Paracrine Engineering of Human Cardiac Stem Cells with Insulin-Like Growth Factor 1 Promotes Cell Survival to Enhance Myocardial Repair

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Table of Contents

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Abstract

Insulin-like growth factor (IGF-1) is a potent pro-survival cytokine that is not robustly expressed by human cardiac stem cells (CSCs). Here, we explore the mechanism underlying IGF-1 enhanced cardiac repair by CSCs. Human CSCs underwent lentiviral-mediated somatic gene transfer of IGF-1 to boost cytokine secretion without adversely blunting the overall cytokine signature of CSCs. *In vitro* studies demonstrated that IGF-1 provided paracrine and autocrine support that reduced apoptosis by CSCs and cardiomyoctes. *In vivo* experiments demonstrated that IGF-1 increased CSC-mediated cardiac repair by enhancing salvage of reversibly damaged myocardium and transplanted cell survival.

List of Tables

List of Figures

List of abbreviations

αSMA	Alpha smooth muscle actin
AKT	(PKB) Protein kinase B
CD90	Cluster of differentiation marker 90
CDC	Cardio-sphere-derived cells
CEM	Cardiac explant media
CHF	Congestive heart failure
CM	Conditioned media
CSC	Cardiac stem cells
cTnT	Cardiac-troponin T
DAPI	4',6-diamidino-2-phenylindole
EPC	Endothelial progenitor cells
ERK	Extracellular signal-regulated kinase
GFP	Green fluorescent protein
HGF	Hepatocyte growth factor
IGF-1	Insulin-like growth factor 1
IGF-1R	Insulin-like growth factor-1 receptor
IR	Insulin receptor
LAD	Left anterior descending coronary artery
LTS	Long term risk stratification model for survivors of an acute coronary syndrome
lv	Lentivirus
LV	Left ventricle
LVEF	Left ventricular ejection fraction
MAPK	Mitogen-activated protein kinase
MI	Myocardial infarction
MSC	Mesenchymal stem cells
NYHA	New York Heart Association
NOD SCID	Non-obese diabetic severely compromised immunodeficient mouse
PCR	Polymerase chain reaction
SDF-1α	Stromal cell-derived factor 1 alpha

TGF1β	Transforming growth factor beta 1
VEGF	Vascular endothelial growth factor
VWF	von Willebrand factor

1.0 INTRODUCTION

Congestive heart failure (CHF) occurs when an injured heart cannot adequately pump blood to the body leading to inadequate forward output and pulmonary congestion.[1] CHF can be further classified as left or right-sided CHF. Left-sided CHF is the most predominant form whereby cardiac output from the left ventricle is reduced leading to fluid backup in the lungs. In the case of right-sided CHF, the right ventricle cannot adequately pump blood to the lungs leading to fluid accumulation in lower extremities. Collectively, CHF is a common cause of hospitalization and death[2] with an estimated 500,000 Canadians currently living with CHF.[3] These numbers are anticipated to rise as the population ages and medical innovations improve cardiac survival.[4] While surgical and pharmaceutical interventions alleviate symptoms and limit adverse myocardial remodeling, they rarely reverse disease progression.[5] As such, new therapies aimed towards replacing lost myocytes are warranted to obtain true-disease reversal and myocardial repair.

1.1 CELL THERAPY FOR HEART REPAIR AND REGENERATION

Myocardial infarction results in large amounts of myocardial cell death followed by fibrotic scar development and adverse remodeling by the remaining myoctes.[6] Regenerative medicine such as the transplantation of autologous stem cells has emerged as a promising means of repairing damaged myocardium. To date skeletal myoblasts,[7] bone marrow mesenchymal cells,[8,9] mononuclear cells,[10,11] adipose tissue-derived cells[12] and more recently stem cells of cardiac origin (c-Kit+ cardiac stem cells (CSC);[13]

cardiosphere-derived cells (CDCs))[14] have been used in clinical trials in the setting of acute or convalescent myocardial infarction.

