potential mediator of this myocardial cellular communication during and after MI is hypoxia inducible factor-1 (HIF-1). HIF-1 is a heterodimeric transcription factor (comprised of HIF-1α and HIF-1β subunits) upstream of over 100 genes [7–9]. Due to its oxygen dependent post-translational regulation, the role of the HIF-1α subunit in protection from ischemic diseases under hypoxic conditions has been studied extensively. However, even though there is evidence that oxidative stress can stabilize HIF-1α [10,11], its regulation under oxidative stress is poorly studied. In addition, the results of many of these studies examining the exact role of HIF-1 or HIF-1α during MI vary widely [12–17]. Some studies showed that HIF-1 enhances myocardial cell survival [12,17], while others showed that it enhances cell death through increased apoptosis [14,18] and increases the infarct size [13]. One of the major reasons for these contradictory outcomes is the complexity of the in vivo environment. In addition, there are often discordances between animal studies and clinical trials due to differences in human and animal physiology and pathology [19]. On the other hand, cell culture studies are an oversimplification of the native phenomena where the cell-cell and cell-extracellular matrix (ECM) interactions are almost entirely misrepresented. As an alternative approach, it is now possible through tissue engineering techniques to fabricate model tissues *in vitro* with better-controlled parameters and using human cells [20–22]. Using such tissue engineered model myocardial tissues with defined cellular composition and microenvironment would be a very powerful research approach to study the role of HIF-1α and the paracrine factors regulated by HIF-1α under RI mimicking oxidative stress conditions. Moreover, it would serve as a platform to study potential therapeutics for RI treatment.

In this study, we developed 3-dimensional (3D) tissue engineered myocardial model tissues using primary neonatal rat CMs and human induced pluripotent stem cell (hiPSC)-derived ECs (iECs). We studied the effect of EC-CM interactions solely through secreted factors as well as cell-ECM interactions on cell survival under oxidative stress conditions mimicking the early onset of RI. We used rat origin CMs and human origin ECs, which allowed us to investigate the changes in their mRNA expression separately yet allowing a successful intercellular communication owing to the high level of protein homology between rats and humans in paracrine factors such as vascular endothelial growth factor (VEGF) [23]. Using these model tissues, we showed that EC-CM interactions, specifically mediated through EC-driven HIF-1α expression, improve cell survival under oxidative stress. We also showed evidence, for the first time in literature, of an alternate possible means of HIF-1α regulation under oxidative stress through HIF-1α antisense RNA1 (HIF1A-AS1), which could have an important role in the cardioprotective effect of EC-CM crosstalk.

2. Materials and methods

An expanded Methods section is available in the Online Data Supplement. All animal experiments were performed according to the guidelines of Institutional Animal Care and Use Committee (IACUC) of University of Notre Dame.

2.1. Cell culture and HIF-1α knockdown

2-day-old Sprague-Dawley rats (Charles River Laboratories) were sacrificed by decapitation and the hearts were immediately excised following the Institutional Animal Care and Use Committee (IACUC) guidelines of the University of Notre Dame, which has an approved Assurance of Compliance on file with the National Institutes of Health, Office of Laboratory Animal Welfare. The hearts were rinsed in ice-cold Hank's Balanced Salt Solution (HBSS, Gibco) immediately and the respective CMs were isolated and cultured following well established protocols [24].

The hiPSCs (line DiPS SevA1016) derived from fibroblasts were differentiated to iECs following a recently established protocol [25]. Briefly, the hiPSCs were cultured on Geltrex (Invitrogen) coated tissue culture flasks with mTeSR1 (StemCell Technologies) and, to induce differentiation, the culture media was switched to N2B27 medium (1:1 mixture of DMEM:F12 (1:1) with Glutamax and Neurobasal media supplemented with N2 and B27) (Life Technologies) supplemented with a glycogen synthase kinase 3β (GSK3β) inhibitor, CHIR (Stemgent) and bone morphogenic protein 4 (BMP4) (R&D Systems). The media was replaced with StemPro-34 SFM medium (Life Technologies) (supplemented with 200 ng/mL VEGF (PeproTech) and 2 μM forskolin (Sigma-Aldrich)) after three days. The media was renewed the following day and at the end of day six, the cells were sorted using magnetic assisted cell sorting (MACS) (autoMACSpro, Miltenyi Biotec, Harvard University) against vascular endothelial cadherin (VE-CAD). The purity of the cell population after sorting was determined using fluorescence assisted cell sorting (FACS) against VE-CAD (MACSQuant, Miltenyi Biotec, Harvard University). The collected cells were then cultured on fibronectin coated tissue culture dishes in endothelial growth media-2 (EGM-2). The endothelial phenotype of the iECs was confirmed using quantitative polymerase chain reaction (qPCR), immunostaining, and tube formation assay, and compared with human umbilical cord vein endothelial cells (HUVECs). In some experiments, when CMs and ECs were required to be monitored separately in the culture, ECs were marked by using Cell Tracker Blue (Invitrogen).

The HIF-1α knockdown was introduced by small hairpin RNA (shRNA) targeting of HUVECs and iECs. The change in HIF-1α expression was then examined on mRNA and protein levels using qPCR and enzyme-linked immunosorbent assay (ELISA), respectively.

2.2. Conventional cell culture and model tissue fabrication

For conventional, 2-dimensional (2D) cell culture experiments, the CMs (1×10^5 cells/well) were seeded with or without HIF-1α shRNA knockdown or control iECs or HUVECs (2.5×10^4 cells/well) into regular 96 well tissue culture plates ($n \geq 6$).

The 3D model tissues were prepared by encapsulating CMs alone (9×10^5 cells/construct), iECs or HUVECs alone (2×10^5 cells/construct) or as co-culture (9×10^5 CMs and 2×10^5 iECs or HUVECs per construct) in Arginine-Glycine-Aspartic acid (RGD) (Bachem) conjugated or pure poly(ethylene glycol) diacrylate (JenKem) (PEG-RGD) hydrogels ($n \geq 6$). The cell densities in 3D tissue constructs were calculated by estimating the number of cell layers throughout the volume of the constructs (one cell layer was estimated to be approximately 12 μm thick, giving 8.3 layers per construct) and each layer was aimed to have approximately equal cell numbers as in 2D culture samples.

2.3. Oxidative stress treatment and determination of cell survival and apoptosis

Conventional cell cultures and the engineered tissue constructs were cultured in minimal media (DMEM supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin) for all experiments unless stated otherwise. Both the cell cultures and model tissues were allowed to pre-incubate for 3 days before being exposed to oxidative stress. The oxidative stress was applied

through 16 h incubation with 0.2 mM H_2O_2 containing media followed by 2 h normoxic media incubation. The control samples were incubated in normoxic media for 18 h with a media change at 16 h. Following this treatment the cell viability was determined using Live/Dead Assay (Life technologies) following manufacturer's instructions and the number of live (green) and dead (red) cells were quantified using ImageJ software (A more detailed explanation of 3D construct imaging can be found in Online Data Supplement, Supplemental Methods). Cell survival was determined by normalizing each oxidative stress treated sample to the corresponding control group. In addition to Live/Dead Assay, apoptosis was assessed through caspase activity using Apo-ONE® Homogeneous Caspase-3/7 Assay (Promega), and through DNA fragmentation using DeadEnd™ Fluorometric Terminal Deoxynucleotidyl Transferase (TdT)-Mediated dUTP Nick End Labeling (TUNEL) System (Promega) following manufacturer's instructions and quantified using ImageJ software. (A more detailed explanation of TUNEL assay analysis in 3D constructs can be found in Online Data Supplement, Supplemental Methods). The results of the caspase 3/7 assay were normalized to the respective cell numbers in each construct type.

2.4. Transcriptome analysis

Total RNA was isolated from the 3D model tissues of CMs with or without iECs cultured under normoxia or oxidative stress conditions (n = 3) following manufacturer's instructions (Promega SV-total RNA kit). Library preparation and sequencing was provided as a custom service by Michigan State University Research Technology Support Facility Genomics Core. Libraries were prepared using the Illumina TruSeq stranded mRNA library preparation kit, and were quality checked and quantitated using a combination of Qubit dsDNA assay, Caliper LabChipGX size determination and Kapa Biosystems Illumina Library qPCR assay. The sequencing was performed in Illumina HiSeq 2500 Rapid Run flow cell (v2) using HiSeq Rapid SBS reagents (v2) in a 2 × 100 bp paired end format. Base calling was done by Illumina Real Time Analysis (RTA) v1.18.64. Significant expression change was defined with a $p < 0.05$ and a fold change (FC) > 2 for upregulation and smaller than 0.5 for downregulation.

2.5. HIF1A-AS1 knockdown and determining its effect on CM survival

The CMs were seeded into 96 well plates (1×10^5 cells/well) immediately after isolation to: 1) prepare the "CM-iEC co-culture pre-conditioned media", and 2) be analyzed using Live/Dead Assay after being cultured in pre-conditioned media. Simultaneously, the iECs were transfected (DharmaFECT, Dharmacon) with siRNA targeting HIF1A-AS1 (Lincode HIF1A-AS1 siRNA-SMARTpool (Human), Dharmacon) (100 nM, 48 h) or non-targeting siRNA (Lincode Non-targeting siRNA, Dharmacon) as a negative control. Transfection efficiency was confirmed using a positive control siRNA (ON-TARGETplus GAPDH Control Pool (Human), Dharmacon). The initial HIF1A-AS1 knockdown level in iECs was determined with qPCR analysis right after transfection (t = 0 h). The transfected iECs were then seeded (2.5×10^4 cells/well): 1) as single cultures to prepare the "single iEC culture pre-conditioned media" and 2) on top of the CMs to prepare the "CM-iEC co-culture pre-conditioned media". The preconditioned media of single iEC cultures and CM-iEC co-cultures were prepared by using either normoxic minimal media preconditioning (pN) or minimal media with 0.2 mM H_2O_2 preconditioning (pS) for 16 h. At the end of 16 h, the media pre-conditioned by single iEC cultures or CM-iEC co-cultures was transferred to single CM cultures. The relative HIF1A-AS1 expression was determined with qPCR analysis at this time point (t = 16 h). The single CM cultures were maintained with the pre-conditioned media (under normoxia (N) or with an additional 0.2 mM H_2O_2 (S)) for 16 h. Following the 16 h incubation the cell viability was determined using Live/Dead Assay following manufacturer's instructions and quantified using ImageJ software to determine live cell percentages.

2.6. Statistical analysis

The results are represented as average ± standard deviation. The statistical analysis was carried out using 1-way ANOVA analysis. Student's *t*-test was used for comparing two individual groups. All *p* values reported were two-sided, and statistical significance was defined as $p < 0.05$. Sample size (n) ≥ 3 for individual experiments and all experiments were repeated 3 times.

3. Results

3.1. Differentiation, characterization and HIF-1α shRNA knockdown of iECs

We differentiated the hiPSCs to iECs and assessed the endothelial phenotype commitment of the iECs using morphological assessment via bright field microscopy, qPCR analysis for EC and iPSC specific markers, and immunostaining for EC specific markers (Fig. 1A–D). In all analyses, HUVECs were used as positive control while undifferentiated hiPSCs were used as negative control. We showed that the mRNA expression level of EC markers, CD31, EDN1, and von Willebrand factor (vWF) in iECs was comparable to HUVECs, confirming the successful differentiation of hiPSCs to endothelial lineage (Fig. 1B). Immunostaining for CD31, vWF, and VE-CAD showed similar levels of expression of these EC markers in iECs and HUVECs whereas their expression was undetectable in the undifferentiated hiPSCs (Fig. 1C). In addition, the expression level of pluripotency marker proteins, OCT-4 and NANOG were significantly lower in iECs and HUVECs compared to the undifferentiated iPSCs (Fig. 1D) ($p \leq 0.01$).

