Cardiac inducing colonies halt fibroblast activation and induce cardiac/endothelial cells to move and expand via paracrine signaling

Samiksha Mahapatraa, Michael V. R. Sharmab, Breanna Brownsona,c, Vaughn E. Galliccano,d, and G. Ian Gallicanoa,b,*

aDepartment of Biochemistry and Molecular Biology, Georgetown University Medical Center, Washington, DC 20057-145; bCelprogen, Torrance, CA 90503; cRye High School, Rye, NY 10580; dThomas Edison High School, Alexandria, VA 22310

ABSTRACT Myocardial fibrosis (MF), a common event that develops after myocardial infarction, initially is a reparative process but eventually leads to heart failure and sudden cardiac arrest. In MF, the infarct area is replaced by a collagenous-based scar induced by “excessive” collagen deposition from activated cardiac fibroblasts. The scar prevents ventricular wall thinning; however, over time it expands to noninfarcted myocardium. Therapies to prevent fibrosis include reperfusion, anti-fibrotic agents, and ACE inhibitors. Paracrine factor (PF)/stem cell research has recently gained significance as a therapy. We consistently find that cardiac inducing colonies (CiCs) (derived from human germline pluripotent stem cells) secrete PFs at physiologically relevant concentrations that suppress cardiac fibroblast activation and excessive extracellular matrix protein secretion. These factors also affect human cardiomyocytes and endothelial cells by inducing migration/proliferation of both populations into a myocardial wound model. Finally, CiC factors modulate matrix turnover and proinflammation. Taking the results together, we show that CiCs could help tip the balance from fibrosis toward repair.

INTRODUCTION

According to the World Health Organization, an estimated 17.9 million people died in 2019 from cardiovascular disease (CVD), representing 31% of all global deaths. Of these deaths, 85% were due to sudden cardiac arrest (SCA), which is a condition where the heart abruptly stops beating. The most significant risk for SCA occurs

This article was published online ahead of print in MBoC in Press (http://www.molbiolcell.org/cgi/doi/10.1091/mbc.E22-02-0032) on June 2, 2022.

Author contributions: S. M.: majority of the writing of manuscript and most of the experimental analyses. M.V.R.S.: analysis and identification of HCMs. B. B.: high school student, performed Western blots for repetitions. V.E.G.: high school student, performed Western blots and immunofluorescence analyses. G.I.G.: senior author, most ideas and authorization of manuscript contents.

*Address correspondence to: G. Ian Gallicano (gig@georgetown.edu).

Disclosure statement: No conflict of interest.

Abbreviations used: CiCs, cardiac inducing colonies; ECs, endothelial cells; ECM, extracellular matrix; HCFs, human cardiac fibroblasts; HCMs, human cardiomyocytes; HF, heart failure; MF, myocardial fibrosis; MI, myocardial infarction; PF, paracrine factor; SCA, sudden cardiac arrest; TGFβ, transforming growth factor β.

© 2022 Mahapatra et al. This article is distributed by The American Society for Cell Biology under license from the author(s). Two months after publication it is available to the public under an Attribution–Noncommercial-Share Alike 4.0 International Creative Commons License (http://creativecommons.org/licenses/by-nc-sa/4.0).

“ASCB®, “ The American Society for Cell Biology®,” and “Molecular Biology of the Cell®” are registered trademarks of The American Society for Cell Biology.
Prabhu and Frangogiannis, 2016; Talman and Ruskoaho, 2016). After clearance, the proinflammatory reaction is repressed by up-regulation of anti-inflammatory players and down-regulation of proinflammatory players marking the end of the first phase (Frangogiannis, 2015; Prabhu and Frangogiannis, 2016; Talman and Ruskoaho, 2016). The second phase of repair is induced by increased mechanical stresses causing inactivated/quiescent cardiac fibroblasts (hHCFs) to migrate into the infarcted area and transdifferentiate to hHCFs (Frangogiannis, 2015; Prabhu and Frangogiannis, 2016; Talman and Ruskoaho, 2016). hHCFs are responsible for secreting excessive amounts of extracellular matrix (ECM) proteins including collagen I and III (Col I/III), eventually resulting in a noncompliant scar (Frangogiannis, 2015; Prabhu and Frangogiannis, 2016; Talman and Ruskoaho, 2016). Subsequently, a majority of hHCFs undergo apoptosis marking the end of the second phase. During the third phase, surrounding resident iHCFs in the noninfarcted myocardium undergo activation, resulting in expansion and maturation of the scar (Frangogiannis, 2015; Prabhu and Frangogiannis, 2016; Talman and Ruskoaho, 2016). One hallmark of scar establishment and expansion is cross-linking of collagen I and III fibers by lysyl oxidase/lysyl oxidase-like II (LOX/LOXL2) along with other ECM proteins such as peroxisome (PER). It is this expansion that leads to loss of elasticity and abnormal contractile function. On the basis of the ultimate outcome of cardiac scarring after an MI, we have proposed to test a logical twofold hypothesis for treating an infarct: 1) attenuating cardiac fibrosis to prevent differentiation of iHCFs into hHCFs can limit scar development and expansion and 2) simultaneously induce surrounding cardiomyocytes (HCM) and endothelial cells (EC) to infiltrate into the infarct and repair the wound.

One approach for treating or curing ischemic heart disease has been stem cell research, which to date has culminated in three basic approaches: the use of induced pluripotent stem cells (iPSCs), pluripotent embryonic stem cells (ESCs), and multipotent adult stem cells (ASCs). The approach of using iPSCs has shown promise in vitro and in vivo; however, problems remain for their use in vivo as teratoma formation, genetic instability, mitochondria aging, and cell memory must yet be resolved (Boudoulas and Hatzopoulos, 2009; Tompkins et al., 2018; Yan et al., 2019). ESCs can be differentiated into cardiomyocytes in vitro and in vivo; however, they pose ethical concerns, immune rejection, genomic instability, and, similar to iPSCs, potential generation of teratomas Boudoulas and Hatzopoulos, 2009; Yan et al., 2019). Multipotent ASCs such as bone marrow mononuclear cells (BMNCs), hematopoietic stem cells (HSCs), mesenchymal stem cells (MSCs), cardiac stem cells (CSCs), endothelial progenitor stem cells (EPSCs) have all been used as potential therapeutics for CVD with various successes and failures (Boudoulas and Hatzopoulos, 2009; Fan and Guan, 2016; Yan et al., 2019). In fact, many clinical trials using ASCs including BOOST, REPAIR-AMI, and PreSERVE-AMI have resulted in increased ventricular function and decreased infarct size (Yu et al., 2017; Muller et al., 2018; Tompkins et al., 2018; Bracco Gartner et al., 2019; Wernly et al., 2019); however, the minuses have slowly diminished the initial excitement for ASC clinical use. For example, MSCs and BMSCs have plasticity issues and can be differentiated only into limited types of cells (Boudolas and Hatzopoulos, 2009; Fan and Guan, 2016; Yan et al., 2019), and CSCs are found in very small numbers and isolating them remains a challenge (Hodgkinson et al., 2016; A futile cycle, 2017; Yu et al., 2017; Tompkins et al., 2018). Consequently, the culmination of unsuccessful trials has led to a long-standing debate as to whether consuming institutional and/or private funding for these trials should continue on patients with serious heart conditions (A futile cycle, 2017).

As a pro argument to this debate, we previously identified that unipotent germline stem cells, when removed from their niche and cultured in the correct medium, endogenously express pluripotent markers including Sox2, Oct4, Lin28, and Klf4, all of which induce them to become human germline pluripotent stem cells (hgPSCs; Golestaneh et al., 2009). Subsequent work revealed that hgPSCs could be induced to become cardiac inducing colonies (CiCs) capable of integrating and fusing with heart tissue via gap junctions (Mahapatra et al., 2018). CiCs were found to express cardiac genes, as well as genes that negatively regulate teratoma formation (Mahapatra et al., 2018). Equally important, Mahapatra et al. (2018) identified that hgPSCs could be indefinitely and quickly expanded in a defined expansion medium without losing their pluripotency. This expansion technology has been key for generating thousands of hgPSCs, potentially solving the cell dosage issues put forth by A futile cycle (2017).

Here, testing our twofold hypothesis, we show that CiCs are capable of secreting 18 cardioprotective paracrine factors that 1) attenuate activation of hHCFs; 2) induce migration/proliferation of human cardiomyocytes (HCMs) and ECs into a model infarct; 3) achieve ECM homeostasis by controlling collagen cross-linking through regulation of LOX/LOXL2; and 4) promote containment and effective resolution of proinflammation.

In vitro activation of HCFs with subsequent treatment with rPF cocktail or CiC-CM

Human cardiac fibroblasts (HCFs) were activated by addition with 10 ng/ml TGFβ for 24/48/72 h. For rPF ([recombinant paracrine factor] cocktail/CiC-CM [conditional media derived from cardiac induced colonies]) treatments, HCFs were activated by addition of TGFβ for 24 h, and subsequently rPF cocktail/CiC-CM was added to 24 h activated HCFs and incubated for an additional 24/48/72 h wherein rPF cocktail/CiC-CM was replenished every 24 h.

qPCR

RNA from 0 h (resident inactivated HCFs) and 24, 48, and 72 h TGFβ- and paracrine factor–induced HCFs was isolated from three different batches (biological replicates; N = 3) using the RNeasy isolation kit from Qiagen (Cat #217004). Using the iScript cDNA synthesis kit (Cat #1708890) from Biorad, we made cDNA from all of the RNA isolated. Primer sequences for Periostin, Collagen Type I, Collagen Type III, CD90, and alpha smooth muscle actin were obtained from IDT Technologies (sequences provided in Table 4). PCRs were carried out in the CFX 96 Biorad thermal cycler machine at 35 cycles, and the qPCR kit used was the Biorad iTaq SYBR green PCR kit (Cat #1725121). Each gene was done in triplicate for each sample/condition (technical replicates; n = 3), and gene expression levels were calculated from DDCT. Each qPCR experiment was repeated three times for each sample/condition. The control gene was GAPDH.

Western blot analysis

All cells were scraped, washed, suspended in sample buffer (62.5 mM Tris-HCl, pH 6.8, 2% SDS [wt/vol], 1 mM β-mercaptoethanol, 10% glycerol), and then placed into a heat block set at 95°C for 5 min. Samples from each condition were isolated from three culture wells (technical replicates; n = 3). Nine different wells of CiC-CM–treated HCFs were from three different batches (biological replicates; n = 3). Nine different wells of CiC-CM–treated HCFs were isolated and plotted as individual bars and averaged as well. Each Western blot was repeated three times for each protein of interest. Samples were loaded into 4–20% polyacrylamide gels (BioRad Cat #4561094) with precision standard ladder (Biorad Cat #1610374).
Proteins were transferred to the polyvinylidene difluoride membrane and blocked with a blocking agent made in 5% dry milk in PBST (1x PBS and 1% Tween) at pH 7.4. The blots were incubated with primary antibody overnight at 1:1000-1:5000 dilutions followed by three washes with PBST. The blots were then incubated with secondary antibody conjugated with horseradish peroxidase (Jackson Laboratories Cat #711-035-152) at 1:5000-1:10000 dilutions for 2 h followed by three washes with PBST. The blots were then visualized under chemiluminescence in an ECL imager (GE Life Sciences) using the Clarity Western ECL Substrate kit (BioRad Cat #170-5061). Primary antibodies were desmin (rabbit) (Cat #5332P; Cell Signaling), peroxisome (Abcam Cat #ab14041), pro-collagen 1A1 (Sigma Cat #ABT257), collagen III (Abcam Cat #ab6310), alpha smooth muscle actin (Sigma Aldrich Cat #A5228), fibronectin EDA (Adipogen Sciences Cat #AG-20B-6001PF-C100), LOX-PP (Novus Biologicals Cat #NBP1-30327), and vimentin (Abcam Cat #ab137321).

**Immunofluorescence**

All cells were fixed with 4% paraformaldehyde for 1 h, followed by permeabilization with 1% Triton X-100 for 30 min. Cells were then washed with 1x PBS and blocked with 2% bovine serum albumin in PBS and 1% Tween 20. Primary and secondary antibodies were diluted in block at 1:100 and 1:800, respectively, and incubated for 1 h at room temperature or overnight at 4°C. Cells were then stained with 2 μg/ml 4,6-diamidino-2-phenylindole (DAPI; Life Technologies Cat #62248) solution for 15 min. Cells were then visualized using an EVOS microscope, and the cells that stained positive were counted and plotted as a bar graph. Primary antibodies: Most the same as for Western blots (above), others: cyclin D2 (Novus Biologicals Cat #NB2-14460-25 ul), phallolidin (Life Technologies Cat #R415), vinculin (ProteinTech Cat #66305-1-1g), Rac-GTP (NewEast Biosciences Cat #26903), EdU (and PCNA) (Santa Cruz Biotech Cat #sc-56). Secondary antibodies: donkey anti-mouse Alexa Fluor 647 nm (Abcam Cat ab150107), donkey anti-rabbit Alexa Fluor 594 nm (Abcam Cat #ab150076) and donkey anti-goat Alexa Fluor 488 nm (Abcam Cat #ab150129).