To date no human clinical trials have been initiated using embryonic stem cells due to both ethical issues as well as possibility of teratoma formation.[15] The use of skeletal myoblasts have also demonstrated safety concerns, as cell transplantation was associated with sustained ventricular tachycardia.[7] Lastly stem cells obtained from bone marrow (e.g. mesenchymal stem cells and mononuclear cells) and heart (CSC and CDCs) have shown greatest therapeutic promise as clinical trials have shown cell transplantation to be safe with hints of improving cardiac performance.[8, 9, 10, 11, 12, 13, 14]

Although questions still remain as to what specific cell type is most effective in repairing the myocardium, an ideal cell candidate should be readily available, easily expandable, non-immunogenic and capable of safely engrafting with proper electrochemical coupling within its host.[16] As such the use of stem cells of cardiac origin (CSCs, CDCs, cardiospheres (CSp)) have gained attention as they posses the ability of *ex vivo* culture expansion, hypoimmunogenic[17] and are intrinsically programed to differentiate into functional myocardium.[18, 19] Therefore, this study will focus on enhancing the regenerative capacity of human cardiac stem cells (CSCs) with the pro-survival cytokine insulin-like growth factor-1 (IGF-1) as a means of improving traditional CSC-mediated cardiac repair.

1.1.1 Cardiac stem cells for cardiac repair

The adult heart is not a terminally senescent organ and exhibits low-grade cardiomyocyte renewal throughout the life cycle.[20] Although still debated, adult cardiomyocytes have an estimate of turnover rate of about 1% per year[21, 22] with rates

known to increase after myocardial damage.[23] The adult heart also contains discreet niches of resident cardiac stem cells (CSCs) predominantly located in the atria and left ventricular apex of the heart.[19] CSCs exhibit stem cell properties of being undifferentiated cells capable of self-renewal, proliferation and can differentiate into multiple lineages.[18, 19]

The c-Kit+ (cardiac progenitor) CSCs give rise to cardiomyocytes *in vivo* and contribute to the cardiomyocyte pool within the heart.[24] The contribution of c-Kit+ cells in routine myocyte renewal through development, ageing or cardiac injury is likely negligible [24, 25] as their abundance is relatively rare (1 in 10000 myocytes).[19] As a result, the direct contribution of c-Kit+ cells to new cardiomyocytes is unlikely to have an affect on cardiac function or meaningful regeneration.[24, 26] However, the autologous infusions of c-Kit+ CSCs into patients with heart failure have demonstrated hints of functional improvements on heart contractility and regeneration.[13] This data along with reports of low cell engraftment and low cardiomyocyte differentiation following CSC transplantation further suggests indirect mechanisms involving the paracrine secretion of factors and their trophic actions are playing a vital role in the therapeutic repair process.[26]

To this end, our lab has developed protocols to proliferate and expand heterogeneous mixture of *ex vivo* proliferated CSCs from myocardial biopsies prior to cell transplantation into damaged myocardial tissue. Our heterogeneous CSC products express markers of stem cell (c-kit, SSEA-1), endothelial progenitor (CD34, CD31) and mesenchymal (CD105, CD90) identity.[27, 28] These CSCs spontaneously release a broad array of cardio-protective and pro-angiogenic cytokines (VEGF, HGF, IGF-1, IL-6, angiogenin, SDF-1) [28, 29] that provide autocrine and paracrine repair with evidence for

differentiation into working myocardium while promoting significant myocardial salvage and scar reduction.[30, 18, 31]

1.1.2 Current limitations to CSC therapy: Overcoming low transplant cell engraftment

Although clinical trials have shown great promise, the efficacy of CSC therapy is largely limited as the transplanted cells rarely persist following injection into an ischemic environment that is often oxygen and nutrient deprived. It follows that the functional benefits conferred by CSCs are largely driven by paracrine stimulated recruitment of endogenous stem cells and rescue of reversibly damaged myocardium.[26] As such, techniques aimed towards improving cell engraftment and survival may have salutatory effects as they can persist to engage in endogenous repair for a sustained period of time or participate in the formation of new cardiomyocytes.[32, 33]