Additionally, we showed that the iECs carry the key mature functional properties of primary ECs by testing the ability of iECs to form tube-like structures similar to those of their primary counterparts (Fig. 1E). Both HUVECs and iECs were observed to spread and form tube-like cellular networks indicating that the iECs function similar to primary ECs. Taken together, we successfully differentiated the hiPSCs to iECs as they show marker gene expression and *in vitro* functionality.

In order to downregulate the HIF-1α expression in HUVECs and iECs, we used shRNA targeting. The infected cells were purified using puromycin selection and the down regulation of HIF-1α at mRNA (Fig. 1F) and protein (Fig. 1G) levels was confirmed using qPCR analysis and ELISA, respectively. The HIF-1α levels were significantly lower in the HIF-1α shRNA knockdown cells compared to controls, at both mRNA ($p \leq 0.01$ for both iECs and HUVECs) and protein levels ($p < 0.01$ for both iECs and HUVECs) showing that the shRNA targeting was successful.

3.2. Effect of HIF-1α and EC-CM interactions on cell survival in conventional cell cultures

In order to examine the effect of EC presence we cultured CM-alone cultures with two different types of conditioned media: iEC-only-conditioned media and iEC-CM co-culture-conditioned media. In addition, we determined the effect of EC specific HIF-1α expression on cell survival under oxidative stress. We compared the survival of CM-alone cultures and their co-cultures with HIF-1α shRNA knockdown or control HUVECs (Suppl. Fig. 1) and iECs under normoxia and oxidative stress. We calculated the live

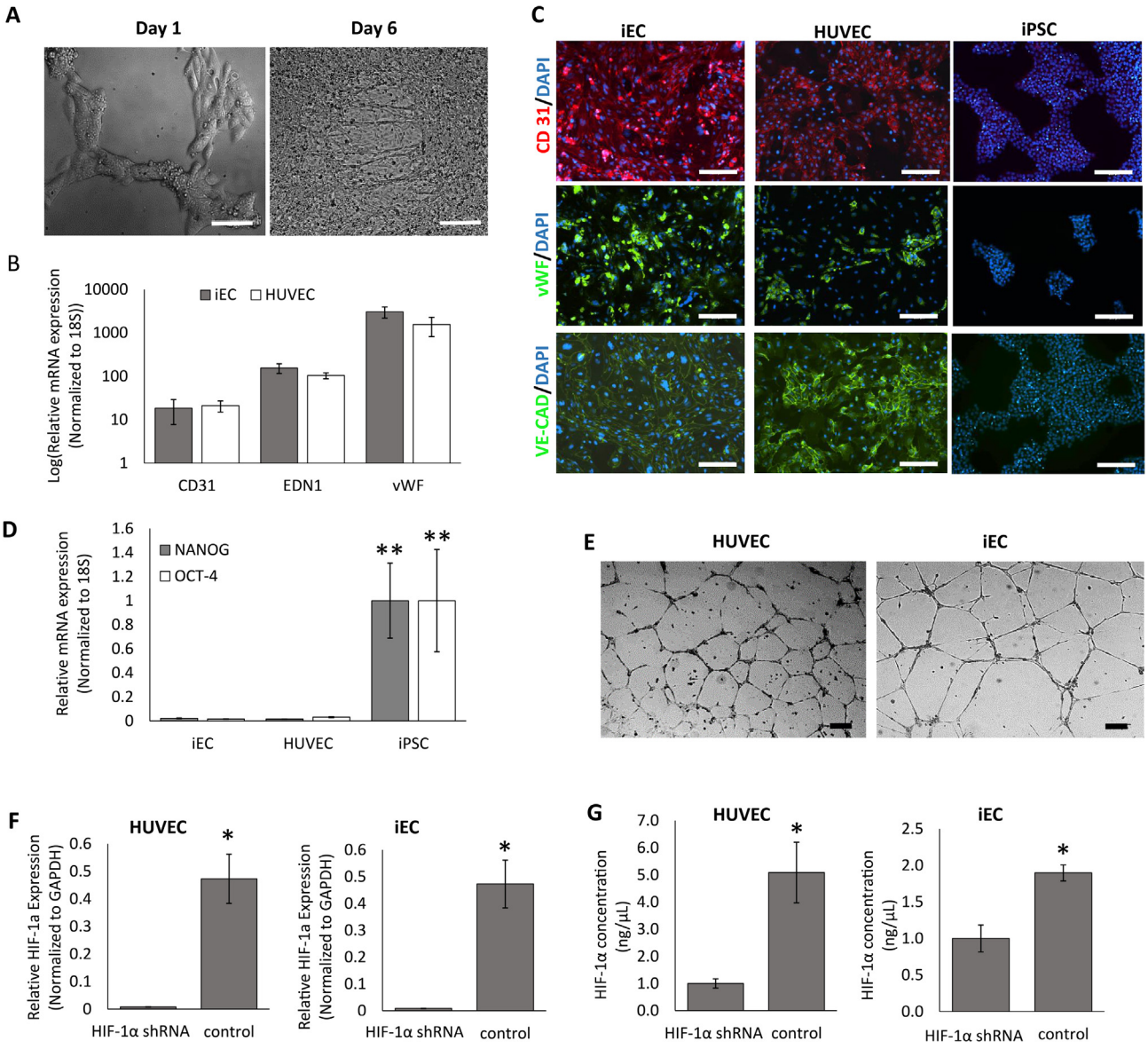

Fig. 1. Characterization of hiPSC-derived ECs (A-E). (A) The bright field images of hiPSCs at days 1 and 6 during differentiation (Scale bar: 100 μm). (B) qPCR analysis for EC marker genes in iECs and HUVECs (positive control). (C) Immunostaining images of iECs, HUVECs, and hiPSCs (negative control) for EC markers: CD31, vWF, and VE-Cadherin (top to down) (Scale bar: 200 μm). (D) qPCR analysis for pluripotency markers NANOG and OCT-4 in HUVECs, hiPSCs and iECs. (E) Bright field images of tube formation assay of iECs and HUVECs (Scale bar: 200 μm). (F) qPCR analysis of shRNA treated HUVECs and iECs showing the downregulation of *Hif-1α* at mRNA level. (G) ELISA results showing the relative HIF-1α protein concentration of HIF-1α shRNA treated HUVECs and iECs. (* indicates statistical significance between two individual groups ($p < 0.05$) and ** indicates significant difference between a single group to all other groups ($p < 0.01$), $n \geq 3$ for all).

cell percentages (Fig. 2B) and cell *survival* (cell viability normalized to untreated control samples for each condition) (Fig. 2C) using Live/Dead Assay images (Fig. 2A) for each group and represented as percentage. We observed that when cultured alone in non-conditioned media, only 38 ± 2% of CMs were alive under the oxidative stress. Live cell percentage increased to 71 ± 2% with iEC-CM co-culture-conditioned media, while it remained at 40 ± 3% with iEC-only-conditioned media (Fig. 2B). We then examined the survival of the CM cultures with or without ECs. We observed that the 60 ± 2% survival of CM-only cultures increased to above 90% upon co-culturing CMs with control HUVECs (91 ± 5%) (Suppl. Fig. 1B) or iECs (96 ± 4%) ($p < 0.01$). The ECs cultured alone were not affected much by the oxidative stress treatment as 94 ± 1.5% of HUVECs (Suppl. Fig. 1B) and 98 ± 0.5% of iECs survived (Fig. 2C). In order to show that the higher survival in co-cultures is not due to ECs overgrowing the CMs, we tracked ECs by labeling them with blue cell tracker. We found that the initial ratio of EC to CM (1 to 4) was 1 to 3 at the end of the treatment, indicating that the majority of surviving cells in the co-culture were CMs (Fig. 2D).

When HIF-1α shRNA knockdown ECs were used for co-culture, we observed a significant decrease in survival in both EC-only cultures and in co-cultures. In HIF-1α shRNA knockdown EC-only cultures, 67.5 ± 2.9% of HUVECs (Suppl. Fig. 1B) and 91.3 ± 0.1% of iECs survived the oxidative stress ($p < 0.01$). In co-cultures of HIF-1α shRNA knockdown ECs with CMs, cell survival decreased by 15%, from 91 ± 5% to 76 ± 1.3%, in HUVEC-CM co-cultures (Suppl. Fig. 1B). Similarly, the cell survival decreased by 13%, from 96 ± 4% to 83 ± 1.1%, in iEC-CM co-cultures. The decrease in survival of co-cultures was not due to the compromised survival of HIF-1α shRNA knockdown ECs themselves, as the EC to CM ratio remained approximately the same throughout the experiment