**Coculture of HCFs and HCMs with the help of ibidi inserts**

Adult human cardiac fibroblasts were purchased from Sigma Aldrich (Cat #306-05A) and grown in adult cardiac fibroblast growth media (Sigma Aldrich Cat #316-50033712). Adult cardiomyocytes were purchased from Celprogen (Cat #M36044-15) and grown in human cardiomyocyte media with serum (Celprogen Cat #M36044-15S). Two chambered cell culture inserts (ibidi Cat #80209) were placed into an ECM-coated plate (Celprogen Cat #E36044-15-24Well). Equal numbers of HCFs and HCMs were seeded into both chambers of the insert for 24 h. The inserts were removed, followed by addition of 10 ng/ml TGFβ to activate HCFs. Paracrine factor cocktail (PF) or conditioned media were then added for 24–96 h depending on the experiment. Rac-1 inhibition was performed by seeding ibidi inserts with 2.0 × 10^5 HCMs overnight, followed by removing the insert and incubating the HCMs in 50 μM NCS23766 (Dwivedi et al., 2010) and CiC-primed media for 24 h.

**Identification of HCMs**

HCMs from Celprogen were analyzed using next-generation RNA sequencing to confirm their cardiac myocyte identity (Supplemental Figure S1, A and B). NoVogene performed all the next-generation sequences, and individual heat maps were generated by Celprogen utilizing GraphPad Prism 9.1.0 software. Data show that Celprogen cardiomyocytes express cardiac genes and are not cardiac fibroblasts (Supplemental Figure S1B).

**ELISAs**

Conditioned media derived from hgPSC-derived cardiac colonies at day 10–12 post–cardiac differentiation (48 and 72 h) isolated from two different patients (31 and 66 yr old [YO]; biological replicates; N = 2) from four different wells (technical replicates; n = 4). The bar graph plotted for each ELISA shows averaged values for each time point. Each time point from both patients was tested along with standards supplied with the kit for various cardioprotective paracrine factors such as NRG1 (RayBiotech Cat #ELH-NRG1b1-1), VEGFA (R&D Systems Cat #DEVE00), angiopoietin-1 (RayBiotech Cat #ELH-Angiopoietin1-1), HGF (Abcam Cat #ab100534), IDO (Novus Biologicals Cat #NBP2-62765), and TGFβ (Life Technologies Cat #BMS249-4). Every ELISA experiment for each patient was repeated three times. Conditional media derived from hgPSCs (hgPSC-CM) was used as a negative control.

**Dot blot analysis**

Conditioned media derived from hgPSC-derived CiCs (CiC-CM) at day 13 of post–cardiac differentiation was collected from three different wells (technical replicates; n = 3) from two different patients (31 YO and 66 YO; biological replicates, N = 2) and added onto the blots supplied by R&D Systems that contained various angiogenic and cardioprotective proteins (angiogenesis array R&D Systems Cat #ARY007 and cytokine array R&D Systems Cat #ARY005B). As negative controls, media straight from the bottle and hgPSC-CM were also tested on this blot. Comparative analysis was done, and each dot that represented a particular protein was quantified on a bar graph. Each dot blot for each patient was repeated two times, and each dot blot has two replicates for a total n of 4.

**HCMs/HUVECs/bead migration immunofluorescence**

HCMs and HUVECs (purchased from the cell culture core at George-town University) were plated separately in one well of the two chambered ibidi inserts placed in wells of a 24-well plate. Ionic beads (Biorad Cat #143-2446) were soaked in regular media (negative control), 10× rPF cocktail, CiC-CM separately, and then plated in the other well of the ibidi insert. After 24 h, the inserts were removed and incubated for another 24 h. All wells were then fixed and stained for vWF (for HUVECs, SCBT Cat #sc-53466), desmin (for HCMs), Rac-GTP, vinculin, and phallolidin. DAPI was used to stain the nuclei of cells.

**EdU staining of CiC-CM–treated and untreated HCMs**

HCMs treated with CiC-CM were stained with EdU from a Click-iT EdU cell proliferation kit (Life Technologies Cat #C10337). Experimental procedures from the supplier were followed.

**Quantification and statistical analyses**

Quantification of Western and dot blots was done using Photoshop and ImageJ. Error bars were defined as SEM, and replicates (minimum N of 3) from each sample/condition were averaged and plotted for each experiment. Comparisons were made between these groups: untreated (0 h HCFs) versus TGFβ treatment and TGFβ treatment versus PF/CiC-CM treatment for Western blot assays and qPCRs. Unpaired nonparametric Student’s t tests were used because we compared only two groups at a time. A p < 0.05 or better was considered significant. P values greater than 0.05 have been stated in the figure legends.

**Schematic representation of CiC differentiation**

The flowchart was created using Biorender.com software (Figure 1J).
RESULTS

Recombinant paracrine factor cocktail significantly reduces fibrotic gene/protein levels in TGFβ-activated HCFs (aHCFs)

The rationale behind this experiment was to create an in vitro cardiac fibrosis model to help mirror the sequence of events that occur after an MI in the adult heart. To achieve this, human cardiac fibroblasts were treated with 10 ng/ml recombinant human TGFβ for 72 h to induce activation (Pan et al., 2013). Quantitative PCR (qPCR) analyses showed evidence that after 72 h of TGFβ treatment, expression levels of all cardiac fibrotic markers tested increased including alpha smooth muscle actin (ASMA) (Figure 1A), periostin (PER) (Figure 1B), and collagen I (COL I) (Figure 1C) when compared with untreated, iHCFs. Cluster of Differentiation 90 (CD90) expression, a marker for iHCFs, is lost in TGFβ-treated aHCFs (Figure 1D). It has been reported that various paracrine factors can counteract genes that mark HCF activation (Gnechi et al., 2008; Fan and Guan, 2016; Hodgkinson et al., 2016). Using this information, we formulated a unique set of paracrine factors to identify the efficacy of a paracrine factor cocktail to suppress HCF activation. We found that activating HCFs with TGFβ for 24 h, followed by culturing in a novel recombinant paracrine factor cocktail (rPF) (Table 1), resulted in decreased fibrotic gene expression after 72 h (Figure 1, A–D). These data provide evidence that activation of HCFs can be prevented or attenuated with treatment with this novel cocktail of rPFs. Furthermore, CD90 expression (a marker for iHCFs) levels lost in TGFβ-treated aHCFs were restored after culture in rPF. It has been reported that various paracrine factors can counteract genes that mark HCF activation (Gnechi et al., 2008; Fan and Guan, 2016; Hodgkinson et al., 2016). Using this information, we formulated a unique set of paracrine factors to identify the efficacy of a paracrine factor cocktail to suppress HCF activation. We found that activating HCFs with TGFβ for 24 h, followed by culturing in a novel recombinant paracrine factor cocktail (rPF) (Table 1), resulted in decreased fibrotic gene expression after 72 h (Figure 1, A–D). These data provide evidence that activation of HCFs can be prevented or attenuated with treatment with this novel cocktail of rPFs. Furthermore, CD90 expression (a marker for iHCFs) levels lost in TGFβ-treated aHCFs were restored after culture in rPF. Confirming the qPCR analyses, a significant decrease in protein expression of fibrotic markers is found in TGFβ-treated aHCFs after culturing in
media containing rPF cocktail (Figure 1, E–I). The flowchart in Figure 1J illustrates how hgPSC-CiCs (human germline pluripotent stem cell–derived cardiac inducing colonies) are acquired and differentiated from human spermatogonial stem cells (hSSCs) isolated from adult testes. Before differentiation, hgPSC colonies are expanded indefinitely in germline expansion media (GEM) as described previously (Mahapatra et al., 2018). Expanded colonies are then differentiated into CiCs.

**CiC-derived conditioned media (CiC-CM) significantly reduces fibrotic gene/protein levels in aHCFs**

Next, we tested whether the effects of CiC-CM are similar to the effects exerted by the rPFs on aHCFs. The flowchart in Figure 1J illustrates how hgPSC-CiCs are acquired and differentiated from human spermatogonial stem cells (hSSCs) isolated from adult testes. Before differentiation, hgPSC colonies are expanded indefinitely in germline expansion media (GEM) as described previously (Mahapatra et al., 2018). Expanded colonies are then differentiated into CiCs. aHCFs treated with CiC-CM show significantly decreased levels of expression of ASMA, PER, and COL I as compared with TGFβ-treated aHCFs (Figure 2, A–C). CD90 expression is also restored in CiC-CM–treated aHCFs (Figure 2D). Fibrotic protein expression levels in aHCFs compared with CiC-CM–treated aHCFs measured in Figure 2, E–J, all showed significantly lower expression of ASMA, PER, and COL I protein. To rule out apoptosis as a cause for the decline in fibrotic proteins, TUNEL assays performed on untreated HCFs, rPF–treated HCFs, and CiC-CM–treated HCFs showed virtually no cell death caused by both treatments compared with positive control (Supplemental Figure S2, A–J).

**Identifying paracrine factors secreted in CiC-CM**

The fact that CiC-CM could attenuate HCF activation suggested that CiCs must express and secrete cardioprotective paracrine factors. To identify the paracrine factors secreted by CiCs, 1.5 ml of cardiac differentiation media was added to 300–350 CiCs in separate wells from days 8–13 of culture and incubated for 24, 48, and 72 h. Subsequently, CiC-CM samples were collected at days 11–14 of cardiac differentiation. CiC-CM (50–100 µl) was then assayed by multiple enzyme-linked immunosorbent assays (ELISAs), and concentrations of paracrine factors were calculated in picograms or nanograms per milliliter and graphed. Figure 3, A–F, shows a cohort of paracrine factors including NRG-1b, TGFβ, VEGFA, ANG-1, HGF, and IGF-1 detected in CiC-CM. From these data, we observed that all paracrine factors were stably secreted from day 11 to day 14 of cardiac differentiation. The secretion levels for all paracrine factors observed at day 14 were significantly higher than levels observed in hgPSC-CM (conditional media derived from hgPSCs) and 0 h media (DMEM+20%SR only). Further analyses were carried out to calculate approximate secretion levels of each paracrine factor from a single CiC at day 14 of cardiac differentiation (Table 2). These results suggested that CiCs continuously secrete increasing levels of all six paracrine factors for multiple days. This observation led to the hypothesis (to be tested below) that CiCs can create a cardioprotective microenvironment within and around the infarcted area.

**Table 1: rPF cocktail (and also detected in CiC-CM).**

| Cardioprotective factor | Function | References |
|------------------------|----------|------------|
| 1) Angiopoietin-1 (ANG-1/ANGPT1) | - It is responsible for interacting with myocytes and mediating cell survival, vessel stabilization, and activating anti-apoptotic pathways. - Along with VEGF, it stimulates migration and proliferation of vascular ECs into the infarct area. - ANG-1 also stabilizes blood vessels and induces vascular homeostasis in the infarct area. | Dallabrida et al., 2005; Arita et al., 2014 |
| 2) Vascular endothelial growth factor-A (VEGFA) | - Plays a role in angiogenesis, vessel stabilization, and EC migration in the infarct area and promotes myocardial regeneration. - Also helps prevent ventricular remodeling and induce neovascularization and cardiomyocyte proliferation in vivo. | Wang et al., 2018; Dallabrida et al., 2005 |
| 3) Neuregulin 1b (NRG1b) | - It is responsible for inducing survival and proliferation in cardiomyocytes via PI3K/AKT and ERK/MAPK pathways through up-regulation of Bcl-2. - It has been shown to stabilize blood vessels and vascular homeostasis through angiopoetin-1. - NRG1b also activates Src/FAK, which is critical for movement of HCMs into the model MI infarct. | Rupert and Coulombe, 2017; Kuramochi et al., 2006; Vermeulen et al., 2017; Liang et al., 2015; Arita et al., 2014 |
| 4) Transforming growth factor β (TGFβ) | - It is known to protect the heart against ischemic myocardial damage during the early phase of MI by activating the ERK that is associated with the RISK pathway. - It also protects myocytes from ischemic reperfusion injury by modulating TNFA and ROS. - During the early phase of MI, TGFβ protects the heart by inhibiting neutrophils from adhering to the endothelium, thereby reducing proinflammatory responses. - In cardiomyocytes, TGFβ is critical for maintaining ion-channel gene expression, sarcomeric kinetics, and myocyte survival through SMAD4-dependent mechanisms. | Ikeuchi et al., 2004; Euler, 2015; Umbarkar et al., 2019; Frangogiannis, 2017 |
| 5) Insulin growth factor-1 (IGF1) | - IGF-1 has been shown to be cardioprotective and possess a regenerative effect on myocardium through activation of prosurvival pathways such as AKT and induce HCM proliferation. - It also induced endogenous stem cell mobilization to the infarcted area. | Rupert and Coulombe, 2017; Mohamed et al., 2018; Khan et al., 2014 |
Dot blot assays were then utilized as a medium-throughput assay to identify a larger cohort of cardioprotective factors secreted by CiCs (Figure 3, G and I). Spot intensity was quantified (using ImageJ), and the resultant bar graph shows the differential protein amounts between 0 h CiCs and day 13 CiC-CM samples (Figure 3, H and J). Factors including thrombospondin-1 (TSP-1), tissue inhibitor of metalloproteinases (TIMP-1), serpin family E member 1 (SERPINE1), serpin family F member 1 (SERPINF1), insulin growth factor binding protein-1–3 (IGFBP 1-3), monocyte chemotactic protein-1 (MCP-1), interleukin-8 (IL-8), and pentraxin-3 (PTX-3) were all significantly increased in day 13 CiC-CM compared with 0 h. Table 3 defines known function/s of each identified protein in resolving cardiac fibrosis.