Genetic engineering of stem cells may provide a means of improving cell survival and their ability to engraft. As demonstrated in Table 1, this approach has been employed using a number of non-cardiac stem cell sources by directly enhancing survival or promoting the secretion of pro-survival cytokines. To date, only one paper has attempted to broaden the paracrine repertoire of CSC using somatic gene transfer of hypoxia-inducible factor-1alpha (HIF-1a).[34] Interestingly, this paper demonstrated that HIF-1 over-expression blunted the benefits conferred by CSC transplantation which may be mediated by: 1) loss of other CSC-sourced paracrine factors (i.e., endothelin-1, VEGF, and IGF-1), 2) lentiviral mediated attenuation of CSC viability, or 3) over-expression of a harmful/ineffective cytokine.[34] Given the success using similar approaches with non-

cardiac stem cell products (Table 1) the full potential of this genetic enhancement warrants further exploration. Modifications, such as selectively targeting CSC sub-populations or more effective transgenes, may avoid these complications and provide a better cell product. Given the success of insulin-like growth factor-1 (IGF-1) transgene expression in EPCs and MSCs,[35, 36] this study we will investigate the therapeutic potential of *ex vivo* proliferated CSCs enriched with IGF-1 to promote transplant cell survival and cell-mediated cardiac repair.

Table 1: Select examples of gene based strategies used to enhance autologous stem cell engraftment and survival.

Cell Type	Gene overexpressed/ Function	Animal model	Gene Delivery	Study Outcome
EPC [37]	hTERT: telomerase activity /proliferation	Mouse hind limb ischemia	Adenovirus	Reduced EPC apoptosis Enhanced vascularization Enhanced limb perfusion
EPC [38]	VEGF: angiogenesis	Mouse hind limb ischemia	Adenovirus	Enhanced EPC engraftment Enhanced vascularization
EPC[39]	eNOS/HO-1: endothelial function and anti-apoptotic	Rat balloon injury	Retrovirus	Enhanced vasculoprotective properties
EPC [35]	IGF-1: cell survival	Rat MI	Adenovirus	Reduced cardiac apoptosis Enhanced myocyte proliferation Enhanced vascularization Enhanced cardiac function
SkM [40]	CSF-1: proliferation/ angiogenesis/survival	Rat MI	Plasmid	Enhanced cell engraftment Enhanced angiogenesis Enhanced cell recruitment Enhanced cardiac function
MSC [41]	HSP20: cell survival/angiogenesis	Rat MI	Adenovirus	Improved transplant cell survival Enhanced vascular density Improved cardiac function
MSC [42]	SDF-1: cell recruitment/survival	Rat MI	Plasmid	Improved myocyte survival Enhanced vascular density
MSC [43]	HGF: angiogenesis/cell recruitment	Rat MI	Adenovirus	Reduced ischemic area and fibrosis Improved vascularization
MSC [44]	Akt: cell survival	Rat MI	Retrovirus	Reduced cardiac

				remodeling Improved cardiac function
MSC [36]	IGF-1: cell survival	Rat MI	Adenovirus	Reduced infarct size Improved stem cell recruitment Enhanced angiogenesis Improved cardiac function

EPC: endothelial progenitor cells; SkM: skeletal myoblasts; MSC: mesenchymal stem cells; MI: myocardial infarction; EF: ejection fraction; hTERT: human telomerase reverse transcriptase; CSF-1: colony stimulating factor-1;eNOs: endothelial nitric oxide synthase; HO-1: heme oxygenase-1; SDF-1: stromal-cell derived factor-1; IGF-1: insulin-like growth factor-1; HGF: hepatocyte growth factor; HSP20: heat shock protein 20.