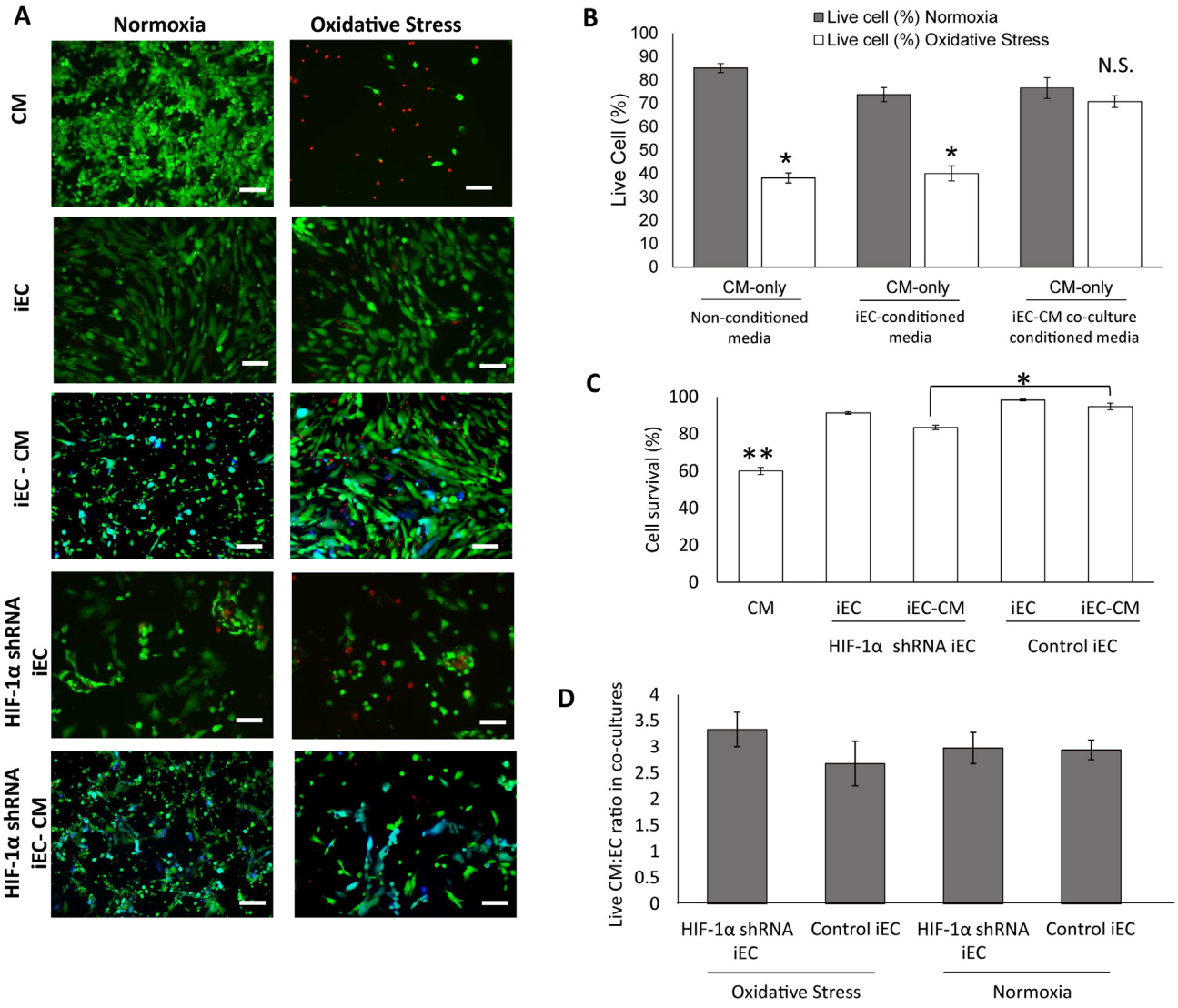

Fig. 2. Conventional culture of CMs with or without iECs under oxidative stress and normoxia. An improvement in cell survival was observed with the presence of ECs and this improvement was compromised with HIF-1α knockdown. (A) The Live/Dead Assay (green: live, red: dead, blue: live iECs) images. (B) The live cell percentages of CM-alone cultures that received iEC-only or iEC-CM co-culture-conditioned media, or non-conditioned media, under normoxia or oxidative stress. (C) The cell survival of single cultures and co-cultures of CMs and HIF-1α shRNA knockdown or control iECs after oxidative stress treatment. (D) The live CM:EC ratio of the different co-cultures at the end of normoxia or oxidative stress treatment. (* indicates statistical significance between two individual groups ($p < 0.05$), and ** indicates significant difference between a single group to all other groups ($p < 0.05$), $n \geq 3$ for all) (Scale bar = 50 μm).

(Fig. 2D). Even though the co-culture with HIF-1α shRNA knockdown ECs resulted in a higher cell survival compared to CMs alone ($p < 0.01$), survival in both control HUVEC-CM (Suppl. Fig. 1B) and iEC-CM co-cultures was significantly higher than these values ($p < 0.05$) (Fig. 2C). This indicates that HIF-1α is one of the important factors in EC-mediated CM survival under oxidative stress.

HIF-1α is known to regulate the expression of growth factors such as VEGF [26], platelet derived growth factor-B (PDGF-B) [27], human epidermal growth factor (hEGF) [28], and insulin-like growth factor-1 (IGF-1) [29]. Therefore we compared the survival in minimal media (DMEM with 10% FBS) to survival in media supplemented with growth factors (EGM-2) including, hFGF-B, hEGF, VEGF, and R3 IGF-1 (Suppl. Fig. 2). We observed that the CM survival improved when cultured in EGM-2 upon oxidative stress ($p < 0.05$), even in the absence of ECs, indicating that the growth factor supplements of EGM-2 have cardioprotective effects themselves, and could directly improve the CM survival. The survival of iECs also improved when cultured in EGM-2, however, CM-iEC co-culture showed a higher survival when cultured in minimal media with 0.2 mM H_2O_2. We did not observe any significant difference in the survival of HUVECs alone with oxidative stress treatment in the two types of media ($p > 0.05$). Similarly, HUVEC-CM co-cultures survived the oxidative stress treatment in either type of media with no statistical difference ($p > 0.05$).

3.3. Effect of HIF-1α and EC-CM interactions on cell survival in 3D model tissues

We constructed 3D model tissues to provide a controllable and physiologically relevant platform to study cell-cell and cell-ECM interactions under oxidative stress. The 3D model tissues were prepared by encapsulating 1) CMs-alone, 2) control or HIF-1α shRNA knockdown iECs or HUVECs alone, and 3) as co-cultures of CMs with control or HIF-1α shRNA knockdown ECs, in photo-crosslinkable PEG-RGD hydrogels (Fig. 3A). The hydrogels were designed to be 100 μm thick to avoid hypoxia. We used a PEG-based hydrogel system aiming to restrict the cells from forming physical cell-cell contact. We determined the stiffness of the plain

hydrogel constructs to be stable at approximately 8 kPa for 2 weeks in culture. (Suppl. Fig. 3). This way we were able to study the protection solely through the secreted factors from ECs and/or CMs. In addition, the 3D models kept the iEC:CM ratio constant throughout the experiment, ensuring that the iECs, which are more resilient to stress, not to take over the culture. Similar to the conventional culture conditions, the presence of control HUVECs (Suppl. Fig. 4) and iECs (Fig. 3B and C) significantly improved the overall survival of their respective co-cultures with CMs (HUVEC-CM: 95 ± 0.5%, iEC-CM: 82 ± 2%) compared to single culture CMs constructs (69 ± 3%) ($p < 0.05$). This indicates that the secreted factors are a major contributor in rescuing CMs under oxidative stress. Importantly, we observed that HIF-1α is involved with at least one of these factors as the cell survival were significantly lower in co-culture constructs with HIF-1α shRNA knockdown ECs (HIF-1α shRNA knockdown HUVEC-CM: 75 ± 1%, HIF-1α shRNA knockdown iEC-CM: 70 ± 2%) ($p < 0.05$) similar to conventional culture results.

3.4. Effect of 3D culture and cell-ECM interactions on cell survival

In order to study the effect of cell–cell and cell-ECM interactions on cell survival under oxidative stress, we compared the survival of conventional cell cultures to cells encapsulated in 3D (Fig. 4A). We observed that CM-only cultures had a 9% higher survival in 3D model tissues compared to 2D conditions, indicating the importance of the 3D biomimetic environment ($p < 0.05$). Interestingly, HUVEC-CM co-culture did not show any change in survival in conventional (91 ± 5%) vs. 3D culture conditions (95 ± 0.5%) ($p > 0.05$), while survival of iEC-CM co-culture was significantly lower in the model tissues (82 ± 2% in 3D culture conditions, compared to 96 ± 4% in conventional culture conditions). ($p < 0.01$). In addition, the survival percentages of single iEC and HUVEC constructs, 78 ± 2%, and 68 ± 1%, respectively, were lower in 3D ($p < 0.01$ for HUVECs and $p < 0.05$ for iECs) compared to the conventional culture conditions. We hypothesized that this was related to the diffusion limitation created due to the increased cell-cell distance in

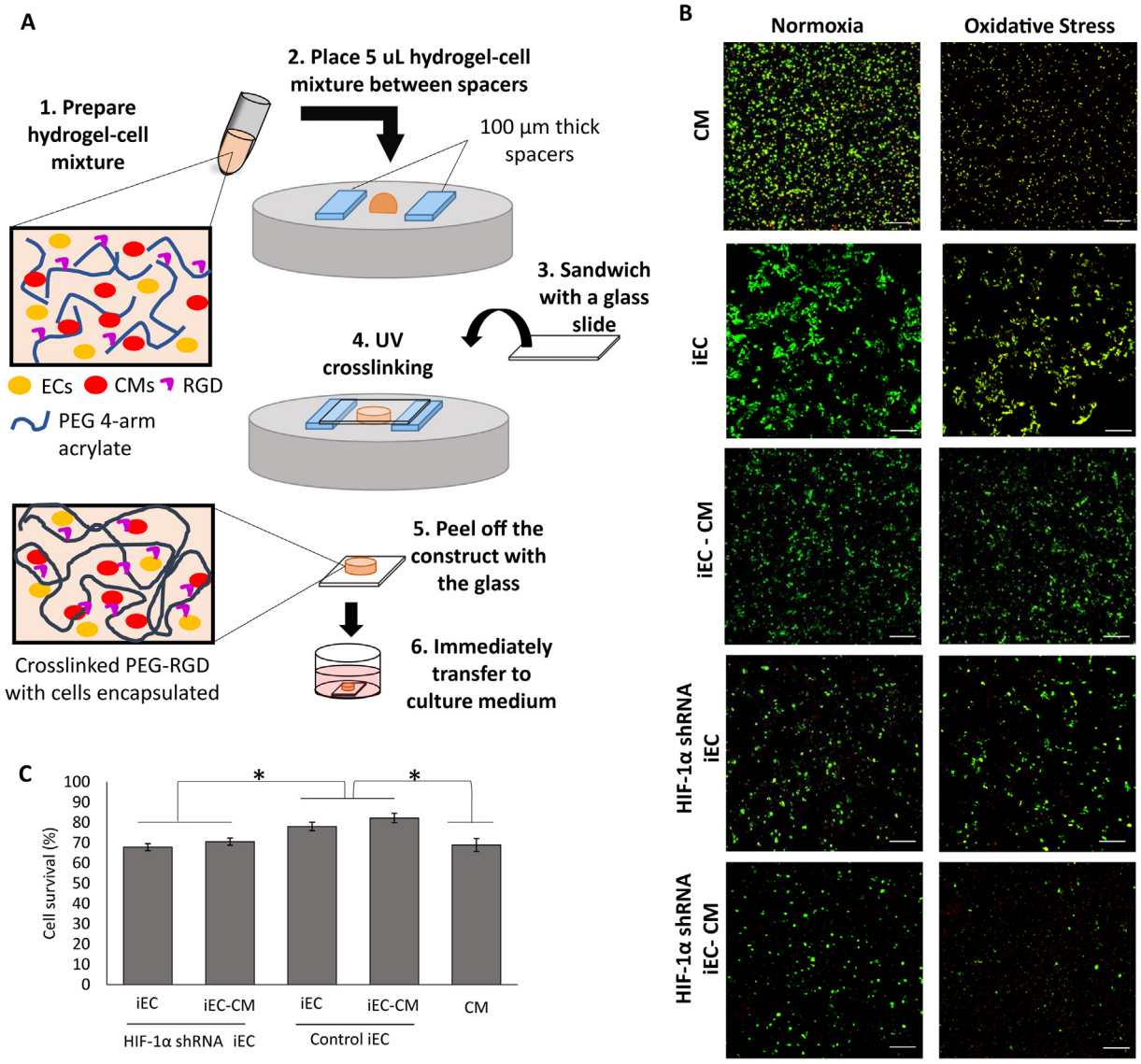

Fig. 3. Response of ECs and CMs to oxidative stress in 3D. 3D environment improved CM survival, however, there was a more significant increase in survival with iEC presence. This cardioprotective effect was compromised with HIF-1α knock down, consistent with the 2D results. (A) The schematic of 3D construct preparation. (B) The Live/Dead Assay images and (C) the cell survival of the single cultures and co-cultures of CMs and HIF-1α shRNA knockdown or control iECs after oxidative stress treatment. (* indicates statistical significance between two individual groups ($p < 0.05$), $n \geq 3$ for all) (Scale bar = 100 μm).