**TABLE 2:** Approximate concentration of each paracrine factor secreted by one CiC at day 14 of cardiac differentiation.

| Factor   | Concentration, pg |
|----------|-------------------|
| TGFβ     | 1.59              |
| HGF      | 0.4               |
| VEGFA    | 9.88              |
| NRG-1b   | 893.32            |
| ANG-1    | 5.83              |
| IGF-1    | 13                |
| IDO      | 167               |
FIGURE 3: ELISAs performed on CiC-CM collected at days 11–14 of cardiac differentiation. Each bar (gray, green, blue, and red) represents 24, 48, and 72 h time points where media was added from days 8–13 of cardiac differentiation. Concentration of each paracrine factor was measured in picograms or nanograms per milliliter (Y-axis) and compared with two negative controls: regular CiC media (0 h) and CM derived from hgPSCs (hgPSC-CM). Significant difference was measured between hgPSC-CM and CiC-CM from day 14. (A) NRG1b ELISA, (B) active TGFβ, (C) VEGF, (D) angiopoietin-1 (Ang-1), (E) hepatocyte growth factor (HGF), and (F) insulin growth factor-1 (IGF-1). Additional cardioprotective factors were identified using angiogenesis dot blot arrays (G) and cytokine dot blot arrays (I) in CiC-CM compared with regular CiC differentiation media (0 h). A pair of black spots signifies expression levels of each cardioprotective factor. Black boxes are positive and negative controls, white boxes are the additional factors found in both arrays, purple boxes are proteins that did not change, and red boxes are proteins that were shown in the ELISAs. (H, J) Black spots were quantified from the angiogenesis array (H) and cytokine array (J) as pixel intensity using Photoshop. Handling of replicates for ELISAs and dot blots is explained in Materials and Methods.
Function in cardiac fibrosis

| Secreted protein name | Function in cardiac fibrosis | References |
|-----------------------|-----------------------------|------------|
| Thrombospondin 1 (TSP1) | • It exerts a barrier function in the infarct border zone to limit propagation of inflammation and fibrosis into the noninfarcted myocardium.  
• It prevents long-term inflammation and excessive remodeling.  
• It inhibits proteolytic activation of MMP2/9.  
• It is also responsible for regulating collagen trafficking, processing, and fibril assembly so TSP binds to collagen in ECM and normalizes the matrix thereby preventing myofibroblast differentiation.  
• TSP-1 has been shown to bind to pro-LOX thereby preventing its activation by BMP-1. | Murphy-Ullrich, 2019; Chistiakov et al., 2017; Stenina-Adognravi, 2014; Rosini et al., 2018 |
| Serpin E1/PAI-1 | • It prevents ECM degradation by blocking MMP activation and helps prevent cardiac rupture.  
• PAI-1–deficient mice have shown elevated collagen I synthesis and increased myofibroblast population. | Ghosh et al., 2013; Iwaki et al., 2012 |
| Serpin F1/PEDF | • Inhibits EndMT through β-catenin dependent pathway and inhibits both activation and translocation of β-catenin in endothelial cells.  
• Reduces infarct size, suppresses vascular permeability and ECM deposition in the infarct border zone and protects vascular integrity.  
• Reduces cardiac fibrosis by reducing collagen III expression. | Zhang et al., 2017 |
| Pentraxin (PTX3) | • Plays a cardioprotective role in acute MI. PTX3-deficient mice show increased myocardial damage associated with increased neutrophil infiltration, decreased number of capillaries, and increased number of apoptotic cardiomyocytes.  
• Induces proliferation and migration of endothelial cells through VEGF.  
• It induces myocyte survival and protection against ischemia via Gα protein and JAK/STAT/MAPK pathway.  
• It also has an anti-apoptotic characteristic through ERK1/2 and BCL-2 family proteins on myocytes. | Salio et al., 2008; Ristagno et al., 2019 |
| Monocyte chemoattractant protein-1 (MCP-1) | • Induces proliferation and migration of endothelial cells through VEGF.  
• It induces myocyte survival and protection against ischemia via Gα protein and JAK/STAT/MAPK pathway.  
• It also has an anti-apoptotic characteristic through ERK1/2 and BCL-2 family proteins on myocytes. | Niyama et al., 2004; Tarzami et al., 2005; Morimoto and Takahashi, 2007 |
| Insulin growth factor binding protein 1–3 (IGFBP 1–3) | • IGFBP 1 is known to stimulate IGF-1 action, protect IGF-1 from degradation, and transport IGF-1 to peripheral tissues from the bloodstream.  
• IGFBP 2 is known to transport IGF-1 from the bloodstream to the endothelial cells (ECs) to facilitate EC migration and proliferation.  
• IGFBP 3 is the primary stabilizer of circulating IGF-1. It binds to it directly thereby prolonging its half-life. It acts as a trophic factor and exerts an autocrine effect on cardiomyocytes. IGFBP-3 also induces EC proliferation. | Kluge et al., 1997; Allard et al., 2018; Delaforetaine et al., 2004 |
| Tissue inhibitor of metalloproteinase-1 (TIMP-1) | • Prevents ECM degradation by inhibiting MMPs thus exerting matrix-preserving actions on cardiac fibroblasts.  
• TIMP1 deficiency leads to accelerated remodeling and LV dilation.  
• TIMP1 also induces proliferation of aortic smooth muscle cells through the PI3K pathway and inhibits myocyte apoptosis. | Roten et al., 2000; Arpino et al., 2015; Lindsey et al., 2015 |
| IL-6 | • It has been shown to induce cardiomyocyte proliferation in neonatal mice through cyclin D expression and STAT3.  
• It also improves ventricular contractility and inflammation in mice and prevents apoptosis. | Xu et al., 2019; Tang et al., 2018 |
| IL-8 | • It is responsible for increased pro-inflammatory reaction by inhibiting neutrophil recruitment.  
• Helps increase capillary density in the infarct area by accelerated re-endothelization in injured arteries via EC recruitment. | Tang et al., 2018; Zhao et al., 2013 |
| SDF-1 | • It plays a central role in stem cell homing, cardiomyocyte survival, proliferation, and angiogenesis.  
• SDF1α/CXCR4 signaling pathway induces acute and prolonged cardiac repair via activation of MAPK/ JAK/ STAT3 signaling.  
• SDF-1A significantly reduced apoptosis and improved overall functional recovery through activation of STAT3. | Ziff et al., 2018; Bromage et al., 2014 |
| MiF (macrophage inhibitory factor) | • Local release of MiF activates the cardioprotective AMPK pathway via CD74/CD44 MiF receptor complex to up-regulate glucose transport and utilization that provides for metabolic adaptation to ischemia.  
• In the ischemic heart, MiF reduces cardiomyocyte apoptosis and cellular oxidative stress. | Tilstam et al., 2017; Voss et al., 2019 |
| Hepatocyte growth factor (HGF) | • It is responsible for cardiomyocyte survival through P13/AKT and p38 MAPK pathways.  
• It is anti-fibrotic as it weakens TGFβ-SMAD signaling in HCFs and induces myofibroblast death. | Gallo et al., 2014 |
| Indoleamine-2,3-dioxygenase (IDO) | • It is an enzyme that catalyzes the degradation of tryptophan, an essential amino acid, along the kynurenine metabolic pathway. Immunosuppression is a result of depletion of tryptophan.  
• IDO helps increase immunosuppression by inhibiting T-cell proliferation, cytokine production, and NK proliferation and mediates T-cell apoptosis. | Talman et al., 2016; Frangogiannis, 2015; Gnecchi et al., 2008; Hodgkinson et al., 2016 |

TABLE 3: Additional PFs detected in both dot blots and ELISAs.
Recombinant paracrine factor cocktail and CiC-CM decreases Col I/III, PER, and ASMA expression in HCFs in a myocardial wound model

The data found in Figure 1 identified the effects of an rPF cocktail on statically grown HCFs. Although informative, those data did not tell us how a rPF cocktail could affect HCFs in an MI setting. As a result, we tested the effects of rPF cocktail on COL I expression within migrating HCFs. Culturing HCFs in ibidi inserts in wells coated with a cardiac-specific ECM was used to mimic the formation of a myocardial wound model post-clearance of dead cardiomyocytes. After plating equal amounts of iHCFs in both chambers, the inserts were removed after 24 h. The space coated with fibronectin/ECM components is analogous to the formation of a 500 µm myocardial wound model area. TGFβ was then added to all wells, and cells were cultured for 48 h. Another set of wells were treated with rPF cocktail and cultured for 48 h. Both sets of wells are fixed and stained for COL I and vimentin. A significant difference is observed and measured for COL/vimentin between TGFβ-treated and rPF-treated HCFs within myocardial wound model (Figure 4, A and B).

In another set of experiments, three sets of cells were tested. Set 1: Untreated iHCFs; Set 2: 72 h TGFβ-treated aHCFs; Set 3: 72 h TGFβ/CiC-CM–treated HCFs. All three sets were fixed and stained for COL I and vimentin (Figure 4, C and D). Again, marked differences were observed between the three sets of cells. The data provide evidence that there is a decrease in expression of COL I and PER in 72 h TGFβ/CiC-CM HCFs compared with 72 h TGFβ aHCFs. Vimentin staining served as a control for HCFs (Figure 4, C’ and D’).