1.2 INSULIN-LIKE GROWTH FACTOR-1

The insulin-like growth factor-1 (IGF-1) protein has many actions within mammals. As a circulatory hormone, it plays a vital role in growth and development with continued anabolic effects in adults.[45, 46] IGF-1 deficiencies in both mice and humans have deleterious effects on growth, perinatal lethality and delayed development.[45, 46] IGF-1 also has pleiotropic actions on myocyte proliferation, regeneration and is a key regulator of cell growth and apoptosis.[47, 48, 49, 50] This would suggest IGF-1 is a logical growth factor to enhance as it may promote cell survival and subsequence engraftment of transplanted cells.

1.2.1 IGF-1 and cardio-protection

IGF-1 has well known cardio-protective effects in mammals. In humans and many laboratory animals, IGF-1 levels significantly decline with age.[51, 52, 53] Numerous epidemiological studies have demonstrated high level of circulating IGF-1 is associated with reduced risk of heart failure and mortality.[54, 55] Furthermore, reductions in

circulating IGF-1 are predictive of heart failure development[56, 57, 58] suggesting that age-associated decline of IGF-1 may be associated with age-related cardiovascular alterations. The cardiovascular protection inferred by IGF-1 may be explained by its ability to positively influence myocyte survival and myofibril hypertrophy; therefore enabling the heart to withstand hemodynamic stressors.[59]

Torella *et al.* (2004) further examined IGF-1 protective actions on the aging heart using a myocyte-specific IGF-1 overexpressing transgenic mouse model. Aging IGF-1 transgenic mice demonstrated elevated IGF-1 pathway stimulation (Akt phosphorylation) with associated reductions in the expression of proteins involved in growth arrest and senescence (p27, P53, p16 and p19).[60] Furthermore the IGF-1 transgenic mice were protected against the age-associated loss in myocyte number and left ventricle function.[60] This data demonstrates the cardio-protective effects of IGF-1 especially within the aging population.

1.2.2 Splice variants of the IGF-1 gene

The synthesis of IGF-1 is controlled by the *IGF-1* gene, which contains six exons that can give rise to multiple mRNA transcripts (Figure 1). As such multiple IGF-1 isoforms can arise by alternative promoter usage, splicing and polyadenylation states.[61, 62] The mature IGF-1 protein comprises 70 amino acids encoded by exons 3 and 4. Immature IGF-1 isoforms can also arise from extensions beyond the mature protein to include the E-peptide (exons 5 and 6). In humans, 3 main isoforms have been identified including IGF-1Ea, IGF-1Eb, and IGF-1Ec (mechano growth factor).[63] The majority of these immature pro-peptides undergo post-translational processing to generate the mature

70 amino acid IGF-1 protein and separate E peptide. However, cells may also skip this processing to secrete IGF-1 with intact E-peptides.[64]

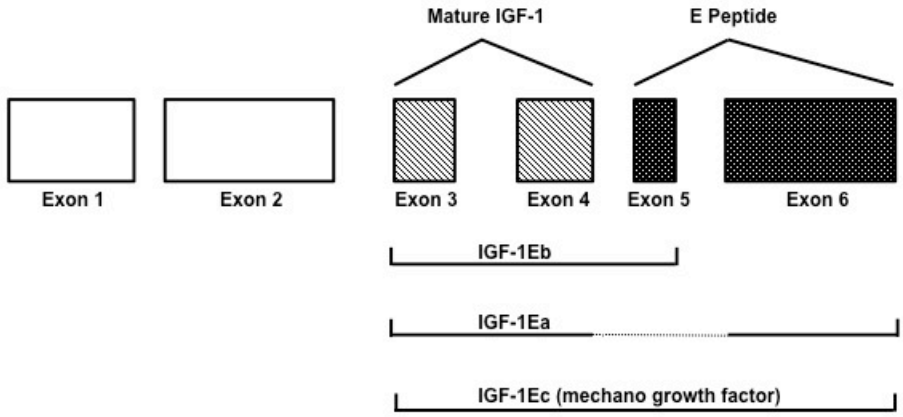


Figure 1. Human *IGF1* gene organization. Exons 3 and 4 give rise to the mature IGF-1 protein. Additional splice variants can arise with E-peptide extensions (IGF1-Eb, IGF1-Ea, IGF-1Ec).