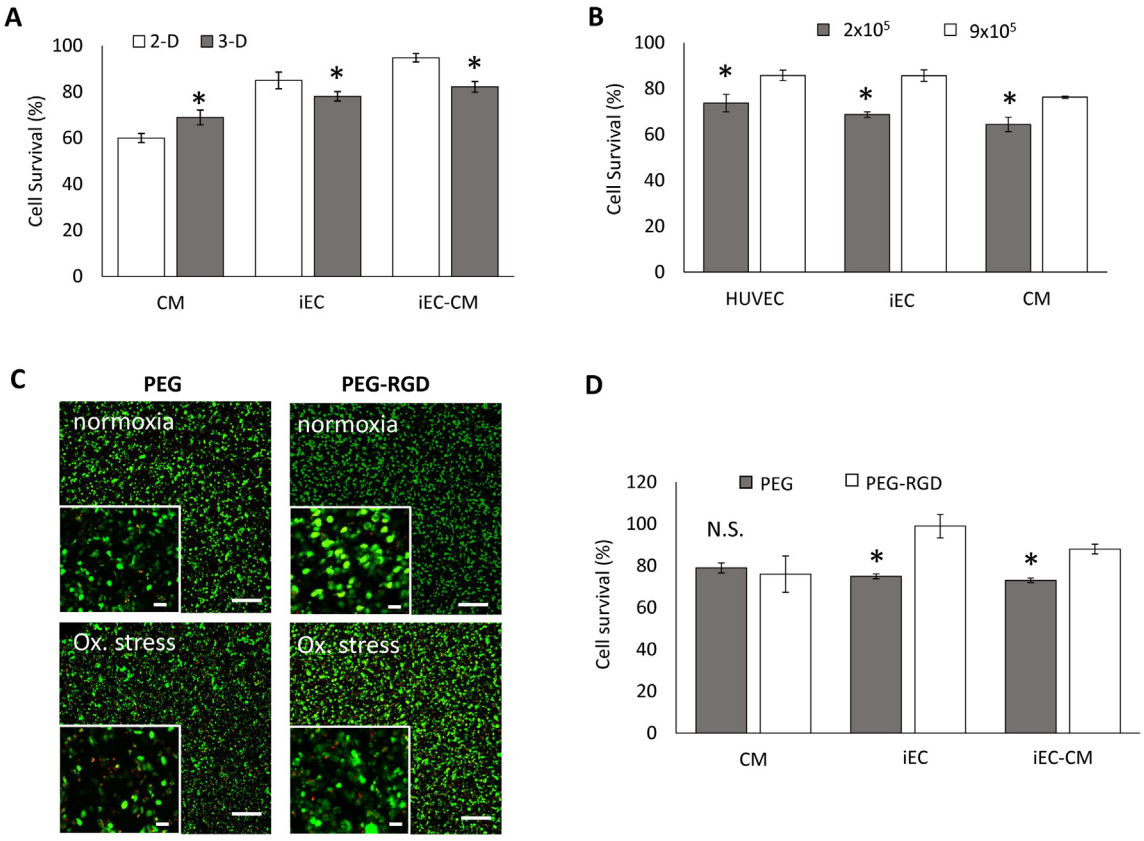

Fig. 4. The effect of cell-ECM interactions on cell survival. The importance of 3D environment and the cell-cell distance is shown with improved survival in 3D cultures with high encapsulation density. (A) The comparison of cell survival in 2D vs. 3D culture conditions. (B) The comparison of cell survival of EC-only and CM-only cultures in 3D with different cell encapsulation densities. (C) The live/dead images of iEC-CM model tissues in PEG and PEG-RGD, under normoxia or oxidative stress (Ox. stress) (Insets show magnified images of the corresponding samples). (D) The cell survival (%) of CMs, iECs and iEC-CM co-cultures encapsulated in PEG vs. PEG-RGD hydrogels in Fn (-) media. (* indicates statistical significance between two individual groups ($p < 0.05$), N.S. indicates "no significance" ($p > 0.05$), $n \geq 3$ for all). (Scale bar = 100 μm, inset scale bar = 20 μm).

the model tissues: We increased the cell number when transitioning from 2D to 3D culture to compensate for the construct volume. However, due to the specific hydrogel system we used in this study the cells were restricted in their encapsulation location and were evenly distributed in the gel. This kept the cells farther from one another, creating a possible diffusion limitation for the secreted factors, whereas, in the conventional culture studies, the cells could spread, divide and move. Therefore, we measured the respective cell-cell distance in different tissue constructs (Suppl. Fig. 5). We found that the 14.2 ± 1.6 μm average cell–cell distance in CM-alone constructs (Suppl. Fig. 5A), and 12.3 ± 0.5 μm cell–cell distance in iEC-CM (Suppl. Fig. 5B) constructs was doubled in iEC-alone constructs due to lower cell encapsulation density and measured as 28.2 ± 3.2 μm ($p < 0.05$) (Suppl. Fig. 5C). In addition, we showed that the oxidative stress treatment did not affect the cell-cell distance in the constructs (Suppl. Fig. 5A–D). Since we observed that the single CM culture survival increased in model tissues, we hypothesized that at this density the corresponding cell-cell distance was small enough to overcome the diffusion limitation. To test this hypothesis we encapsulated CMs or ECs at a density of 9×10^5 and 2×10^5 cells per construct and assessed their survival (Fig. 4B). We observed that for both HUVECs and iECs survival was significantly higher when the cell density was increased to 9×10^5 ($p < 0.01$, and $p < 0.001$, respectively) suggesting that the diffusion limitation for secreted factors was overcome. In support of this the average cell-cell distance was measured to be 13.2 ± 0.5 μm in these constructs, showing no significant difference compared to that of the CM-alone constructs at the same cell encapsulation density ($p > 0.05$) (Suppl. Fig. 5E). On the other hand, when the CM density was lowered to 2×10^5 per construct, the cell-cell distance increased to 25.7 ± 1.1 μm and their survival decreased significantly ($p < 0.01$) (Suppl. Fig. 5F), suggesting that in addition to paracrine factors, autocrine factors secreted by both ECs and CMs could also be contributing to cell survival (Suppl. Fig. 5G).

Cell-ECM interactions occur through specific surface proteins and these interactions are crucial for tissues to function properly in vivo. In order to test if the cell-ECM interactions improve cell survival under oxidative stress, we compared the cell survival in pure PEG hydrogels with RGD-functionalized PEG hydrogels using Live/Dead Assay (Fig. 4C). The results showed that the cell survival under oxidative stress was not altered due to the absence of RGD in the cell microenvironment for single cell type or co-culture constructs ($p > 0.05$) when standard minimal media was used (Suppl. Fig. 6). However, the minimal media used in the experiment is supplemented with FBS which usually contains soluble fibronectin. Therefore, in order to eliminate any contribution of soluble fibronectin, we depleted fibronectin from FBS and used this FBS in media preparation (Fn (−) media). We then cultured the model tissues in FN (-) media during the 3 day pre-stress incubation as well as the normoxia and oxidative stress treatments. This resulted in a significant decrease in the survival of iECs and iEC-CM co-culture ($p < 0.001$) in pure PEG constructs compared to that of PEG-RGD constructs (Fig. 4D). This indicates that the presence of RGD, and thus cell-ECM interactions, improve cell survival under oxidative stress.

3.5. Effect of EC-CM interactions and HIF-1α expression on apoptosis in model tissues

To study the effect of CM-EC interactions on apoptosis under oxidative stress, we assessed the DNA fragmentation and the caspase 3/7 activity of 3D CM-only and iEC-CM tissue constructs. To determine the specific role of HIF-1α in these interactions, these studies were performed either with control or HIF-1α shRNA knockdown iECs. We used TUNEL assay to determine the effect of EC presence on oxidative stress-induced apoptosis (Fig. 5A). Under normoxic conditions the CM-only and iEC-CM model tissues did not have a significant difference in apoptotic cell percentage ($p > 0.05$) (Fig. 5B). However, the number of apoptotic CMs increased significantly upon exposure to oxidative stress in CM-only models ($p < 0.05$) while it did not in the co-culture constructs ($p > 0.05$), indicating the involvement of EC-driven soluble factors in CMs' rescue from apoptosis. As HIF-1α is known to have a protective effect against apoptosis under hypoxic conditions [14], we also measured the caspase 3/7 activity in the constructs with iEC-CM and HIF-1α shRNA knockdown iEC-CM co-cultures (Fig. 5C). We observed significantly higher caspase activity in CM-only model tissues compared to the co-culture constructs regardless of their HIF-1α expression ($p < 0.05$). This indicates that CMs are protected from apoptosis in the presence of ECs, supporting the TUNEL assay results. In addition, we found that the HIF-1α shRNA knockdown iEC co-culture presented a higher caspase activity compared to the control iEC co-culture ($p < 0.05$) showing the involvement of HIF-1α in reducing apoptosis under oxidative stress.

3.6. Transcriptome analysis

Whole genome transcriptome analysis was performed in order to identify the possible factors involved in the cardioprotection under oxidative stress. We discuss the results from the full transcriptome analysis in detail in another publication [52]. Here, we will only highlight some of the gene expression changes we observed that are relevant to HIF-1α and oxidative stress response (Table 1). We performed this analysis on 4 types of 3D model tissue constructs: 1) CM-only constructs under normoxia, 2) CM-only constructs under oxidative stress, 3) iEC-CM co-culture constructs under normoxia, and 4) iEC-CM co-culture constructs under oxidative stress. Using rat origin CMs and human origin ECs allowed us to determine the changes in gene expression of the two cell types of the co-culture constructs separately through sequence specificity. This way we were able to show the effects of EC presence and oxidative stress on CM gene expression, individually. Interestingly, TGF-β3 and latent transforming growth factor β1 binding protein were upregulated in CMs that were co-cultured with ECs under both normal and stress conditions, indicating the presence