CiC-CM effects on migration of HCFs and HCMs into the myocardial wound model

On the basis of the HCF activation data, we next wanted to identify the effects of HCF activation on HCM infiltration into an MI model. This result would be important because it would identify an in vitro model for a myocardial scar. To test this idea, equal numbers of HCFs and HCMs were cocultured in both chambers of ibidi inserts on ECM. Three sets of cells in a 24-well dish were tested. Set 1: TGFβ-treated coculture; Set 2: TGFβ+rPF–treated coculture; Set 3: TGFβ+CiC-CM added to coculture. After 24 h, the inserts were removed from all the wells. Forty-eight hours later, all the wells were fixed and stained with vimentin and desmin. In the 48 h TGFβ-treated coculture, similar numbers of vimentin (green) HCFs migrated into the myocardium compared with rPF- and CiC-CM–treated coculture (Figure 5A). However, a greater number of desmin (red) HCMs migrated into the area in both rPF- and CiC-CM–treated coculture when compared with TGFβ-treated coculture. To quantify HCM/HCF distribution, desmin (red) and vimentin (green) cells were counted in the myocardial wound model (500 µm) and a deeper 200 µm area and plotted as ratios (HCMs:HCFs) from all three treatments (Figure 5, B and C). Normalizing the number of HCFs to 1, it was found that in both rPF- and CiC-CM treatments, significantly more HCMs migrated deep into the infarct (200 µm area). To confirm their cardiac myocyte identity, HCMs express cardiac genes and not cardiac fibroblast genes as shown in the RNA sequencing data in Supplemental Figure S1B.

| Gene name                              | Forward and reverse sequences | Product size, base pairs |
|----------------------------------------|------------------------------|--------------------------|
| Alpha smooth muscle actin (ASMA/ACTA2) | F-GTGCTGTCTCTCTATGCCT R-AATCTCAAGCGTCAAGCTGA | 210                       |
| Collagen type I (COL I)                | F-AATGAAGGACACAGAGGTT R-CACAGTAGACCACTATT | 200                       |
| Periostin (PER/POSTN)                  | F-ACGGTGCGATTCACATTATCC R-GAAGAGCATTTGTGTCCT | 234                       |
| Cluster of Differentiation 90 (CD90)   | F-CTTCCCGCCTTCACACGCAA R-ATCTCTAGCAGTCAACTT | 267                       |
| Cyclin dependent kinase 1 (CDK-1)      | F-ACAAAATCTACAGGTAAAGTTG R-TGCTACTGACCAGGAGGATA | 231                       |
| Cyclin B                               | F-AACACTGGAAGAGCAAG R-ATAACATGCGTACGACC | 225                       |
| Cyclin D                               | F-GGTCTCTAAGTAGAGGAGGAGA R-TTGCCCGATGATGATGTCCT | 214                       |
| Myosin light chain 2A (MLC2A)          | F-ACTTCAAGAGCGCTCAGC R-AACCTACCTTGTCCACCAC | 274                       |
| Desmin (DES)                           | F-CCATCGGGCTAAGACATTT R-GCCATGATGATGATGACCC | 256                       |
| Myosin heavy chain 7 (MYH7)            | F-ACACACTTGGAGTCGCCCCAG R-CCCGAGTAGGGATGACCC | 383                       |
| Dead end gene 1 (DND 1)                | F-AAACGCGGATTGTAGCTGTTG R-CCCGATGACACCCCTGACC | 180                       |
| Cyclin dependent kinase inhibitor 2C   | F-AAAAATGGGGCGGCTTTTC TGCAGTACAGTACGACC | 270                       |
| Cyclin dependent kinase inhibitor 2D   | F-CGCGAGGAGAGGGGA R-GGTTGTCAGGAAAATCCAGTG | 301                       |

TABLE 4: Primer sequences for genes tested.
Based on the data in Figure 5, it was surmised that cells could enter the MI model by cell migration, cell division, or both. To analyze the possibility that CiC-CM could up-regulate cell cycle components within HCMs, we plated HCMs into ibidi inserts on ECM, followed by incubation in CiC-CM. After 48 h, they were fixed and stained for proliferating cell nuclear antigen (PCNA) (Supplemental Figure S3, A–D). PCNA staining identified ∼22% ± 2.5% of PCNA (+) nuclei in CiC-CM treatment and 20% ± 2.0% of PCNA (+) nuclei in rPF treatment. Both treatments were significantly higher than the ∼4% ± 0.5% of PCNA (+) nuclei observed in untreated HCMs (Supplemental Figure S3D). Cell cycle activity was also observed with a different proliferation marker Ki67. CiC-CM treatment resulted in significantly higher numbers of Ki67 (+) nuclei compared with iHCMs (Supplemental Figure S3, E–H). EdU staining on CiC-CM–treated HCMs compared with untreated HCMs also confirmed PCNA and Ki67 results (Figure 6).

Adult human cardiomyocytes peripheral to a recently occurring MI have been shown in vivo to reenter the cell cycle by activation of nuclear cyclin D2 (Mohamed et al., 2018). As a result, a logical experiment is to test whether CiC-CM could induce cyclin D2 expression in HCMs. Using the same experimental setup as in Figure 5, we observed significantly more cyclin D2 (+) HCMs within the myocardial wound model after treatment with CiC-CM for 24 h compared with untreated HCMs (Figure 6, E–K). These data suggest that the paracrine factors secreted from CiCs can induce HCMs to reactivate cell cycle genes.

In addition to cyclin D2, recent work done in Srivastava’s group (Mohamed et al., 2018) provided evidence that four cell cycle regulators (cyclin D1, cyclin B1, CDK1, and CDK4) could in combination induce cell division in postmitotic human, adult cardiomyocytes when added exogenously. This finding led us to two hypotheses: 1) CiC-CM possesses the ability to induce expression of some or all of these cell cycle regulators in cultured HCMs, leading to repopulation of the...
CiCs promote cardioprotection

CiCs promote cardioprotection by affecting cell cycle progression. Upon removal of CiC-CM, HCMs revert or exit the cell cycle once they have repopulated the myocardial wound model. Figure 6L shows significant increases in cyclin D1, which is a marker for cell cycle reentry. The increase in cyclin D1 is evident in both the 500 µm area (middle left panel) and the 200 µm area (bottom right). The increase is more pronounced in the 500 µm area (middle left panel) compared to the 200 µm area (bottom right).

**Figure 5:** HCM and HCF interactions were analyzed in response to rPF cocktail and CiC-CM. (A) Equal amounts of HCFs and HCMs were plated in ibidi inserts in wells of a 24-well plate. After removal of inserts, a set of wells (top panel) were treated with TGFβ; a set of wells (middle panel) were treated with TGFβ followed by recombinant paracrine factor cocktail (rPF), and a set of wells (last panel) were treated with TGFβ followed by CiC-CM. Wells were then fixed and stained with antibodies against vimentin (green) and desmin (red). White lines outline the 500 µm area (myocardial wound model) that was created after inserts were removed. Another area was marked with black lines (left panel), which was the 200 µm area. Top panel: TGFβ-treated coculture; more vimentin (+) HCFs (green) and fewer desmin (red) (+) HCMs migrated into the 500 µm area (left), and these HCFs migrated deeper into the myocardial wound model (higher magnification of 200 µm area, top right panel). Middle panel: rPF-treated coculture: presence of vimentin (+) HCFs; however, more desmin (+) HCMs that migrated into the 500 µm area (middle left panel) and 200 µm area (higher magnification, middle right panel). Bottom panel: CiC-CM–treated coculture: presence of vimentin + HCFs, but increased number of desmin + HCMs in the 500 µm area and deeper into the myocardial wound model (200 µm area) (bottom right). Desmin (+) HCMs and vimentin (+) HCFs were counted from the 500 µm area (A–C) and 200 µm area (C) from all treatments and plotted as a ratio. N = 5 for the experiment represented in this figure.

We next delved further into HCM migration potentials in response to CiC-CM. To do so, equal amounts of HCMs were plated in ibidi inserts on ECM for 24 h. After the inserts were removed, HCMs were treated with and without CiC-CM for 24 h; the HCMs were then fixed and stained for vinculin and rhodamine phalloidin (Figure 7, A–F). Although we found that some untreated HCMs could move into the myocardial wound model and that they stained positive for both vinculin and phalloidin at leading and trailing edges (Figure 7, A–C), significantly more HCMs were found in the wound area after treatment with CiC-CM. All cardiomyocytes were positive for vinculin/phalloidin at leading and trailing edges (Figure 7, D–F, quantified in G). We then used an antibody to active Rac1 (anti–GTP-Rac1) to identify a signaling mechanism for actin dynamics. HCMs treated for 24 h with CiC-CM exhibited active Rac1 expression around the leading edges as they moved into the myocardial wound model area (Figure 7, H and I). Four different experiments (N = 4) identified significantly more GTP-Rac1–positive HCMs deep in the myocardial wound model (within 200 µm area) after CiC-CM treatment as compared with untreated HCMs (Figure 7J). To further ensure that Rac1 was the signaling mechanism for HCM movement, NCS23766 was used to specifically inhibit Rac1 signaling (Dwivedi et al., 2016). Figure 7, K and L, shows HCM migration after only 16 h in CiC-CM. GTP-Rac1 was detected at the leading and trailing edges of virtually all HCMs within the wound area; however, cotreating HCMs with 50 µM NCS23766 and CiC-CM resulted in the virtual complete suppression of HCM movement into the wound even after 24 h of CiC-CM treatment (Figure 7, M and N). As a result, the data suggest that CiC–CM–treated HCMs migrate into the myocardial wound model through activation of Rac1.

We then devised a set of experiments to confirm the ability of CiCs to chemically "attract" HCMs. Specifically, HCMs were plated in one chamber of the ibidi insert. Twenty-four hours later ionic beads were soaked for 1 h in either rPF cocktail (rPF), CiC-CM, or control DMEM+20%SR (Figure 8, A–I). The beads were then added to the other chamber. After incubation for 12–24 h, we observed a marked number of desmin (+) HCMs surrounding each bead in rPF treatment (Figure 8, A–C); however, beads soaked in CiC-CM resulted in virtually every bead surrounded by desmin (+) HCMs (Figure 8, D–F). As a negative control, beads soaked in regular DMEM+20% SR media for 1 h resulted in few HCMs attached to the beads (Figure 8, G–I). Quantifying HCMs/bead for each treatment revealed significantly more HCMs attached to CiC-CM– and rPF–soaked beads compared with control DMEM+20%SR–soaked beads (Figure 8J). To show that these soaked ionic beads have the capability to secrete factors from our rPF or factors from CiC-CM causing HCMs to migrate toward them, we performed a proof of point ELISA experiment (Figure 8K). In this experiment, beads were soaked in 100 ng/ml VEGF for 1 h, followed by two washes in phosphate-buffered saline (PBS), and then tested for secretion. After 15 min, the third PBS wash results in beads secreting 18.52 pg/ml VEGF into the myocardial wound model; 2) Upon removal of CiC-CM, HCMs will revert or exit the cell cycle once they have repopulated the myocardial wound model. Figure 6L shows significant increases in cyclin D1, cyclin B1, and CDK-1 gene expression in HCMs after 24 h of CiC-CM when compared with untreated HCMs. Upon removal of CiC-CM from cultured HCMs (24 h CM, 24 h no CM), gene expression levels of all three cell cycle regulators decrease to levels similar to those of untreated HCMs. Taken together, these data suggest that CiC-CM not only induces expression and nuclear translocation of cyclin D2 but also reversibly increases gene expression levels of cyclin D1, cyclin B1, and CDK-1 in HCMs, resulting in reentry into the cell cycle and contributing to repopulation of the myocardial wound model.
PBS. This amount doubled to ~34.57 pg/ml after 2 h (Figure 8K). Based on these data, the ionic bead model system provides good evidence that CiCs themselves could be used to attract HCMs into the myocardial wound model.

Next, we tested the mechanism of our CIC-like model system to attract HCMs into the myocardial wound model. Repeating the experiment setup from Figure 8, A−I, inserts were removed and HCMs and beads were incubated for 12 h (as opposed to 24 h previously; Figure 8, L−O). HCMs and soaked beads were fixed and probed for vinculin and actin. Few HCMs were observed migrating toward the control beads (Figure 8, L′ and M′); however, significantly more were observed physically attached and/or migrating toward the CiC-CM beads (Figure 8, N′ and O′). Numerous focal adhesion sites were seen within HCMs near to CiC-CM–soaked beads as compared with HCMs near control media-soaked beads. Actin fibers are also observed in the leading edge of HCMs that are near CiC-CM–soaked beads in Figure 8, N and O. HCMs with control beads were positive for vinculin; however, its staining pattern in relation to beads was much more randomly localized. Combining the data within all of Figure 8, it can be concluded that CiCs secrete factors that can attract surrounding HCMs into a myocardial wound model.

To take this a step further, we used the same model system and experimental setup to test whether CiC-CM could also attract HUVEC migration into the myocardial wound model. Supplemental Figure S4 shows that CiC-CM and rPF cocktail–soaked beads attract more vWF (+) HUVECs compared with control media-soaked beads (Supplemental Figure S4, E and F).

**CiCs prevent collagen cross-linking (scar formation) by reducing LOXL2 expression using qRT-PCR and Western blot**

Excessive collagen deposition and hyper cross-linking of collagen fibers is a process conditioned by LOX enzymes LOX and LOXL2. This process leads to ventricular stiffness, dysfunction, and HF. We first tested the effect of CiC-CM on reducing LOX and LOXL2 RNA expression. HCMs were cultured in CiC-CM or control DMEM for 24 h, then RNA was extracted and qRT-PCR was performed to determine the expression levels of CDK-1, cyclin D1, and cyclin B1. The results showed a significant decrease in LOXL2 expression in HCMs treated with CiC-CM compared to control DMEM. Furthermore, Western blot analysis confirmed the reduction in LOXL2 protein levels in HCMs treated with CiC-CM.