Emerging data suggests that the immature forms of IGF-1 may have distinct actions.[63] IGF-1Ea has been associated with local IGF-1 autocrine/paracrine actions such as muscle hypertrophy and regeneration [65] whereas IGF-1Eb has been linked to the endocrine effects of IGF-1 with high expression in the liver.[66] IGF-1Ec is up-regulated following muscle damage or exercise to initiate tissue repair, hypertrophy and remodeling via satellite cell activation and myoblast proliferation.[61, 67]

The therapeutic importance of the E-peptide moiety is debatable but recent reports have suggested it may act as an independent growth factor without need for the IGF-1 ligand.[68] Rosenthal and colleagues suggest the E-peptide moiety promotes local retention of IGF-1 at the sites of synthesis through the interaction of positively charged amino

acids with the extracellular matrix.[64] This group also demonstrates that macrophages are a main source of these differentially spiced IGF-1 variants which may play a role in M2 macrophage activation and injury resolution [69](pinto 2014). However, the effects of E peptide glycosylation on IGF-1 receptor binding is controversial as some studies suggest E peptides limits IGF-1 receptor (IGF-1R) signaling [69, 70] and others find that E peptides increase the proportion of IGF-1R at the cell surface.[71]

Further work to understand of the fundament effect of E-peptides is warranted. As such, this thesis will limit E peptide variability by strictly focusing on the mature human IGF-1 transcript, which has already consistently been shown to induce cell proliferation, reduce apoptosis, and improve post-ischemic cardiac function. [35, 36, 47, 48, 49, 50, 72, 73]

1.2.3 IGF-1 mediates its action through the IGF-1 receptor

At the cell surface, IGF-1 signaling is transmitted by the IGF-1 receptor (IGF-1R) or to a much lower degree, the insulin receptor (IR). The three IGF family members (IGF-1, IGF-2 and to a much lower affinity, insulin) bind to the IGF-1R, which is present on may cell types including cardiac cells.[53] As demonstrated in Figure 2, IGF-1R activation by IGF-1 ligand initiates the receptor tyrosine kinase auto-phosphorylation and the recruitment of docking proteins that transduce actions of cell survival, migration, cell growth and proliferation.[74]