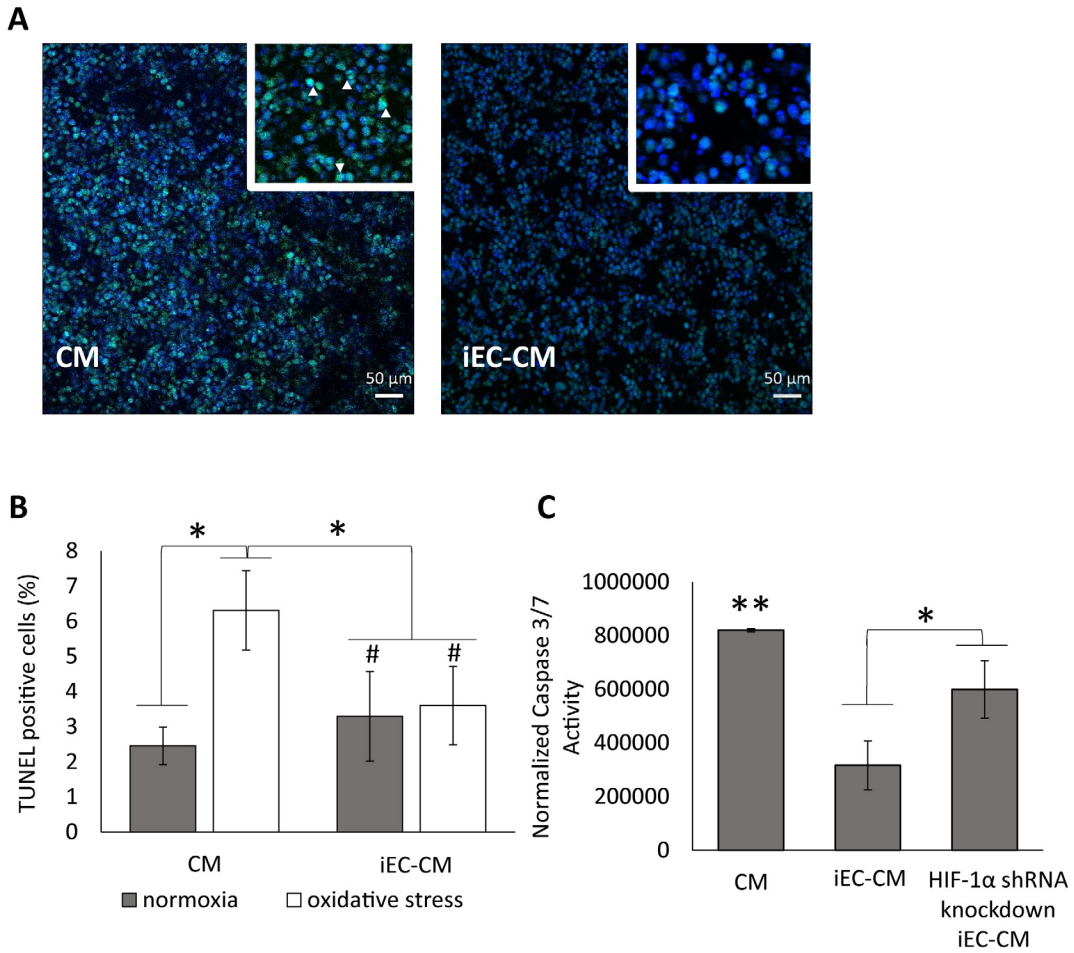

Fig. 5. The effect of EC presence and the role of HIF-1α on apoptosis under oxidative stress. (A) TUNEL assay images of CM and iEC-CM model tissues under oxidative stress (Blue: DAPI; Green: fragmented DNA) (white triangles indicate TUNEL positive cells) (B) TUNEL positive cell percentages of CM and iEC-CM model tissues under normoxia and oxidative stress. (C) Caspase 3/7 activity of CM, iEC-CM, and HIF-1α shRNA knockdown iEC-CM model tissues under oxidative stress (* indicates statistical significance between two individual groups and ** indicates significant difference between a single group to all other groups ($p < 0.05$), # indicates no statistical significance compared to "CM under normoxia" group ($p < 0.05$), $n \geq 3$ for all).

Table 1
Expression change in HIF-1α/apoptosis/oxidative stress related genes.

Gene Description	Fold Change*	p value
CMs in Co-culture vs. CMs In Single Culture: Oxidative Stress		
Transforming Growth Factor, Beta 3 (TGF-β3)	**2.386688**	7.40×10^{-12}
Latent Transforming Growth Factor Beta Binding Protein 1	**3.288170**	7.00×10^{-23}
Bcl2-Interacting Killer (Apoptosis-Inducing) (BIK)	0.357369	0.001003
Reactive Oxygen Species Modulator 1 (Romo-1)	0.300652	8.28×10^{-5}
CMs in Single Culture Under Stress vs. Normoxia		
Reactive Oxygen Species Modulator 1 (Romo-1)	**3.034063**	0.000258
CMs in Co-culture vs. CMs in Single Culture: Normoxia		
Transforming Growth Factor, Beta 3 (TGF-β3)	**2.024318**	1.43×10^{-8}
iECs in Co-culture Under Stress vs. Normoxia		
HIF-1α Antisense RNA 1 (HIF1A-AS1)	0.235003	0.015684

* Fold change of upregulated genes are indicated in bold type.

of ECs has an inductive role in the expression of both factors regardless of oxidative stress. Although there was no significant change in HIF-1α, von Hippel-Lindau factor, or prolyl hydroxylase (PHD) expression under any conditions, HIF1A-AS1 expression was downregulated by 73% in iECs co-cultured with CMs (FC:0.23) in response to oxidative stress, suggesting that its expression may play a role in promoting CM survival through HIF-1α regulation. In addition, BIK was downregulated in CMs when co-cultured with ECs (FC:0.36). We also observed that one of the mitochondrial membrane proteins, Romo-1, was downregulated (FC:0.30) in CMs cultured with ECs while it was upregulated (FC:3.03) in the absence of ECs under oxidative stress (Table 1).

We confirmed the transcriptome results for two relevant targets: HIF1A-AS1 as the results suggest that it might be a novel regulator of HIF-1α under oxidative stress conditions and for TGF-β3, as its expression has been shown to be directly correlated with HIF-1α expression. To confirm and further test the HIF1A-AS1 expression, we prepared iEC-CM co-culture and iEC-alone constructs and determined the relative HIF1A-AS1 expression under normoxia (N) or oxidative stress (S) (Fig. 6A). Interestingly, we observed that the HIF1A-AS1 mRNA expression was significantly lower in co-culture constructs compared to iEC-alone ones. This suggests that HIF1A-AS1 is downregulated in co-culture conditions through the bidirectional crosstalk between iECs and CMs, possibly stabilizing HIF-1α, as shown by the improved viability in co-culture conditions. Next, we performed ELISA to determine TGF-β3 levels in the media samples of the same CM-alone constructs that were used for transcriptome analysis, before and after the 16 h H_2O_2 and 2 h normoxia treatments (Fig. 6B). We also determined the TGF-β3 levels in the non-conditioned, iEC-only-conditioned or iEC-CM co-culture-conditioned media samples before or after they were incubated with 2D CM cultures under normoxia (N) or oxidative stress (S) conditions (Fig. 6C). We observed that the oxidative stress did not cause any change in TGF-β3 expression in CM-alone constructs, supporting the

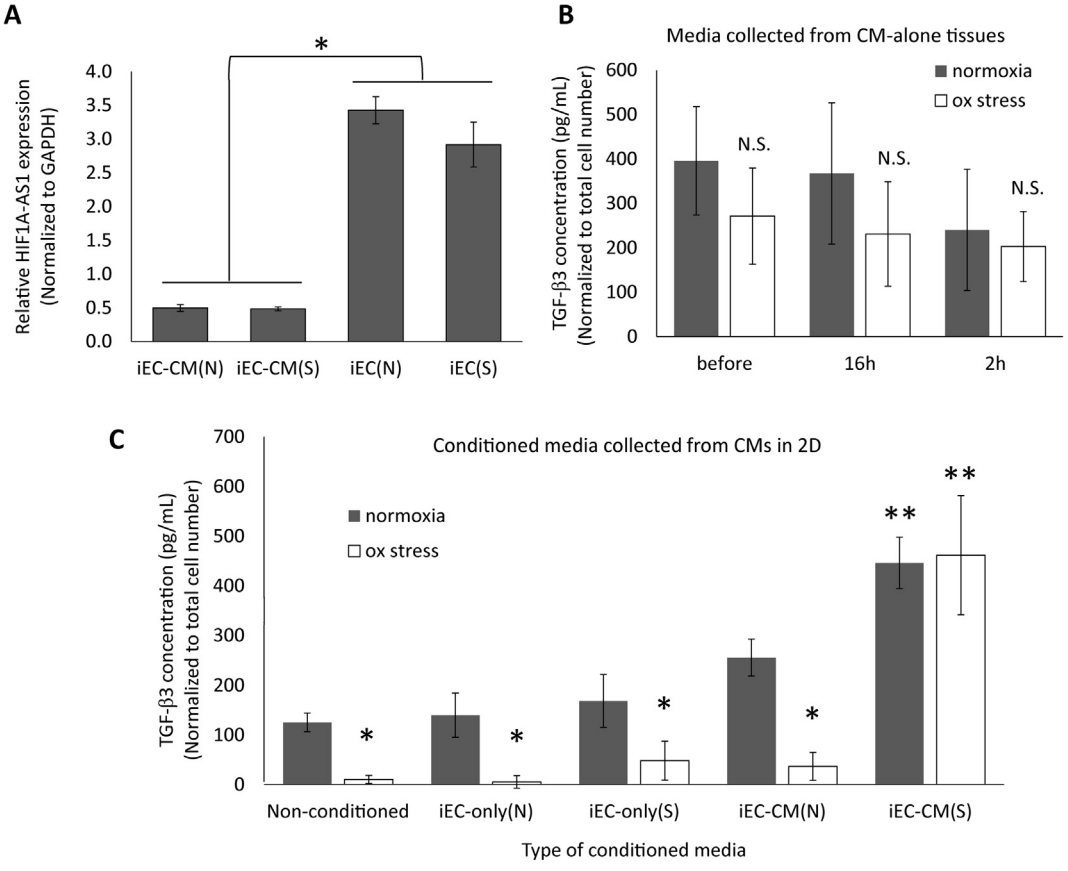

Fig. 6. Changes in expression of HIF-1α related factors with oxidative stress treatment and EC presence. The results confirm the bidirectional crosstalk between iECs and CMs as the most drastic changes were observed as a result of the co-culture conditions. (A) Relative HIF1A-AS1 mRNA expression in iEC-only or iEC-CM co-culture tissue constructs after normoxia (N) or oxidative stress treatment (S). (B) Normalized TGF-β3 protein concentrations in CM-alone tissue constructs before oxidative stress treatment (before), after 16 h H_2O_2 treatment (16 h) and after the subsequent 2 h normoxia treatment (2 h). (C) Normalized TGF-β3 protein concentrations in CM-alone cultures that received non-conditioned or iEC-only or iEC-CM co-culture-conditioned media, under normoxia (N) or oxidative stress (S). (* indicates statistical significance between two individual groups and ** indicates significant difference between a single group to all other groups ($p < 0.05$), N.S. indicates "no significance" ($p > 0.05$), $n \geq 3$ for all).

transcriptome results (Fig. 6B). Also supporting the transcriptome results, we observed an increased expression only in the co-culture pre-conditioned media. In addition, the increase was more prominent under oxidative stress (Fig. 6C).

3.7. Role of endothelial HIF1A-AS1 in CM survival under oxidative stress

To further confirm that the significant downregulation in HIF1A-AS1 in iECs (only seen when iECs were co-cultured with CMs as described in the previous section) has a cardioprotective effect under oxidative stress, we knocked down HIF1A-AS1 in iECs using siRNA targeting. Initially, we aimed to keep the 3 day long pre-culture period constant, however, we observed that the siRNA knockdown was effective for a limited time for our target and 3 days was too long to keep the knockdown condition (Suppl. Fig. 7A and B). Therefore, to prepare the co-culture-conditioned media, iECs were transfected and seeded on top of CMs right after transfection to preserve the knockdown condition as much as possible. The CMs were seeded 48 h before the iEC seeding so that the 3-day pre-culture period is kept constant for CMs (Fig. 7A). We achieved a 69% knockdown of HIF1A-AS1 through siRNA transfection as the qPCR analysis showed a 3.2-fold decrease in siRNA treated samples at t = 0 h (Fig. 7B). After 16 h the relative knockdown level decreased to 30% when iECs were maintained in oxidative stress (1.4-fold decrease in siRNA treated samples), and to 57% in normoxia (2.3-fold decrease in siRNA treated samples) (Fig. 7C). At this time point, the conditioned media (pN or pS) from iEC-only cultures and CM-iEC co-cultures were transferred to CM-only cultures. Then the CM-only cultures were maintained for 16 h with the respective conditioned media, with or without adding 0.2 mM H_2O_2, yielding 4 different conditions: 1) pN + N, 2) pS