---

**FIGURE 6:** HCM and HCF cell cycle activities were analyzed in response to rPF cocktail or CiC-CM. (A−C) HCMs show increased EdU labeling when cultured in CiC-CM. (A) EdU labeling in HCMs after 30 min of culture. Arrows point to a few representative positive nuclei (top left panel). DAPI and Edu merge confirms nuclear staining (A, top middle) while phase contrast image (A, top right) shows healthy cells. (B, C) EdU staining in HCMs first incubated in CiC-CM from a 31 YO (B) or a 66 YO (C) individual. Both show increased EdU staining (arrows), which was quantified and graphed (D). There are significantly more EdU-positive nuclei in CiC-CM–treated HCMs. (E−K) The cyclin D2+ nuclei from F−K were counted from the 500 µm region for both treatments. Cyclin D2 was analyzed in HCMs moving into a myocardial wound model using immunofluorescence after culturing for 24 h in CiC-CM or control DMEM. (F−H) Few cyclin D2−positive cells (red) are present after 24 h within the 500 µm region in control DMEM treatment. (I−K) Twenty-four hours after CiC-CM treatment, Cyclin D2−positive cells are markedly more observable. (L) qPCR analyses were done on untreated HCMs, 24 h CiC-CM HCMs, and 24 h CiC-CM treatment, Cyclin D2−positive cells are markedly more observable. The graph in E shows that the number of cyclinD2+ nuclei was significantly higher in CiC-CM treatment than control DMEM treatment. (L) qPCR analyses were done on untreated HCMs, 24 h CiC-CM HCMs, and 24 h CiC-CM 24 h no CiC-CM HCMs probed for CDK-1 (blue), cyclin D1 (orange), and cyclin B1 (gray) genes. Gene expression levels were plotted relative to GAPDH, and expression levels seen in untreated HCMs were normalized to 1. N = 3 for the experiment represented in this figure.
CiCs promote cardioprotection by quantitative reverse transcription-Polymerase chain reaction (qRT-PCR). Figure 9A shows that after 72 h TGFβ treatment, LOX and LOXL2 gene expression in aHCFs significantly increased when compared with that in resident iHCFs (0 h). In contrast, 72 h CiC-CM–treated HCFs results in a significant decrease in LOX and LOXL2 expression when compared with 72 h TGFβ-treated aHCFs.

FIGURE 7: Equal amounts of HCMs were plated in ibidi inserts placed in wells of a 24-well dish. After removal of ibidi inserts, wells were treated with two different CiC-CM (CM #60 and CM #35) for 24 h. Consequently, treated and untreated wells were fixed and stained for antibodies against rhodamine phalloidin (red) and vinculin (green). (A) Vinculin staining of untreated HCMs. (B) Rhodamine-phalloidin staining of untreated HCMs. (C) Merged (vinculin/rhodamine) staining of untreated HCMs. Fewer rhodamine/vinculin (+) HCMs migrated in the 500 µm area in A–C. (D) Vinculin staining of CiC-CM (CM #60)-treated HCMs. (E) Rhodamine-phalloidin staining of CiC-CM (CM #60)-treated HCMs. (F) Merged staining of CiC-CM (CM #60)-treated HCMs. Increased vinculin/rhodamine staining on the leading edges of HCMs that migrated into the 500 µm area compared with untreated HCMs. (G) Vinculin (+) HCMs found in the 500 µm area were counted in all three treatments, plotted as a bar graph, and averaged. Statistical analyses showed that there was a significant increase in migration of vinculin (+) HCMs in both CiC-CM treatments compared with untreated. (H, I) CiC-CM–treated HCMs were stained with an antibody against Rac1 GTP. Several HCMs migrating deeper into the myocardial wound model (200 µm) expressed Rac1 GTP on the leading edges (arrowheads). (J) GTP Rac1-positive HCMs were counted in the 200 µm area in untreated, CiC-CM (CM 31 YO)-, and CiC-CM (CM 60 YO)-treated samples, plotted as a bar graph, and averaged. Statistical analyses showed that there was a significant increase in GTP Rac1 (+) HCMs in both CiC-CM treatments compared with untreated. (K–N) Low (K) and high (L) magnification of HCMs show Rac1-GTP staining (green) cells primarily moving into the wound after only 12 h of CiC-CM treatment. Arrows point to leading/trailing edges of cells. (M, N) Low and high magnification of HCMs treated with CiC-CM and the Rac1 inhibitor 50 µM NCS23766. Few cells move into the wound even after 24 h of CiC-CM treatment. N = 3 for the experiment represented in this figure.
FIGURE 8: CiC-CM was tested for its ability to attract HCMs using ionic beads. HCMs were grown in an ibidi insert to localize cells to a single region of a 24-well dish. Ionic beads were then placed in the opposite well of the ibidi insert. (A–C) Beads that were soaked for 1 h in PBS + all five paracrine factors, followed by three washes in PBS, revealed HCMs surrounding each bead (arrowheads) after 24 h of culture. The focal plane is roughly on the equator of the beads. (D–F) Beads were soaked in CiC-CM for 1 h, followed by 24 h of culture with HCMs. Virtually every bead was surrounded by HCMs (arrowheads). (G–I) Beads (arrows) soaked in DMEM (+20% serum replacement) showed virtually no HCMs attached to them. (J) Graph shows the average number of HCMs/bead measured roughly around the bead equator. Significantly more HCMs were attached to CiC-CM–treated beads compared with DMEM (20%SR) only. (K) Proof of point ELISA experiment shows that the beads soaked in VEGF for 1 h secrete that paracrine factor into the medium in picogram amounts after 15 min. After 2 h, ELISA showed that the amount of VEGF detected in the medium triples. (L–O) Directional movement of HCMs was tested using CiC-CM–soaked ionic beads. HCMs were grown in an ibidi insert to localize cells to a single region of a 24-well dish. Twenty-four hours later, CiC-CM–soaked ionic beads were placed in the opposite chamber of the ibidi insert, followed by removal of the insert. Beads were cultured in DMEM (20% SR) control medium (L, M) or CiC-CM (N, O) for 24 h and then probed for vinculin (green) and rhodamine phalloidin (red). HCMs were observed near control beads, but few were observed aligned with or physically attached to them. However, HCMs cultured with beads soaked in CiC-CM (N, O) clearly showed HCMs trekking toward or near beads. Insets show vinculin-positive puncta (arrows) representing focal adhesion contact sites. The focal plane for this experiment was toward the bottom of the beads using an inverted TEVO microscope. This focal plane shows the attachment of HCMs to the dish and to the bottom of the beads. Actin fibers were also observed at the leading edge of HCMs in the direction of the CiC-CM–soaked beads (C', D'). N = 3 for the experiment represented in this figure.
Confirming our qRT-PCR data, both LOX pro-enzyme (~50 kDa) and mature form (Lox pro peptide LOX-PP) (~35 kDa) protein expression increases 24, 48, and 72 h after TGFβ treatment. Again, in contrast, both proteins were suppressed in at least nine different samples of CIC-CM–treated HCFs. (Figure 9B). We next wanted to determine LOX-PP localization and collagen fibril assembly and physiology. Untreated HCFs showed a modest network of COL I fibers and LOX-PP associated with Col 1 fibers within cells. Whereas 72 h TGFβ-treated HCFs exhibited increased COL I fibers and elevated Lox-PP expression. CIC-CM–treated HCFs exhibited markedly reduced LOX-PP expression and were associated with a very loose network of Coll 1 fibers (Figure 9, C–E). Taken together these data show that CIC-CM possesses the ability to prevent "excessive" collagen fiber deposition and cross-linking by decreasing both PRO-LOX and active LOX expression in TGFβ-treated aHCFs.

### CiC-CM effect on short-term signal transducers and activators of transcription (STAT3) activation in HCMs

During heart development, Stat3 expression increases during late cardiac differentiation and is necessary for cardiomyocyte differentiation and development. STAT3 has also been shown to be

---

**FIGURE 9:** Culturing HCFs in TGFβ results in progressive increase in expression of LOX and LoxPP, which is suppressed by CiC-CM. (A) Western blot provides evidence of both LOX proenzyme and mature enzyme in response to TGFβ. In contrast, culturing in TGFβ followed by CiC-CM for 72 h results in suppression of both LOX ProE and LoxPP. N = 9 different CiC-CMs. (B–D) Images illustrate LoxPP expression, localization, and resultant physiology of collagen fibers. (B) Untreated HCFs show collagen 1 expression within cells and a modest network of collagen 1 fibers (arrows); B (middle and right) shows modest staining of LoxPP (arrowheads) associated with collagen 1 fibers. (C) HCFs cultured for 72 h in TGFβ resulted in an increase in collagen 1 fibers (left image) when compared with B and clear, elevated staining for LoxPP (arrowheads in middle and right images). Arrowheads within white boxes (D′–D′′) in the merged image identify the close association of LoxPP and collagen 1 fibers. (D) HCFs treated with TGFβ for 24 h followed by TGFβ and CiC-CM for 72 h resulted in a loose, anastomosing network of collagen 1 fibers (left image). (D) (middle and right images) LoxPP expression is markedly decreased but not completely absent (arrowheads). LoxPP is observed associated with fibers (right merged image) but clearly at lower levels when compared with C. N = 3 for C–E.
a key cardioprotective player that is activated under acute and chronic stress conditions. Short-term activation of STAT3 in myocytes is very important as genes like VEGF, B-cell lymphoma extra-large (Bcl-XL) are turned on during the subacute phase of MI (Enomoto et al., 2015; Zouein et al., 2015). However, long-term activation of STAT3 is detrimental for the heart because it can lead to aberrant inflammation, which can result in ventricular dysfunction and potential activation of embryonic genes not conducive to adult cardiac physiology (Foshay and Gallicano, 2008; Enomoto et al., 2015; Harhous et al., 2019).

Untreated HCMs were compared with HCMs cultured for 72 h in CiC-CM and HCMs removed from CiC-CM and cultured in basal media (not CiC-CM) for 24 and 96 h. After fixation, HCMs in each treatment were stained for P-STAT3 and DAPI (Supplemental Figure S5, A–D). Increased expression of P-STAT3 is detected in nuclei at about 72 h post–CiC-CM treatment. This expression persists and even increased up to ∼24 after replacing the CiC-CM with normal media. However, 96 h after removing HCMs from CiC-CM, P-STAT3 expression decreases dramatically. All treatments were analyzed, counted, and plotted in Supplemental Figure S5E. Consequently, this set of data suggests that CiC-CM can briefly induce P-STAT3 in HCMs, serving as a cardioprotective event.

Effect of CiC-CM on inflammation and proinflammatory players
Prolonged inflammation during the first phase of myocardial fibrosis has been shown to extend ischemic insult, leading to detrimental conditions including chamber dilation, adverse cardiomyocyte injury, and heart failure. We wanted to identify the effects of paracrine factors found in CiC-CM on inflammation and proinflammatory proteins. Fibronectin-EDA, also known as alternatively spliced exon-encoding type III repeat extra domain A (EDA), is highly expressed in activated HCFs. After MI, EDA is known to function by activating leukocytes and causing up-regulation of proinflammatory cytokines after ischemic injury. Upon treatment of HCFs with TGFβ, a gradual up-regulation of EDA expression (24, 48, 72 h time points) was consistently observed; however, rPF-treated HCFs show a gradual down-regulation in expression (Figure 10A). EDA expression levels were quantified as band density ratios relative to untreated HCFs (Figure 10B). Interestingly, we also observed similar results when HCFs were treated with CiC-CM. We observed gradual down-regulation in EDA protein expression in 48 and 72 h CiC-CM-treated HCFs compared with TGFβ-treated aHCFs (Figure 10C). Data quantification showed significant changes in EDA expression (Figure 10D).

Another paracrine factor, IDO (indoleamine-2,3-dioxygenase), was also identified in CiC-CM. IDO is known to suppress T-cell responses to autoantigens and modulate the immune reaction post–myocardial infarction (Fan and Guan, 2016; Wang et al., 2018). ELISA in Figure 10E show that IDO secretion was 49.5 ng at day 14 from ∼300 CiCs. Taken together these data suggest that down-regulation of EDA and up-regulation of IDO can prevent prolonged proinflammation, keeping the process more tightly controlled and preventing extended ischemic insult.

DISCUSSION
Here, we show that hgPSC-derived CiCs secrete 18 cardioprotective paracrine factors not only prevent HCF activation but also help induce surrounding myocytes and endothelial cells to migrate into a model infarct. Additionally, CiCs also help preserve ECM homeostasis and modulate proinflammation. Although CiCs express crucial cardiac genes, we previously showed that they did not beat like bona fide cardiomyocytes (Mahapatra et al., 2018). However, differentiating hPGCs into a cardiac lineage enables them to integrate into the surrounding cardiac niche (Mahapatra et al., 2018). We also previously showed that thousands of CiCs could be generated using a specific expansion protocol potentially solving cell dosage issues that may be needed for next step initiatives such as clinical trial testing of CiCs (Hodgkinson et al., 2016; Yu et al., 2017; Mahapatra et al., 2018; Tompkins et al., 2018).