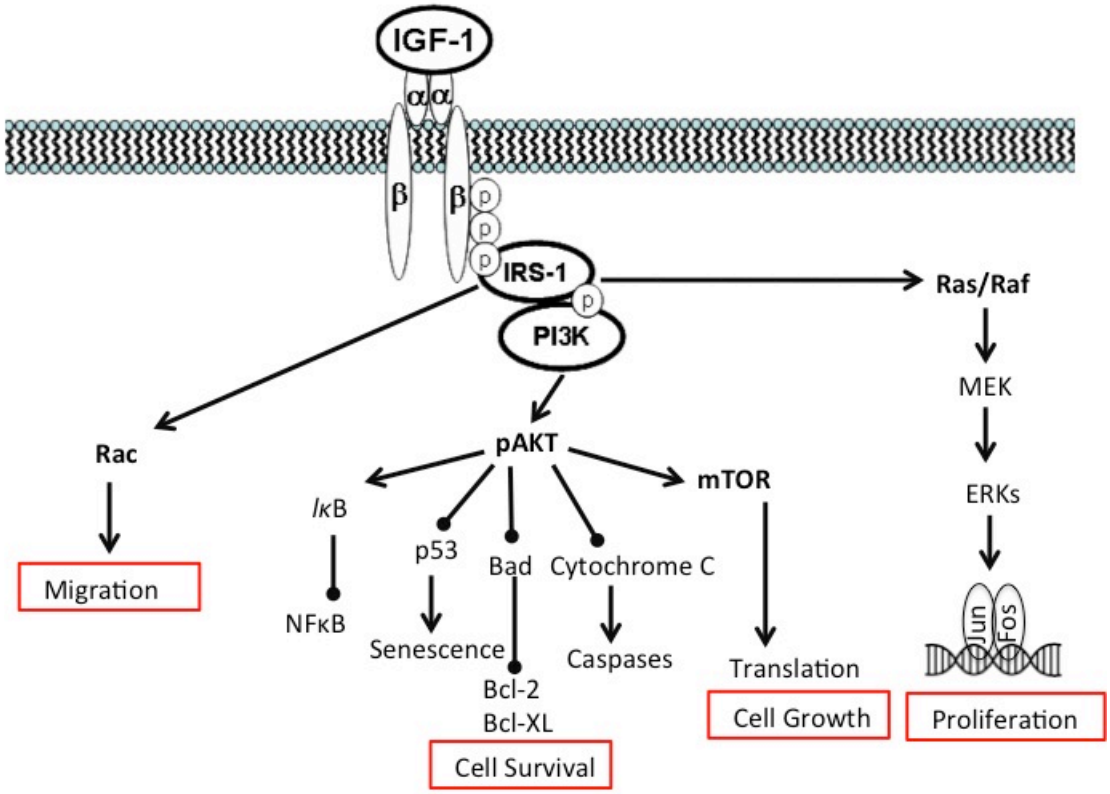


Figure 2. IGF-1R is a tyrosine kinase receptor that mediates cell survival, migration, growth and cell proliferation.

Interestingly, D'Amario *et al.* (2011) have identified a subset of IGF-1R positive CSCs to have superior regenerative potential for myocardial repair.[75] The presence of the IGF-1R was associated with a "younger CSC" phenotype consisting of a highly proliferative cell population with long telomeres and high telomerase activity.[75] Furthermore, the intra-myocardial injection of the IGF-1R enriched CSC population after experimental MI in rats resulted in superior regeneration including enhanced myocyte and ventricular structure compared to the unselected CSCs product.[75] This data supports

the notion that the IGF-1/IGF-1R axis may be an ideal target for repairing the damaged myocardium.

1.2.4 Anti-apoptotic actions of IGF-1

Downstream IGF-1R stimulation can act at different levels of the apoptotic machinery through Ras/MAPK/ERK, PI3/Akt and PI3K/mTOR signaling pathways (Figure 2).[72] These pathways are known to alter early gene expression, stimulate myogenesis, inhibit apoptosis, and enhance cell cycle progression. Although these pathways have overlapping anti-apoptotic effects, the PI3K/Akt cascade is thought to be the canonical pathway involved in IGF-1 mediated inhibition of apoptosis.[76] PI3K is necessary and sufficient for Akt/PKB activation, a serine threonine kinase and powerful survival signal in many systems.[77]Activated Akt though IGF-1 signaling is crucial for inhibiting apoptosis via inactivation of pro-apoptotic proteins such as Bad, Bax and caspases.[78, 79] Other known pro-survival actions of IGF-1 include up-regulation of anti-apoptotic factors such as Bcl-2 and Bcl-x to prevent the initiation of apoptosis.[79] Evidently IGF-1 signaling provides powerful and widespread anti-apoptotic and pro-survival activities making it a logical candidate for improving transplanted cell survival.