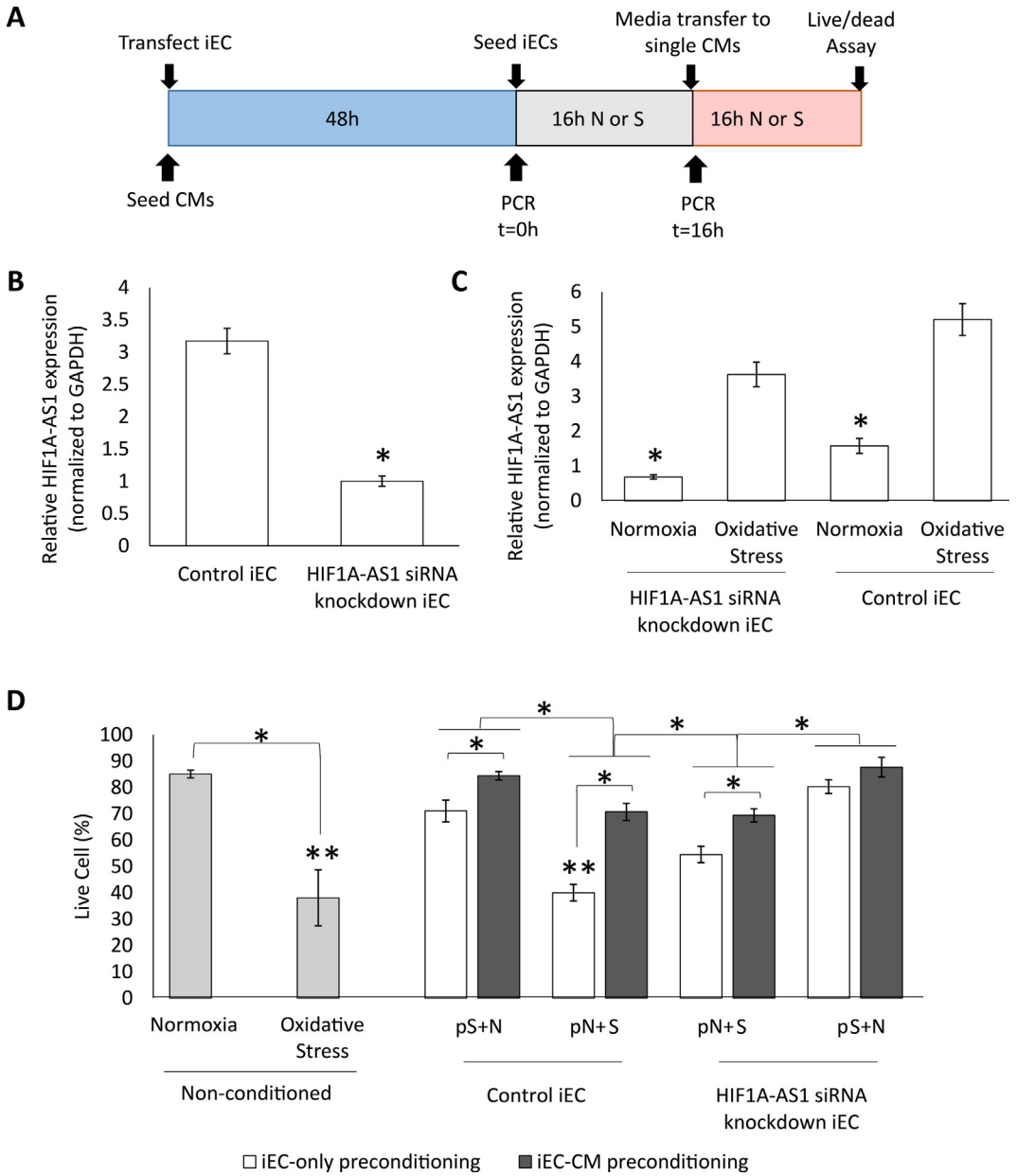

Fig. 7. Effect of HIF1A-AS1 expression and CM-EC interactions on cell survival. (A) The time table for the experiment ("N" stands for normoxia and "S" stands for oxidative stress). The RT-qPCR analysis showing HIF1A-AS1 mRNA expression of control or HIF1A-AS1 siRNA transfected iECs (B) right after transfection, and (C) 16 h after transfection. (D) Live cell (%) of single CM cultures incubated for 16 h in iECs only or CM-iEC co-culture pre-conditioned media prepared under normoxia (N) or oxidative stress (S). (* indicates statistical significance between two individual groups and ** indicates significant difference between a single group to all other groups ($p < 0.05$), $n \geq 3$ for all).

+ N, 3) pN + S, and 4) pS + S. At the end of the 16 h incubation with conditioned media, we determined the live cell percentages of the single CM cultures (Fig. 7D) using Live/Dead Assay (Suppl. Figs. 8 and 9). In cultures treated with pN + S, the viability of CMs was approximately 15% higher when cultured in HIF1A-AS1 knockdown iEC-only-conditioned media (54.5 ± 3%) compared to control iEC-only-conditioned media (40 ± 3%). Importantly, the low viability we observed with control iEC-only-conditioning was not significantly different than the viability of CMs in non-conditioned media (38 ± 10%). This suggests that conditioned media from only iECs does not improve CM survival under oxidative stress. When co-culture-conditioned media was used, however, the viability was significantly higher than that of the CMs in non-conditioned media, regardless of HIF1A-AS1 expression in iECs (HIF1A-AS1 knockdown iEC-CM co-culture: 69 ± 3%; control iEC-CM co-culture: 70 ± 2%). When the CMs were treated with pS + N, the viability was higher than pN + S condition for both iEC-only and co-culture-conditioned media. Similarly, the viability was higher with co-culture conditioning compared to iEC-only conditioning under normoxia regardless of HIF1A-AS1 expression of iECs. The 71 ± 4% viability achieved with control iEC-only-conditioned media was increased to 84 ± 2% when control iEC-CM co-culture-conditioned media was used. Interestingly, the CM viability was 80 ± 3% with HIF1A-AS1 knockdown iEC-only-conditioned media, showing that the viability increased approximately 9% with the sole effect of the knockdown. However, we did not observe an as drastic increase when we compared the co-culture conditioning with or without the knockdown: there was only a 3% increase in viability with HIF1A-AS1 knockdown iEC-CM co-culture conditioning (87 ± 3%). This result suggests that although a lower HIF1A-AS1 expression in iECs improves CM viability, there should be several other factors mediating the crosstalk between ECs and CMs under oxidative stress that contributes to the remarkable CM rescue we observed in this study.

4. Discussion

Understanding the cellular interactions in healthy and diseased myocardium and identifying potential molecular mechanisms that could result in enhanced cell survival and function under pathophysiological conditions is important in preventing and treating cardiovascular diseases. HIF-1 is known to be a major player in oxygen homeostasis in cells and tissues, and as such has been studied for its role in ischemic heart diseases focusing mostly on its well-known regulation under hypoxia [8,9,30]. Similar to hypoxia, oxidative stress is involved in the pathology of the ischemic heart diseases [31], and there is evidence that the HIF-1α level in the cells is regulated in presence of reactive oxygen species, causing it to escape proteasomal degradation [32]. However, the role of HIF-1α in cell survival under oxidative stress has yet to be investigated. In this study, we controlled the cell-cell and cell-ECM interactions by manipulating the functionalization of the hydrogel and the encapsulation density. We eliminated the direct cell-cell contact, which was inevitable in traditional culture conditions, while providing cell-ECM contact. This allowed us to examine the direct effect of soluble factors in EC-mediated CM survival under oxidative stress. The conditions we used (16 h oxidative stress followed by 2 h normoxia) were selected to study the conditions that mimic early MI events. Our results show that iECs do not produce or secrete the factors that result in CM protection under oxidative stress when they are alone: They need to be interacting with CMs directly or indirectly suggesting a bidirectional cross talk that governs the cardioprotective effect that we observed in this study. Furthermore, the CM survival increased similarly in the presence of ECs whether they were in physical contact or were interacting solely through paracrine factors suggesting that the key to the bidirectional crosstalk that leads to cardioprotection is secreted factors. In addition, we observed that the decreased HIF-1α expression in ECs has interfered with this EC-mediated improvement in cell survival. Our results are in line with studies that showed cardioprotective effects of EC-CM interactions and the role of HIF-1 in cardioprotection under hypoxic conditions, using conventional cell culture or animal models [10,33]. Although animal models are widely preferred for such studies, they present limitations on controlling cell type-specific gene expression [33]. This emphasizes the advantage of using model tissues that have cell type specific control over gene expression (i.e. HIF-1α), yet providing higher complexity compared to conventional cell culture methods as presented in this study. In addition, even though a cross-species platform, the model system used in this study can be readily applied in the future for investigating cardiovascular pathophysiology as a fully human-origin platform. Since the characterization and consistent production of fully mature and functional hiPSC-derived CMs is yet to be achieved, and that the cross-species platforms of rat and human cells are widely used in literature [10,19], we co-cultured iECs with well-characterized rat-origin CMs to take the first step towards a completely personalized platform.

Identifying the possible factors involved in CM-EC crosstalk will present a more detailed understanding of the molecular events that promote CM survival under oxidative stress. This will also help identify potential molecular targets to develop new therapeutic agents and provide more effective treatment before and after heart attack. Therefore, we performed a whole genome transcriptome analysis to identify these possible molecular targets. Most strikingly, we observed that HIF1A-AS1, a recently characterized [34] long non-coding RNA (lncRNA) was downregulated in ECs only, when co-cultured with CMs. Although little is known about HIF1A-AS1, it was shown that suppression of HIF1A-AS1 by siRNA targeting reduced palmitic acid induced apoptosis and promoted proliferation in vascular smooth muscle cells [35,36] and HUVECs [37] *in vitro*. The fact that we did not observe any changes in HIF-1α or PHD expression levels might suggest that HIF1A-AS1 may be an alternative means of HIF-1α regulation under oxidative stress. Since oxidative stress is rather a transient event, it is plausible for HIF-1α under oxidative stress to be performed through a long non-coding RNA. Therefore, we investigated this further to gain more mechanistic insight to the possible role of HIF1A-AS1 in cardioprotection under oxidative stress. Although the protective effect is improved with HIF1A-AS1 knockdown, this effect is overridden by the presence of CMs: the most significant improvement in survival was observed when conditioned media from CM-EC co-culture was transferred to single CM cultures, regardless of the HIF1A-AS1 expression. This is in line with the high survival of ECs under oxidative stress; we showed that ECs are not affected by the stress as much as CMs, therefore, it supports the finding that iEC-only conditioned media, especially without the HIF1A-AS1 knockdown, is insufficient to rescue CMs. Overall, 1) the drastic downregulation we observed in transcriptome analysis and the follow up PCR, 2) improved CM survival with HIF1A-AS1 knockdown iEC conditioned media, and 3) impaired CM survival in HIF-1α shRNA knockdown iEC co-cultures suggest that CM-EC interactions and HIF-1α expression in ECs is crucial for CM survival and HIF1A-AS1 is a means of HIF-1α regulation under oxidative stress. Here our primary tests indicate that although HIF1A-AS1 is a potential means of HIF-1α regulation under oxidative stress, it is not the key factor in CM-EC crosstalk. However, the conditions tested here are not sufficient to fully understand the role of HIF1A-AS1 in HIF-1α regulation and its potential as a therapeutic for treatment of cardiovascular diseases. Due to the limited effectiveness of the knockdown used in this study the tests were conducted in 2D con-

ventional cell cultures. These tests should be repeated in controlled 3D tissue models with stable knockdown and knockout models.