In the current field of paracrine–stem cell research, MSCs have been the most popular stem cell type used; however, in the more successful cases, they had to be first engineered through AKT overexpression to induce secretion of cardioprotective factors such as vascular growth factor-A (VEGF) and hepatocyte growth factor (HGF) (Gnecchi et al., 2008). In contrast to MSCs, we show that hgPSC-derived CiCs do not need genetic manipulation to secrete a powerful cocktail of cardioprotective factors.

The work here focused on the cell biology, molecular biology, efficacy, and physiology of primary HCFs, HCMs, and ECs in response to a novel cocktail of paracrine factors and the paracrine factors released from CiCs. Figure 10F summarizes our data showing the model of an infarct and how injected CiCs would affect the dead area by eliciting surrounding myocyte proliferation and migration to the infarct area through secretion of cardioprotective factors.

To simulate the responses to an MI, we used well-characterized protocols similar to those of other laboratories (e.g., TGFβ protocol to activate HCFs). Incubating primary HCFs in 10 ng/ml TGFβ faithfully causes HCFs to overexpress hallmark fibrotic genes. This treatment models HCF activation after ischemic insult where inactive TGFβ bound to latent associated protein (LAP) present in the adult myocardium becomes active upon its release from LAP. Although TGFβ facilitates myofibroblast conversion (i.e., iHCF to aHCF) through the TGFβ-SMAD2/3/4 pathway, it also activates noncanonical pathways including p38 MAPK, RhoGTPase and TAK-1 (Pasumarthi et al., 2005). The canonical TGFβ/TGFβR I/II/SMAD pathway results in nuclear translocation of SMAD4 followed by transcription of ECM genes such as ASMA, COL I, III, PER, endothelin-1 (ET-1), and connective tissue growth factor (CTGF) (Pasumarthi et al., 2005). TGFβ-induced amplification of fibrillar and nonfibrillar collagen causes scar formation and contributes to ECM remodeling, which ultimately leads to heart failure (Pasumarthi et al., 2005). However, in our work, TGFβ seems to also play a cardioprotective role when secreted in combination with other paracrine factors from CiCs. Because latent forms of TGFβ are stored in the myocardium; it is possible that low levels of TGFβ activity are crucial to preserve overall cardiac function (Hanna and Frangogiannis, 2019). TGFβ has been shown to be anti-inflammatory as it promotes M2 polarization and suppresses proinflammatory cytokines (Hanna and Frangogiannis, 2019; López et al., 2021). We present evidence showing that although CiCs secrete TGFβ, the 17 other paracrine factors counteract the detrimental effects of elevated TGFβ alone on HCFs, that is, attenuating their activation thereby tipping the balance from fibrosis toward repair.

Role of CiCs in preservation of ECM homeostasis
Secreted proteins found in the CiC-CM including TSP-1, TIMP1, SERPINP1, and SERPINE1 are responsible for preventing ECM breakdown. which would otherwise lead to cardiac rupture and ventricular dysfunction (Roten et al., 2000; Zhang et al., 2017). TSP-1 interacts with procollagen and potentially helps normalize the collagen matrix by regulating collagen trafficking, processing, and fibril assembly. TSP-1 that is bound to collagen in ECM not only helps preserve ECM homeostasis but also prevents myofibroblast differentiation.
From Murphy-Ullrich (2019), it was found that TSP-1 binds to lysyl oxidase (an enzyme that cross-links mature collagen to form fibrils that contribute to scar formation) and prevents its activation. Similarly, SERPINE1 prevents ECM degradation by inhibiting matrix metalloprotein (MMP) activation, whereas SERPINF1 reduces ECM deposition in the infarct border zone (Ghosh et al., 2013;
Zhang et al., 2017). qPCR analyses confirm that CiC-CM decreased both LOX (lysyl oxidase) and LOXL2 (lysyl oxidase 2) gene expression in CiC-CM–treated HCFs. This decrease could be due to expression of TSP-1 in CiC-CM as observed in the dot blot analyses. There is evidence of increased LOX in both scar area and the border zone leading to increased cardiac stiffness derived from “excessive” collagen cross-linking (Rodriguez and Martinez-Gonzalez, 2019; Laczko and Csiszar, 2020). LOX and LOXL2 contribute to cardiac ECM cross-linking, leading to increased deposition of insoluble collagen in the scar area and border zone (Ghosh et al., 2013; Rodriguez and Martinez-Gonzalez, 2019). Western blot data shown in Figure 9B also confirm that CiC-CM markedly reduces LOX proenzyme and LOX-PP, which is the mature form of LOX enzyme, similar to levels observed in untreated HCFs. Furthermore, CiC-CM induces a very loose collagen fiber assembly as opposed to the collagen network seen in aHCFs. From these observations, we conclude that CiC-CM would help inhibit collagen buildup/cross-linking caused by HCF activation and potentially help restore the balance between soluble and insoluble collagen, which is important for restoring ECM homeostasis.

Role of CICs in endothelial cell migration and cardiomyocyte proliferation/migration

Early on, we surmised that the CiC-CM would not only affect HCFs but also induce effects in the other cell types within the heart such as endothelial cells and HCMs. "Healing" an infarcted region of the heart would require endothelial cells and cardiomyocytes to migrate and perhaps proliferate into the infarct. In our dot blot data, we observed increased expression of SERPINE1, which has been shown to promote endothelial cell migration (Iwaki et al., 2012; Ghosh et al., 2013). Other highly secreted proteins such as MCP-1, PTX3, SERPINF1, interleukin-6 (IL-6), and IL-8 have all been shown to induce reendothelialization of injured arteries via EC recruitment and suppression of capillary permeability (Enomoto et al., 2015). Concrete evidence that VEGF is expressed and secreted by CiCs helped to solidify the ability of CiCs to recruit ECs (Niiyama et al., 2004; Tarzami et al., 2005). IL6 and IL-8 are known to induce myocyte survival through the Janus kinase/signal transducers and activators of transcription (JAK/STAT/MAPK) pathway acting as an anti-apoptotic protein on myocytes (Xu et al., 2019). Angiopoietin-1 (ANG-1) detected in the CiC-CM is a very essential cardioprotective factor that is responsible for interacting with myocytes through integrins to mediate cell survival and angiogenesis and activate anti-apoptotic signaling pathways (Mohamed et al., 2018). It has been reported that Ang-1 enhances migration of vascular ECs with the help of VEGFA and proliferation of subepicardial immature ECs (Dallabrida et al., 2005). Similarly, HGF, also found in the CiC-CM, has been shown to promote cardiomyocyte survival, preventing them from undergoing apoptosis via activation of PI3/AKT and p38 MAPK pathways (Wang et al., 2018). Additionally, HGF is anti-fibrotic as it weakens TGFβ-SMAD signaling in cardiac fibroblasts (Wang et al., 2018). Along with VEGF, HGF helps endothelial cell mobilization, proliferation, and neovascularization in vivo (Wang et al., 2018).

We consistently observed HCMs not only entering in the myocardial wound model area but also moving deeper into the myocardial wound model area (200 µm). One of the important observations made in the HCFs–HCMs coculture experiments was that upon treatment with TGFβ, higher ratios of HCFs migrated into the myocardial wound model compared with HCMs. In contrast, cocultures treated with CiC-CM enabled both HCFs and HCMs to migrate into the myocardial wound model. We conclude that CiC-CM suppresses activation of HCFs, thereby enabling HCMs to enter the myocardial wound model area.

Another important paracrine factor detected in the CiC-CM was NRG-1b, which is essential for cardiomyocyte proliferation and survival. In addition to NRG-1b, CiCs also secrete insulin growth factor-1 (IGF-1) and stromal derived factor-1 (SDF-1). Both have been shown by independent laboratories to promote cardiac protection and/or regeneration when tested separately or in various combinations. Several papers have shown that NRG1b binds to Erb-B2 receptor tyrosine kinase 4 (ErbB4) receptors on surviving myocytes, resulting in proliferation and survival via phosphoinositide 3 kinase/AKT (PI3/AKT) and extracellular signal–regulated kinase ½ (ERK)/MAPK pathways, followed by up-regulation of anti-apoptotic gene Bcl-2 (Gallo et al., 2014). NRG1b has been shown to stabilize blood vessels and vascular homeostasis through paracrine expression of vessel stabilizing factors like Ang-1 (Arita et al., 2014). Reports demonstrate that NRG1b also activates steroid receptor coactivator/focal adhesion kinase (Src/FAK), which is critical for cell motility and focal adhesion complex (FAC) formation, suggesting that NRG1b could possibly induce myocytes to move into the model infarct. There has been recent evidence that the NRG-ErbB4 pathway controls cell mobility in addition to proliferation and survival through the PI3/AKT pathway (Rupert and Coulombe, 2017). NRG-1b has also been shown to stimulate mononuclear mature cardiomyocytes to reenter the cell cycle by inducing cytokinesis through cyclin-dependent kinase 4 (CDK4/Cyclin D), and it has shown positive results in HF animal models (Liang et al., 2015; Rupert and Coulombe, 2017).

Similarly, IGF-1 has also been shown to induce or enhance cardiomyocyte proliferation and survival. IGF-1 works individually as well as synergistically with NRG1 during heart development via the PI3/AKT pathway, and we surmise that both IGF-1 and NRG1b may be working together to induce proliferation of HCMs into the infarct area (Vermeulen et al., 2017). IGF-1 along with SDF-1 induces endogenous stem cell mobilization to the infarcted area and subsequently contributes to myocardial repair. SDF-1 has been shown to induce migration of cardiomyocytes in zebrafish hearts via SDF1A/C-X-C chemokine receptor 4b (CXCR4b) signaling (Mohamed et al., 2018). It is known to be a chemoattractant that stimulates migration of cardiomyocytes and arterial endothelial cells because both express CXCR4 receptors (Mohamed et al., 2018). From our immunofluorescence data, we show that CiC-CM increases Rac-GTP expression in the leading edges of HCMs as they move into the myocardial wound model area (Figure 8, H–J). Activation of Rac leads to assembly of vinculin (+) focal complexes distributed around the entire leading edge of the induced lamellipodium of HCMs that were treated with CiC-CM. Rac has been shown to stimulate new actin polymerization through the actin-related protein 2/3 (Arp 2/3) complex, which binds to actin nucleation factors to form a branching actin network (Nores and Hall, 1995). We believe that CM-treated HCMs migrate into the myocardial wound model through actin polymerization orchestrated by activation of Rac.

Role of CICs in cardiac fibroblast activation and cardiomyocyte cell cycle

In TGFβ-activated cocultures of HCFs and HCMs, HCFs migrate into the infarct and become activated, preventing myocytes and ECs from migrating in. However, CiC-CM almost completely reversed this phenotype. HCFs still migrated, but they were not active in the infarct and become activated, preventing myocytes and ECs from migrating in. CiC-CM-induced activation of HCFs is the primary candidate for preventing myofibroblast differentiation/activation.
It was evident that HCMs could use two processes to enter the myocardial wound model: movement and/or proliferation. From the cell cycle analyses data, Ki67 and PCNA both showed significantly more stained nuclei in CiC-CM–treated HCMs compared with untreated HCMs. We further showed that CiC-CM–treated HCMs are positive for nuclear cyclin D2, implying that CiC-CM could induce HCMs to enter the cell cycle. Cyclin D2 has been shown to become activated in HCMs in a myocardial wound model (Pasumarti et al., 2004). Our data support Pasumarti et al. (2004) and highly suggest that HCMs can be coaxed to repair an MI region partly through proliferation. Further support for this hypothesis is provided by the work shown by Srivastava et al. (Mohamed et al., 2018), who showed that exogenous expression of distinct CDKs and cyclins in adult cardiomyocytes was clearly enough to drive them into the cell cycle. Here, we show that CiC-CM endogenously turns on three of these cell cycle regulators (CDK1, cyclin D1, cyclin B1) in HCMs that are located within the myocardial wound model. More importantly, once they repopulate the area and following withdrawal of CiC-CM, those genes are down-regulated, implying that HCMs exit the cell cycle.

From the cytokine array dot blot, we also showed that IL-6 is found in the CiC-CM. IL-6 has been shown to regulate the cell cycle in cardiomyocytes through the cyclin D-STAT3 pathway (Tang et al., 2018). CiC-CM is turning on the same pathway in HCMs. During heart development, STAT3 is turned on during late cardiac differentiation and binds to promoters of T-box transcription factor 5 (Tbx-5), GATA binding factor 4 (GATA4), and NK homeobox 5 (Nkx 2.5), which are responsible for cardiomyocyte differentiation and development (Zhou et al., 2003; Foshay et al., 2005; Foshay and Galliano, 2009, Larabee et al., 2015; Hanna and Frangogiannis, 2019). STAT3 is a key cardioprotective player known to be activated under acute and chronic stress conditions. Short-term activation of STAT3 in myocytes is very important, as key target genes of STAT3 include VEGF, Bcl-XL are turned on during the subacute phase of MI (Enomoto et al., 2015). It has been shown that deletion of STAT3 during the first 11–24 d after MI results in exacerbated cardiac function, cardiac fibrosis, and up-regulation of fibrotic genes due to increased death of myocytes (Buja and Frangogiannis, 2007). However, STAT3 activation must be kept under control. Long-term activation of STAT3 in myocytes seems to be detrimental for the heart because it can lead to disproportionate inflammation, which can result in ventricular dysfunction. STAT3 has also been shown to be responsible for timely resolution of inflammation by polarizing M1 macrophages to reperative M2 macrophages (Harhous et al., 2019). Consequently, evidence suggests that it is important to have an intrinsic level of STAT3 activity present so as to control fibroblast proliferation and prevent adverse cardiac remodeling (Zouein et al., 2015). Furthermore, STAT3 can be linked to the previously mentioned cyclin D2, which is known to be a STAT3 target gene (Shabbir et al., 2010; Park et al., 2019).

We speculate that from all the paracrine factors detected in the conditioned media, NRG1β, SDF-1, and IL-6 would possibly be inducing HCMs to proliferate and migrate into the 500 µm area. Additionally, VEGFA, ANG-1, SerpinE1, MCP-1, pentraxin, SERPINF1, and IL-8 found in the conditioned media would possibly play a role in EC migration, angiogenesis, and vessel stabilization in the myocardial wound model area.

Role of CiCs in timely resolution of proinflammation

We believe that CiCs could also play a role in inducing timely resolution and containment of proinflammation in cardiac fibrosis. During the inflammation phase, necrotic cells release danger signals known as DAMPs (danger associated molecular patterns). One such DAMP, EDA (fibronectin extra domain III), binds to PRRs (pattern recognition receptors) of the immune system of surviving parenchymal cells and infiltrating leukocytes (Morimoto and Takahashi, 2007). EDA is an alternate splice form of fibronectin, which is a glycoprotein present in the ECM and produced by cardiac fibroblasts in response to injury (Morimoto and Takahashi, 2007). Once the immune system is alerted following injury, these infiltrating leukocytes clear up the dead cells from the infarct area, which leads to activation of a robust inflammation cascade. Aberrant regulation of this step causes ventricular dysfunction and adverse cardiac remodeling. From the Western blot data, we showed that TGFβ–treated HCFs express significantly higher amounts of EDA compared with untreated HCFs; however, upon treatment with CiC-CM, EDA expression decreases significantly. These data suggest that CiCs help control proinflammation by limiting macrophage migration (key players in adverse cardiac remodeling) to the infarct. EDA expression is up-regulated at days 3 and 7 post-MI (Morimoto and Takahashi, 2007). It binds to toll-like receptor 2/4 (TLR2/4) on surviving cardiac fibroblasts and activates downstream signaling pathways like mitogen activated protein kinase (MAPK) and nuclear factor kappa light chain enhancer of activated B-cells (NFκB), which in turn drives expression of a large panel of proinflammatory genes such as interleukin-1B (IL-1B), tumor necrosis factor alpha (TNFA, and interleukin-18 (IL-18) (Morimoto and Takahashi, 2007). Additionally, EDA is responsible for inducing myofibroblast differentiation and function (Morimoto and Takahashi, 2007). According to Morimoto and Takahashi (2007), EDA levels come back to normal after long-term survival, which is 28 d after MI first occurred. The fact that CiC-CM reduces EDA expression provides further evidence that CiCs could induce repair rather than scarring.

The switch from pro- to anti-inflammation is characterized by polarization of M1 macrophages (proinflammatory macrophages) to M2 (reparative/anti-inflammatory macrophages), which causes down-regulation of TNFα, IL1β with simultaneous up-regulation of IL-8, TGFβ, HGF, and TSP-1 within the infarct microenvironment. TSP-1, TGFβ, and IL-8 are found in the CiC-CM as shown in our immunoblot and ELISA data (Figure 3). TSP-1 has been shown to help limit propagation of inflammation into the noninfarcted myocardium and prevent long-term inflammation (Adognnavi, 2014; Murphy-Ullrich, 2019). IL-8 prevents neutrophil recruitment and helps polarize M1 to M2 macrophages. Additionally, TGFβ can also act as an anti-inflammatory player helping to mediate polarization of M1 to M2 macrophages in the MI zone. NRG-1 plays a role in inflammation by activating ErbB4 receptors on macrophages and down-regulating PI3/AKT signaling and phosphorylation of STAT3 in macrophages (Kuramochi et al., 2006). This further leads to a decrease in release of pro-inflammatory genes, which will ultimately suppress inflammation. NRG-1 not only decreases activity of macrophages but also decreases recruitment of new monocytes, thereby promoting timely resolution of proinflammation (Kuramochi et al., 2006).

Indoleamine 2,3 dioxygenase (IDO) is another cardioprotective factor that has been shown to modulate immune suppression and promote polarization of M1 to M2 macrophages by other laboratories (Fan and Guan, 2016; Yan et al., 2019). It is an enzyme that catalyzes the degradation of tryptophan, along the tryptophan-kynurenic metabolic pathway, and is responsible for subsiding the T-cell response to autoantigens by inducing T-cell apoptosis (Arslan et al., 2011). There has been evidence that IDO is responsible for preventing recruitment and activation of proinflammatory lymphocytes (Wang et al., 2018) and expansion of regulatory factors.
T-cells to induce allograft tolerance (Zhang et al., 2017). Our ELISA data show that CiCs secrete IDO. Immunosuppression is the result from depletion of T-lymphocytes and the accumulation of tryptophan metabolites (Arslan et al., 2011). IDO could possibly help modulate the proinflammatory reaction in addition to the others mentioned here. 

**Future directions**

To make CiCs clinically feasible, we will be testing various freezing protocols to determine whether frozen CiCs could be sent to the clinics and then be thawed and injected into the MI of a patient. Upon culturing frozen CiCs in differentiation medium, CiC-CM samples would be collected at day 14 of cardiac differentiation, subjected to ELISA and dot blot tests for quality control, and compared with CiC-CM samples from unfrozen CiCs. Furthermore, PCR analyses of freeze/thawed CiCs probing for cardiac genes and negative regulators of teratoma will also be done to ensure continued expression of these key genes.

No animal products are used in our culture protocol. Although CiCs could be used as an allogeneic cell therapy, we anticipate the patient to be on an immunosuppressive regimen for a certain period of time. One of the commonly used immunosuppressants that have shown significant successes in organ transplantation is cyclosporine (Kapturczak et al., 2004). Cyclosporine is usually administered in two doses from 8 wk to up to 3 mo after transplantation (Oh et al., 2015).

By proteome and transcriptome analyses of freeze/thawed CiCs, we have seen that cyclosporine has also been successful in stem cell transplant survival in spinal cord injury (Kapturczak et al., 2004; Oh et al., 2015). We expect that after transdifferentiation of CiCs, the patient would be administered daily doses of cyclosporine (or alternative steroid-free agents [Nakaoka et al., 2007; Arita et al., 2014]), and because CiCs express IDO, which is known to prevent T-cell activation as well, it would also help prolong transplant survival. The patient would be gradually weaned off cyclosporine, depending on the response.

In summary, the cardioprotective microenvironment set up by CiCs influences surviving myocytes and endothelial cells within the infarct as well as the surrounding myocardium to undergo myocardial repair and regeneration. In vivo, as we observe an improvement in ejection fraction and other vitals, we expect to see CiCs undergoing apoptosis and ultimately cleared from the infarct. As mentioned before, these CiCs can be frozen and thawed, which would benefit MI patients as CiCs would be sent overnight to any facility. While we highlight 18 cardioprotective factors that work together to induce myocardial repair, we plan to not only fully characterize other unknown factors secreted in the CiC-CM but also experimentally narrow down which factors are essential and test different combinations of the cardioprotective factors at different stages of myocardial fibrosis. We would also investigate the effects of CiC-CM on immune cells and overall containment of proinflammation in in vivo studies. Given the unique features of this cell type, we believe that CiCs could be an alternative cell therapy treatment for myocardial fibrosis.

**REFERENCES**

A futile cycle (2017). Editorial. A futile cycle in cell therapy. Nat Biotechnol 35, 291.

Allard J, Duan C (2018). IGF-binding proteins: why do they exist and why are there so many? Front Endocrinol (Lausanne) 9, 117.

Arita Y, Nakaoka Y, Matsunaga T, Kidoya H, Yamamizu K, Arima Y, Kataoka-Hashimoto T, Ikeoka K, Yasui T, Masaki T, et al. (2014). Myocardium-derived angiopoietin-1 is essential for coronary vein formation in the developing heart. Nat Commun 5, 4552.

Arpino V, Brock M, Gill SE (2015). The role of TIMPs in regulation of extra-cellular matrix proteolysis. Matrix Biol 44–46, 247–254.

Arslan F, Smeebs MT, Vis Riem PW, Karper JC, Quax PH, Bongartz LG, Peters JH, Hoefere IE, Doevendans PA, Pasterkamp G, De Kleijn DP (2011). Lack of fibronectin-EDA promotes survival and prevents adverse remodeling and heart function deterioration after myocardial infarction. Circ Res 108, 582–592.

Boudoulas KD, Hatzopoulos AK (2009). Cardiac repair and regeneration: the Rubik’s cube of cell therapy for heart health. Dis Model Mech 2, 344–358.

Bracco Gartner TCL, Deddens JC, Mol EA, Magin Ferrer M, van Laake LW, Bouten CVC, Khademhosseini A, Doevendans PA, Suyker WJL, Sluijter JPM (2019). Anti-fibrotic effects of cardiac progenitor cells in a 3D-model of human cardiac fibrosis. Front Cardiovasc Med 6, 52.

Bromage DI, Davidson SM, Yellon DM (2014). Stromal derived factor 1α: a chemokine that delivers a two-pronged defence of the myocardium. Pharmacol Ther 143, 305–315.

Bujak M, Frangogiannis NG (2007). The role of TGF-beta signaling in myocardial infarction and cardiac remodeling. Cardiovasc Res 74, 184–195.

Chen H, Xia R, Li Z, Zhang L, Xia C, Ai H, Yang Z, Guo Y (2016). Mesenchymal stem cells combined with HGF therapy for attenuating ischemic myocardial fibrosis: assessment using multimodal molecular imaging. Sci Rep 6, 33700.

Chistiakov DA, Melnichenko AA, Myasoedova VA, Grechko AV, Orekhov AN (2017). Thrombospondin-1: a role in cardiovascular disease. Int J Mol Sci 18, 1540.

Dallabrida SM, Ismail N, Oberle JR, Himes BE, Rupnick MA (2005). Angiopoietin-1 promotes cardiac and skeletal myocyte survival through integrins. Circ Res 96, e8–e24.

Delafontaine P, Song YH, Li Y (2004). Expression, regulation, and function of IGF-1, IGF-1R, and IGF-1 binding proteins in blood vessels. Arterioscler Thromb Vasc Biol 24, 435–444.

Dwivedi S, Pandey D, Khandoga AL, Randell R, Siess W (2010). Rac-mediated signaling plays a central role in secretion-dependent platelet aggregation in human blood stimulated by atherosclerotic plaque. J Transl Med 8, 128.

Enomoto D, Obana M, Miyakawa A, Maeda M, Nakayama H, Fujo Y (2015). Cardiac-specific ablation of the STAT3 gene in the subacute phase of myocardial infarction exacerbated cardiac remodeling. Am J Physiol Heart Circ Physiol 309, H471–H480.

Euler G (2015). Good and bad sides of TGF-β signaling in myocardial infarction. Front Physiol 6, 66.

Fan Z, Guan J (2016). Anti-fibrotic therapies to control cardiac fibrosis. Biomater Res 20, 13.

Foshay K, Rodriguez G, Hoel B, Narayan J, Gallicano GI (2005). JAK2/STAT3 directs cardiomyogenesis within murine embryonic stem cells in vitro. Stem Cells 23, 530–543.

Foshay KM, Gallicano GI (2008). Regulation of Sox2 by STAT3 initiates commitment to the neural precursor cell fate. Stem Cells Dev 17, 269–278.