We also observed that TGF-β3 mRNA and protein expression was upregulated in CMs that were co-cultured with ECs under stress, but there was no change in their TGF-β1 and TGF-β2 expression, which were shown to be upregulated early in myocardial infarct models [38,39]. Therefore, observing an increase in the expression of only TGF-β3 further supports that the response observed in this study is a prolonged protective response. This also suggests that this prolonged cardioprotection is related to HIF-1α. Caniggia et al. showed that in trophoblasts TGF-β3 expression increased in parallel with HIF-1α expression and both were suppressed when antisense oligonucleotides targeting HIF-1α were introduced [40].

Another important outcome we observed in this study was that the CMs survived the oxidative stress better in a 3D environment compared to conventional culture conditions, even in the absence of physical cell-cell contact. One possible explanation is that in the 3D environment CMs have more neighboring cells compared to the 2D conditions as the cells are surrounded with other cells in all directions throughout the volume of the construct, enabling rapid diffusion of soluble factors among individual cells. Furthermore, the ECM-like hydrogel environment helps concentrate the paracrine factors released by the cells to their surrounding environment. These two physical constraints that come with a 3D ECM-like environment effectively increase the local concentration of the secreted factors compared to 2D conventional cell culture conditions where the cell-cell interactions are limited to one single plane and they can only receive the factors secreted from the cells that are in close proximity and at a limited concentration due to constant diffusion in the culture media. Therefore, the improved CM-CM interactions in 3D might have improved the overall survival of the CMs. The contradicting outcome of lower EC survival in 3D culture compared to the 2D culture conditions was remedied by increasing the encapsulation density. This indicates that the autocrine factors and their concentration, as defined by cell-cell proximity, plays an important role in intercellular communication and that this communication is crucial for EC and CM survival under oxidative stress.

In addition to provide control over cell-cell interactions, 3D model tissues allow us to manipulate the cell-ECM interactions. A common way to control this interaction is conjugating cell adhesive proteins or peptides and matrix metalloproteinase sequences that would allow the cells to attach and degrade through the hydrogels [41]. Unless tailored with peptides or proteins, pure PEG does not have any biochemical components that could be recognized by the cells, thus eliminating any potential biochemical effect coming from the hydrogel component of the tissue construct, while it has biophysical properties close to that of the natural myocardial ECM including being fully hydrated and having 8 kPa elastic modulus (natural myocardial stiffness is 10 kPa at the beginning of diastole) [42,43]. In this study, we functionalized PEG by incorporating the RGD peptide, a cell recognition peptide derived from fibronectin. When we cultured the model tissues in the absence of soluble fibronectin the survival in iEC-only and iEC-CM co-culture constructs was improved in the presence of RGD functionalization. This suggests that, cellular cascades activated with cell attachment to the ECM molecules are important in cell survival under oxidative stress, emphasizing the necessity of 3D culture models, as also supported by the literature [44]. It is known that under hypoxia HIF-1α mediated pathways promote integrin production [45], and, similar to hypoxic conditions, HIF-1α mediated pathways might be activated under oxidative stress conditions, improving cell survival. The detailed mechanism behind possible effects of HIF-1α mediated cell-ECM interactions on cell survival under oxidative stress should be further investigated.

Finally, we tested the effect of EC-CM interactions, specifically the effect of HIF-1α expression, on oxidative stress induced apoptosis in the model tissues. We report that iEC-CM co-culture constructs did not show an increase in the number of cells positive for DNA fragmentation, while the number of apoptotic cells increased significantly in the CM-only model tissues, indicating that EC-CM interactions help prevent apoptosis under oxidative stress. In addition, apoptosis is known to be regulated through HIF-1α dependent pathways [14]. Under hypoxic conditions HIF-1α activates the transcription of anti-apoptotic proteins such as B-cell lymphoma 2 (Bcl-2) family proteins, thus protecting cells against apoptosis [46,47]. The protective effect of HIF-1 was also shown under oxidative stress [48]. Zaman et al. studied the protection from oxidative stress-induced apoptosis in cortical neurons and concluded that the protection was regulated by HIF-1 activity. They cultured the cortical neurons in standard culture conditions and the cells were shown to escape apoptosis by addition of iron chelators, which in turn improved HIF-1 activity. Here, we show the direct effect of HIF-1α mediated secreted factors on escaping oxidative stress-induced apoptosis in our physiologically relevant model tissues. We measured the increase in caspase activity, a common indicator of apoptosis, and observed an increase in caspase activity in CMs when they were encapsulated alone or co-encapsulated with HIF-1α shRNA knockdown iECs, compared to control iEC-CM co-cultures. These results also correlate with the cell survival; although the presence of ECs decreased the number of apoptotic cells regardless of their HIF-1α expression, this protective effect was impaired when HIF-1α was knocked down in ECs.

Further supporting these results, the transcriptome analysis showed that the BIK, the activity of which is suppressed in the presence of survival-promoting proteins [49], is downregulated in CMs that were in the co-culture constructs compared to CM-only constructs, under oxidative stress. Our results showed that, Romo-1, a mitochondrial membrane protein that induces production of reactive oxygen species [50], was upregulated in CMs under oxidative stress when cultured alone. However, Romo-1 expression was downregulated when CMs were co-cultured with ECs, further supporting the protective role of EC-mediated paracrine factors as Romo-1 expression was shown to cause oxidative stress-induced apoptosis in lung epithelial cells and its siRNA knockdown improved cell survival [51].

5. Conclusion

In conclusion, here we showed that the EC-CM interactions are crucial for CM protection and for overall cell survival following oxidative stress and subsequent normoxia treatment, resembling early MI conditions. We also present proof that the EC-driven soluble factors, at least partially regulated by HIF-1α, play a major role in cell survival and escaping apoptosis. In addition, the cell-cell communication is highly dependent on cell-cell proximity and cell-ECM interactions, contributing to cell survival by taking advantage of the highly controlled yet flexible microenvironment that engineered model tissues provide. Importantly, we showed, for the first time to our knowledge, that HIF-1α expression is possibly mediated by HIF1A-AS1 under oxidative stress and its downregulation in ECs aids CM survival, especially through CM-EC crosstalk. Overall, the results of this study stress the importance of 3D engineered model tissues as suitable platforms to study cardiovascular diseases on molecular, cellular and tissue level. With the introduction of hiPSC-derived cells such as the ones used in this study, these models can be used as personalized platforms for disease diagnostics, treatment and drug screening. In addition, using engineered models, such as the one presented in this study, the potential of iPSC-derived cells to be used in cell therapy can be

tested in a biomimetic environment. In our future studies we will replace the CMs from rat origin with hiPSC-derived CMs to construct and study model tissues with entirely human origin. We also aim to investigate the EC-mediated cardioprotection under conditions that mimic the late events in MI by varying the stress exposure period and using stiffer hydrogel systems to mimic the ECM of infarct tissues that has undergone fibrosis.

Disclosures

No conflicts of interest are declared.

Acknowledgements

We thank Dr. C. Cowan, Dr. K. Musunuru, and Dr. L. Challet-Meylan, Harvard University, for providing human induced pluripotent cells and reagents, and for their helpful suggestions and scientific insight on stem cell culture and differentiation. This work was supported by a Project Development Team within the Indiana Clinical and Transitional Sciences Institute, National Institutes of Health/ National Center for Research Resources (ICTSI NIH/NCRR) [grant number UL1TR001108], and NSF CBET CAREER Award [grant number 1651385].

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.actbio.2017.06.023.