Foshay KM, Gallicano GI (2009). miR-17 family miRNAs are expressed during early mammalian development and regulate stem cell differentiation. Dev Biol 326, 431–443.

Frangogiannis NG (2015). The inflammatory response in myocardial injury, repair, and remodeling. Nat Rev Cardiol 11, 255–265.

Frangogiannis NG (2017). The role of transforming growth factor (TGF)-β in the infarcted myocardium. J Thorac Dis 9(Suppl 1), S52–S63.

Galindo CL, Kasasbeh E, Murphy A, Ryzhov S, Lenihan S, Ahmad FA, Bromage DI, Davidson SM, Yellon DM (2014). Stromal derived factor 1α: a chemokine that delivers a two-pronged defence of the myocardium. Pharmacol Ther 143, 305–315.

Galerlo CL, Kasasbeh E, Murphy A, Ryzhov S, Lenihan S, Ahmad FA, Williams P, Nunnally A, Adcock J, Song Y, et al. (2014). Anti-fibrotic and anti-fibrotic effects of the neuroregulin-1β glial growth factor 2 in a large animal model of heart failure. J Am Heart Assoc 3, e00773.

Gallo S, Sala V, Gatti S, Crepaldi T (2014). HGF/Met axis in heart function and cardioprotection. Biomedicines 2, 247–262.

Ghosh AK, Murphy SB, Kishore R, Vaughan DE (2013). Global gene expression profiling in PAI-1 knockout murine heart and kidney: molecular basis of cardiac-selective fibrosis. PLoS One 8, e63825.

Gnecciu M, Zhang Z, Ni A, Dzuv VI (2008). Paracrine mechanisms in adult stem cell signaling and therapy. Circ Res 103, 1204–1219.

**MATERIALS AND METHODS**

**Request a protocol** through Bio-protocol.

**ACKNOWLEDGMENTS**

We thank the American Heart Association (AHA) for funding S.M. with a predoctoral award (Grant #19PRE34430192) and G.I.G with an Innovative Grant (Grant #18IPA34170394).
Golestaneh N, Kokkinaki M, Pant D, Jiang J, DeStefano D, Fernandez-Bueno C, Rone JD, Haddad BR, Gallicano GI, Dym M (2009). Pluripotent stem cells derived from adult human testes. Stem Cells Dev 18, 1115–1126.

Hanna A, Frangogiannis NG (2019). The role of the TGF-β superfamily in myocardial infarction. Front Cardiovasc Med 6, 140.

Harthous Z, Booz GW, Ozize M, Bidaux G, Kurdil M (2019). An update on the multifaceted roles of STAT3 in the heart. Front Cardiovasc Med 6, 150.

Hirano T, Ishihara K, Hibi M (2000). Roles of STAT3 in mediating the cell growth, differentiation and survival signals relayed through the IL-6 family of cytokine receptors. Oncogene 19, 2548–2556.

Hodgkinson CP, Bareja A, Gomez JA, Dauz VJ (2016). Emerging concepts in pericardic mechanisms in regenerative cardiovascular medicine and biology. Circ Res 120, 104–117.

Ikeuchi M, Tsutsui H, Shiomi T, Matsusaka H, Matsumi S, Wen J, Kubota T, Takeshita A (2004). Inhibition of TGF-beta signaling exacerbates early cardiac dysfunction but prevents late remodeling after infarction. Cardiovasc Res 64, 526–535.

Iwaki T, Urao T, Umemura K (2012). PAL-1, progress in understanding the clinical problem and its etiology. Br J Haematol 157, 291–298.

Kapturczak MH, Meier-Kriesche HU, Kaplan B (2004). Pharmacology of calcinurin antagonists. Transplant Proc 36(2 Suppl), 255–325.

Khan RS, Martinez MD, Sy JC, Pendergrass KD, Che PL, Brown ME, Cabigas EB, Dasari M, Murthy N, Davis ME (2014). Targeting extracellular DNA to deliver IGF-1 to the injured heart. Sci Rep 4, 4257.

Kluge A, Zimmerman R, Weihrach D, Mohri M, Sack S, Schaper J, Schaper W (1997). Coordinate expression of the insulin-like growth factor system after microembolization in porcine heart. Cardiovasc Res 33, 324–331.

Kuramochi Y, Guo X, Sawyer DB (2006). Neuregulin activates erbB2-dependant src/FAK signaling and cytokoskeletal remodeling in isolated adult rat cardiac myocytes. J Mol Cell Cardiol 41, 228–235.

Laczko R, Csazar K (2020). Lysyl oxidase (LOX): functional contributions to signaling pathways in heart. Cardiovasc Res 111, 563–581.

Larabee SM, Coia J, Jones S, Cheung E, Gallicano GI (2015). miRNA-17 members that target Bmp2 influence signaling mechanisms important for embryonic stem cell differentiation in vitro and gastrulation in embryos. Stem Cells Dev 24, 354–371.

Leong YY, Ng WH, Ellison-Hughes GM, Tan JJ (2017). Cardiac stem cells for myocardial regeneration: they are not alone. Front Cardiovasc Med 14, 10–26.

Miao C, Lei M, Hu W, Han S, Wang Q (2017). A brief review: the therapeutic potential of bone marrow mesenchymal stem cells in myocardial infarction. Cell Death Dis 6, e1765.

Mikaei ML, Yabluchansky A, Ma Y (2015). Tissue inhibitor of metalloproteinase-1: actions beyond matrix metalloproteinase inhibition. J Mol Cell Cardiol 67, 683–699.

Mizock B, Ravassa S, Moreno MU, Jose GS, Beaumont J, Gonzalez A, Diez-Tejedor T (2010). Potential of bone marrow mesenchymal stem cells for cardiovascular repair and regeneration. Genes Dis 1, 113–119.

Nobes CD, Hall A (1995). Rho, rac, and cdc42 GTPases regulate the assembly of multimeric focal complexes associated with actin stress fibers, lamellipodia, and filopodia. Cell 81, 53–62.

Oh J, Lee KI, Kim HT, You Y, Yoon DH, Song KY, Cheong E, Ha Y, Hwang DY (2015). Human-induced pluripotent stem cells generated from intervertebral disc cells improve neurologic functions in spinal cord injury. Stem Cell Res Ther 6, 125.

Pan X, Chen Z, Huang R, Yao Y, Ma G (2013). Transforming growth factor β1 induces the expression of collagen type I by DNA methylation in cardiac fibroblasts. PLoS One 8, e60335.

Park SY, Lee CJ, Choi JH, Kim JH, Kim JW, Kim YJ, Nam JS (2019). The JAK2/STAT3/CCND2 axis promotes colorectal cancer stem cell persistence and radioresistance. J Exp Clin Cancer Res 38, 399.

Panguruth KB, Nakajima H, Nakajima HO, Soopama MH, Field LU (2005). Targeted expression of cyclin D2 results in cardiomyocyte DNA synthesis and infarct repair in transgenic mice. Circ Res 96, 110–118.

Prabhu SD, Frangogiannis NG (2016). The biological basis for cardiac repair after myocardial infarction from inflammation to fibrosis. Circ Res 119, 91–112.

Ristagno G, Fumagalli F, Bottazzi B, Mantovani A, Olvani D, Novelli D, Latini R (2019). Pentraxin 3 in cardiovascular disease. Front Immunol 10, 823.

Rodriguez C, Martinez-Gonzalez J (2019). The role of lysyl oxidase enzymes in cardiac function and remodeling. Cells 8, 1483.

Romagnuolo R, Masoudpour H, Sanchez-Porta A, Giang B, Barry J, Laskary A, Qi X, Masse S, Magtbay K, Kawajiri H, et al. (2019). Human embryonic stem cell-derived cardiomyocytes regenerate the infarcted pig heart but induce ventricular tachyarrhythmias. Stem Cell Rep 12, 997–981.

Rosini S, Pugh N, Bonna AM, Hulmes DJ, Farndale RW, Adams JC (2018). Thrombospondin-1 promotes matrix homeostasis by interacting with collagen and lysyl oxidase precursors and collagen-cross linking sites. Sci Signal 11, eaar2566.

Roten L, Nemoto S, Simsic J, Coker ML, Rao V, Baici S, Defrery G, Soloway PJ, Zile MR, Spinaile FG (2000). Effects of gene deletion of the tissue inhibitor of the matrix metalloproteinase-type 1 (TIMP-1) on left ventricular geometry and function in mice. J Mol Cell Cardiol 32, 109–120.

Rupert CE, Coulombe KL (2017). IGFR1 and NRG1 enhance proliferation, metabolic maturity, and the force-frequency response in HESC-derived engineered cardiac tissues. Stem Cells Int 2017, 7648409.

Salio M, Chimenti S, De Angelis N, Molla F, Maina V, Nebuloni M, Pasqualini F, Latini R, Garlanda C, Mantovani A (2008). Cardioprotective function of the long pentraxin PTX3 in acute myocardial infarction. Circulation 117, 1055–1064.

Shabbir A, Zisa D, Lin H, Mastri M, Roloff G, Suzuki G, Lee T (2010). Activation of host tissue trophic factors through JAK-STAT3 signaling: a mechanism of mesenchymal stem cell-mediated cardiac repair. Am J Physiol Heart Circ Physiol 299, H1428–H1438.

Shapiro AM, Ricordi C, Hering BJ, Auchincloss H, Lindblad R, Robertson RP, Secchi A, Brendel MD, Berney T, Brennan DC, et al. (2006). International trial of the Edmonton protocol for islet transplantation. N Engl J Med 355, 1318–1330.

Snyder M, Huang YI, Zhang JJ (2010). Stat3 directly controls the expression of Tbx5, Nkx2.5, and GATA4 and is essential for cardiomyocyte differentiation of P19C16 cells. J Biol Chem 285, 23639–23646.

Song N, Scholtemeijer M, Shah K (2020). Mesenchymal stem cell immunomodulation: mechanisms and therapeutic potential. Trends Pharmacol Sci 41, 653–664.

Stenina-Adognravi O (2014). Invoking the power of thrombospondins: regulation of stress fibroblasts. PLoS One 8, e60335.

Tarzami ST, Calderon TM, Deguzman A, Lopez L, Kitzis RN, Berman JW (2014). Targeting extracellular DNA to signal pathways in smooth muscle cells. Expert Opin Ther Targets 21, 671–683.

Talman V, Ruskoaho H (2016). Cardioblasts in myocardial infarction— from repair and remodeling to regeneration, Cell Physiol Biochem 335, 1008–1016.

Teruya-Feldstein C, Bercovitch F, Nelson C, Dolan M, Engler JA, Johnstone BM, et al. (2006). International best practices for the clinical translation of advanced cell and gene therapies. Cancer Res 66, 661–666.
Tompkins BA, Balkan W, Winkler J, Gyöngyösi M, Goliash G, Fernández-Avilés F, Hare JM (2018). Preclinical studies of stem cell therapy for heart disease. Circ Res 122, 1006–1020.

Umbarkar P, Singh AP, Gupte M, Verma VK, Galindo CL, Guo Y, Zhang Q, McNamara JW, Force T, Lal H (2019). Cardiomyocyte SMAD4-dependent TGF-β signaling is essential to maintain adult heart homeostasis. JACC Basic Transl Sci 4, 41–53.

Van Belle T, Von Herrath M (2008). Immunosuppression in islet transplantation. J Clin Invest 118, 1625–1628.

Vermeulen Z, Hervent AS, Dugaucquier L, Vandekerckhove L, Rombouts M, Beyens M, Schrijvers DM, De Meyer GRY, Maudsley S, De Keulenaer GW, Segers VFM (2017). Inhibitory actions of the NRG-1/ErbB4 pathway in macrophages during tissue fibrosis in the heart, skin, and lung. Am J Physiol Heart Circ Physiol 313, H934–H945.

Wang M, Yuan Q, Xie L (2018) Mesenchymal stem cell-based immunomodulation: properties and clinical application. Stem Cells Int 2018, 3057624.

Zhang H, Hui H, Li Z, Pan J, Jiang X, Wei T, Cui H, Li L, Yuan X, Sun T, et al. (2017). Pigment epithelium-derived factor attenuates myocardial fibrosis via inhibiting endothelial-to-mesenchymal transition in rats with acute myocardial infarction. Sci Rep 7, 41932.

Zouein FA, Altara R, Chen Q, Lesniewsky EJ, Kurdi M, Booz GW (2015). Pivotal importance of STAT3 in protecting the heart from acute and chronic stress: new advancement and unresolved issues. Front Cardiovasc Med 2, 36.