References

[1] D.J. Hausenloy, D.M. Yellon, Myocardial ischemia-reperfusion injury: a neglected therapeutic target, J. Clin. Invest. 123 (2013) 92–100.
[2] E. Braunwald, R.A. Kloner, Myocardial reperfusion: a double-edged sword?, J Clin. Invest. (1985) 1713–1719.
[3] D.L. Carde, D.N. Granger, Pathophysiology of reperfusion injury, J. Pathol. 190 (2000) 255–266.
[4] H.M. Piper, D. Garcia-Dorado, M.A. Ovize, A fresh look at reperfusion injury, Cardiovasc. Res. 38 (1998) 291–300.
[5] D.M. Yellon, D.J. Hausenloy, Myocardial reperfusion injury, N. Engl. J. Med. 357 (2007) 1121–1135.
[6] D.L. Brutsaert, Cardiac endothelial-myocardial signaling: its role in cardiac growth, contractile performance, and rhythmicity, Physiol. Rev. 83 (2003) 59–115.
[7] D. Tekin, D.A. Dursun, L. Xi, Hypoxia inducible factor 1 (HIF-1) and cardioprotection, Acta Pharmacol. Sin. 31 (2010) 1085–1094.
[8] S.G. Ong, D.J. Hausenloy, Hypoxia-inducible factor as a therapeutic target for cardioprotection, Pharmacol. Ther. 136 (2012) 69–81.
[9] S.N. Heyman, S. Rosen, C. Rosenberger, Hypoxia-inducible factors and the prevention of acute organ injury, Crit. Care 15 (2011) 2.
[10] T.M. Leucker, M. Bienengraeber, M. Muravyeva, I. Baotic, D. Weihrauch, A.K. Brzezinska, D.C. Warltier, J.R. Kersten, P.F. Pratt Jr., P.F Jr. , Endothelial-cardiomyocyte crosstalk enhances pharmacological cardioprotection, J. Mol. Cell. Cardiol. 51 (2011) 803–811.
[11] A.A. Qutub, A.S. Popel, Reactive oxygen species regulate hypoxia-inducible factor 1α differentially in cancer and ischemia, Mol. Cell. Biol. 28 (2008) 5106–5119.
[12] R.S. Adluri, M. Thirunavukkarasu, N.R. Dunna, L. Zhan, B. Oriowo, K. Takeda, J.A. Sanchez, H. Otani, G. Maulik, G.H. Fong, N. Maulik, Disruption of hypoxia-inducible transcription factor-prolyl hydroxylase domain-1 (PHD-1-/-) attenuates ex vivo myocardial ischemia/reperfusion injury through hypoxia-inducible factor-1α transcription factor and its target genes in mice, Antioxid. Redox Signal. 15 (2011) 1789–1797.
[13] M. Bonios, C.Y. Chang, J. Terrovitis, A. Pinheiro, A. Barth, P. Dong, M. Santaularia, D.B. Foster, V. Raman, T.P. Abraham, M.R. Abraham, Constitutive HIF-1α expression blunts the beneficial effects of cardiosphere-derived cell therapy in the heart by altering paracrine factor balance, J Cardiovasc Transl Res. 4 (2011) 363–372.
[14] A.E. Greijer, E. Van Der Wall, The role of hypoxia inducible factor 1 (HIF-1) in hypoxia induced apoptosis, J. Clin. Pathol. 57 (2004) 1009–1014.
[15] G. Loor, P.T. Schumacker, Role of hypoxia-inducible factor in cell survival during myocardial ischemia-reperfusion, Cell Death Differ. 15 (2008) 686–690.
[16] R. Malhotra, D.W. Tyson, H.M. Rosevear, F.C. Brosius III, Hypoxia-inducible factor-1alpha is a critical mediator of hypoxia induced apoptosis in cardiac H9c2 and kidney epithelial HK-2 cells, BMC Cardiovasc Disord. 8 (2008) 9.
[17] T. Eckle, D. Kohler, R. Lehmann, K.C.E. Kasmi, H.K. Eltzschig, Hypoxia-inducible factor-1 is central to cardioprotection a new paradigm for ischemic preconditioning, Circulation 118 (2008) 166–175.
[18] X. Wang, S. Ma, G. Qi, Effect of hypoxia-inducible factor 1-alpha on hypoxia/reoxygenation-induced apoptosis in primary neonatal rat cardiomyocytes, Biochem. Biophys. Res. Commun. 417 (2012) 1227–1234.
[19] P. Perel, I. Roberts, E. Sena, P. Wheble, C. Briscoe, P. Sandercock, M. Macleod, L. E. Mignini, P. Jayaram, K.S. Khan, Comparison of treatment effects between animal experiments and clinical trials: systematic review, BMJ 334 (2007) 197.
[20] M. Skolimowski, M. Weiss Nielsen, F. Abeille, P. Skafte-Pedersen, D. Sabourin, A. Fercher, D. Papkovsky, S. Molin, R. Taboryski, C. Sternberg, M. Dufva, O. Geschke, J. Emnéus, Modular microfluidic system as a model of cystic fibrosis airways, Biomicrofluidics 6 (2012) 34109.
[21] G. Wang, M.L. McCain, L. Yang, A. He, F.S. Pasqualini, A. Agarwal, H. Yuan, D. Jiang, D. Zhang, L. Zangi, J. Geva, A.E. Roberts, Q. Ma, J. Ding, J. Chen, D.Z. Wang, K. Li, J. Wang, R.J. Wanders, W. Kulik, F.M. Vaz, M.A. Laflamme, C.E. Murry, K.R. Chien, R.I. Kelley, G.M. Church, K.K. Parker, W.T. Pu, Modeling the mitochondrial cardiomyopathy of Barth syndrome with induced pluripotent stem cell and heart-on-chip technologies, Nat. Med. 20 (2014) 616–623.
[22] M.A. Israel, S.H. Yuan, C. Bardy, S.M. Reyna, Y. Mu, C. Herrera, M.P. Hefferan, S. Van Gorp, K.L. Nazor, F.S. Boscolo, C.T. Carson, L.C. Laurent, M. Marsala, F.H. Gage, A.M. Remes, E.H. Koo, L.S. Goldstein, Probing sporadic and familial Alzheimer's disease using induced pluripotent stem cells, Nature 482 (2012) 216–220.
[23] S. Kashida, N. Sugino, S. Takiguchi, A. Karube, H. Takayama, Y. Yamagata, Y. Nakamura, H. Kato, Regulation and role of vascular endothelial growth factor in the corpus luteum during mid-pregnancy in rats, Biol. Reprod. 64 (2001) 317–323.
[24] M. Tanaka, H. Ito, S. Adachi, H. Akimoto, T. Nishikawa, T. Kasajima, F. Marumo, M. Hiroe, Hypoxia induces apoptosis with enhanced expression of Fas antigen messenger RNA in cultured neonatal rat cardiomyocytes, Circ. Res. 75 (1994) 426–433.
[25] C. Patsch, L. Challet-Meylan, E.C. Thoma, E. Urich, T. Heckel, J.F. O'Sullivan, S.J. Grainger, F.G. Kapp, L. Sun, K. Christensen, Y. Xia, M.H. Florido, W. He, W. Pan, M. Prummer, C.R. Warren, R. Jakob-Roetne, U. Certa, R. Jagasia, P.O. Freskgård, I. Adatto, D. Kling, P. Huang, L.I. Zon, E.L. Chaikof, R.E. Gerszten, M. Graf, R. Iacone, C.A. Cowan, Generation of vascular endothelial and smooth muscle cells from human pluripotent stem cells, Nat. Cell Biol. 17 (2015) 994–1003.
[26] J.A. Forsythe, B.H. Jiang, N.V. Iyer, F. Agani, S.W. Leung, R.D. Koos, G.L. Semenza, Activation of vascular endothelial growth factor gene transcription by hypoxia-inducible factor 1, Mol. Cell. Biol. 16 (1996) 4604–4613.
[27] D. Yoshida, K. Kim, M. Noha, A. Teramoto, Hypoxia inducible factor 1-alpha regulates of platelet derived growth factor-B in human glioblastoma cells, J. Neurooncol. 76 (2006) 13–21.
[28] X.H. Peng, P. Karna, Z. Cao, B.H. Jiang, M. Zhou, L. Yang, Cross-talk between epidermal growth factor receptor and hypoxia-inducible factor-1alpha signal pathways increases resistance to apoptosis by up-regulating survivin gene expression, J. Biol. Chem. 281 (2006) 25903–25914.
[29] J. Yu, J. Li, S. Zhang, X. Xu, M. Zheng, G. Jiang, F. Li, IGF-1 induces hypoxia-inducible factor 1α-mediated GLUT3 expression through PI3K/Akt/mTOR dependent pathways in PC12 cells, Brain Res. 1430 (2012) 18–24.
[30] R.V. Shohet, J.A. Garcia, Keeping the engine primed: HIF factors as key regulators of cardiac metabolism and angiogenesis during ischemia, J. Mol. Med. Berl. Ger. 85 (2007) 1309–1315.
[31] C. Ceconi, A. Boraso, A. Cargnoni, R. Ferrari, Oxidative stress in cardiovascular disease: myth or fact?, Arch Biochem. Biophys. 420 (2003) 217–221.
[32] N.S. Chandel, E. Maltepe, E. Goldwasser, C.E. Mathieu, M.C. Simon, P.T. Schumacker, Mitochondrial reactive oxygen species trigger hypoxia-induced transcription, Proc. Natl. Acad. Sci. U.S.A. 95 (1998) 11715–11720.
[33] K. Sarkar, Z. Cai, R. Gupta, N. Parajuli, K. Fox-Talbot, M.S. Darshan, F.J. Gonzalez, G.L. Semenza, Hypoxia-inducible factor 1 transcriptional activity in endothelial cells is required for acute phase cardioprotection induced by ischemic preconditioning, Proc. Natl. Acad. Sci. U.S.A. 109 (2012) 10504–10509.
[34] D. Bertozzi, R. Iurlaro, O. Sordet, J. Marinello, N. Zaffaroni, G. Capranico, Characterization of novel antisense HIF-1α transcripts in human cancers, Cell Cycle 10 (2011) 3189–3197.
[35] Y. Zhao, G. Feng, Y. Wang, Y. Yue, W. Zhao, Regulation of apoptosis by long non-coding RNA HIF1A-AS1 in VSMCs: implications for TAA pathogenesis, Int J Clin Exp Pathol. 7 (2014) 7643–7652.
[36] S. Wang, X. Zhang, Y. Yuan, M. Tan, L. Zhang, X. Xue, Y. Yan, L. Han, Z. Xu, BRG1 expression is increased in thoracic aortic aneurysms and regulates proliferation and apoptosis of vascular smooth muscle cells through the long non-coding RNA HIF1A-AS1 in vitro, Eur. J. Cardiothorac. Surg. 47 (2015) 439–446.
[37] J. Wang, L. Chen, H. Li, J. Yang, Z. Gong, B. Wang, X. Zhao, Clopidogrel reduces apoptosis and promotes proliferation of human vascular endothelial cells induced by palmitic acid via suppression of the long non-coding RNA HIF1A-AS1 in vitro, Mol. Cell. Biochem. 404 (2015) 203–210.
[38] A. Deten, A. Holzl, M. Leicht, W. Barth, H.G. Zimmer, Changes in extracellular matrix and in transforming growth factor beta isoforms after coronary artery ligation in rats, J. Mol. Cell. Cardiol. 33 (2001) 1191–1207.
[39] O. Dewald, G. Ren, G.D. Duerr, M. Zoerlein, C. Klemm, C. Gersch, S. Tincey, L.H. Michael, M.L. Entman, N.G. Frangogiannis, Of mice and dogs: species-specific differences in the inflammatory response following myocardial infarction, Am. J. Pathol. 164 (2004) 665–677.

[40] I. Caniggia, H. Mostachfi, J. Winter, M. Gassmann, S.J. Lye, M. Kuliszewski, M. Post, Hypoxia-inducible factor-1 mediates the biological effects of oxygen on human trophoblast differentiation through TGF beta(3), J. Clin. Invest. 105 (2000) 577–587.
[41] M.P. Lutolf, J.L. Lauer-Fields, H.G. Schmoekel, A.T. Metters, F.E. Weber, G.B. Fields, J.A. Hubbell, Synthetic matrix metalloproteinase-sensitive hydrogels for the conduction of tissue regeneration: engineering cell-invasion characteristics, Proc. Natl. Acad. Sci. U.S.A. 100 (2003) 5413–5418.
[42] S.F. Nagueh, G. Shah, Y. Wu, S. Lahmers, Altered titin expression, myocardial stiffness, and left ventricular function in patients with dilated cardiomyopathy, Circulation 110 (2004) 155–162.
[43] J.H. Omen, Stress and strain as regulators of myocardial growth, Prog. Biophys. Mol. Biol. 69 (1998) 559–572.
[44] T. Sun, S. Jackson, J.W. Haycock, S. MacNeil, Culture of skin cells in 3D rather than 2D improves their ability to survive exposure to cytotoxic agents, J. Biotechnol. 122 (2006) 372–381.
[45] K.D. Cowden Dahl, S.E. Robertson, V.M. Weaver, M.C. Simon, Hypoxia-inducible factor regulates αvβ3 integrin cell surface expression, Mol. Biol. Cell 16 (2005) 1901–1912.
[46] J.W. Dong, H.F. Zhu, W.Z. Zhu, H.L. Ding, T.M. Ma, Z.N. Zhou, Intermittent hypoxia attenuates ischemia/reperfusion induced apoptosis in cardiac myocytes via regulating Bcl-2/Bax expression, Cell Res. 13 (2003) 385–391.
[47] R. Blum, J. Jacob-Hirsch, N. Amariglio, G. Rechavi, Y. Kloog, Ras inhibition in glioblastoma down-regulates hypoxia-inducible factor-1alpha, causing glycolysis shutdown and cell death, Cancer Res. 65 (2005) 999–1006.
[48] K. Zaman, H. Ryu, D. Hall, K. O'Donovan, K.I. Lin, M.P. Miller, J.C. Marquis, J.M. Baraban, G.L. Semenza, R.R. Ratan, Protection from oxidative stress-induced apoptosis in cortical neuronal cultures by iron chelators is associated with enhanced DNA binding of hypoxia-inducible factor-1 and ATF-1/CREB and increased expression of glycolytic enzymes, p21(waf1/cip1), and erythropoietin, J. Neurosci. 19 (1999) 9821–9830.
[49] L. Coultas, P. Bouillet, E.G. Stanley, T.C. Brodnicki, J.M. Adams, A. Strasser, Proapoptotic BH3-only Bcl-2 family member Bik/Blk/Nbk is expressed in hemopoietic and endothelial cells but is redundant for their programmed death, Mol. Cell. Biol. 24 (2004) 1570–1581.
[50] Y.M. Chung, J.S. Kim, Y.D. Yoo, A novel protein, Romo1, induces ROS production in the mitochondria, Biochem. Biophys. Res. Commun. 347 (2006) 649–655.
[51] J.A. Shin, J.S. Chung, S.H. Cho, H.J. Kim, Y.D. Yoo, Romo1 expression contributes to oxidative stress-induced death of lung epithelial cells, Biochem. Biophys. Res. Commun. 439 (2013) 315–320.
52 P. Zorlutuna, X. Yue, A. Acun, Transcriptome profiling of 3D co-cultured cardiomyocytes and endothelial cells under oxidative stress using a photocrosslinkable hydrogel system, 2017, in press.