1.2.5 Anti-necroptotic effects of IGF-1

Necroptosis is a recently described from of programmed necrotic cell death implicated in the pathogenesis of ischemic injury.[80] Morphologically, necroptosis resembles necrosis where cells increase volume with swelling of organelles and eventual loss of plasma membrane integrity.[81] The stimuli for necroptosis include exocitotoxicity,

oxidative stress, mitochondrial dysfunction or inflammatory receptor ligand binding (TNF, Fas Ligand, TRAIL ligand). Although similar stimuli trigger both apoptosis and necroptosis, cell entry into the necroptotic pathway is regulated by the controlled involvement of receptor kinases (receptor-interacting protein kinases 1 (RIP1) and 3 (RIP3)) in the absence of caspases.[82, 83, 84] As demonstrated in Figure 3, apoptotic cell death would prevail in the presence of Fas-Associated protein with Death Domain (FADD) and subsequently activated caspase-8, whereas necroptosis and RIP1/RIP3 activation would prevail in the absence of FADD and caspases.[84]

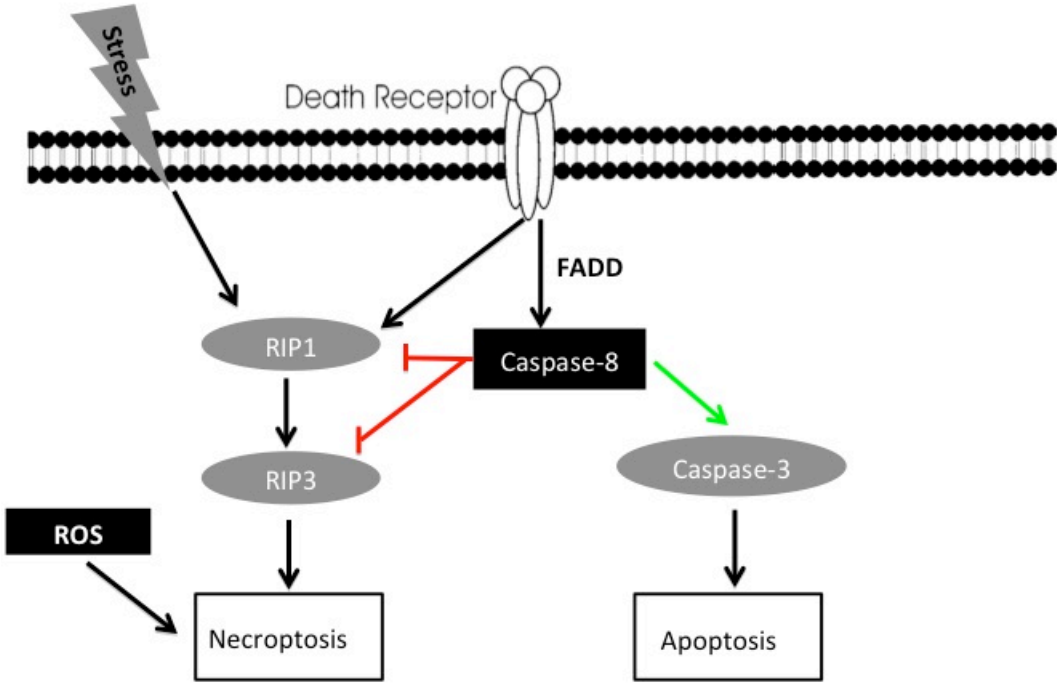


Figure 3. The regulation of necroptosis and apoptosis depends on activity of caspases.

Necroptosis of cardiac myocytes plays an important role to the pathogenesis of myocardial infarction[85, 86] and perhaps even heart failure.[87] As such, pharmacological agents that target the necroptotic pathway are of clinical interest as they may inhibit necrosis and improve cardiac function. Furthermore, the application of an anti-

necroptotic small molecule (necrostatin) has shown therapeutic promise following myocardial infarction by reducing infarct size and rescuing damaged myocytes from necrotic cell death.[88, 89] The effect of IGF-1 signaling on necroptosis is unclear as studies that directly evaluate the effects of IGF-1 on the necroptotic pathways are limited.

In support of IGF-1 having anti-necroptotic actions, Shavlakadze *et al.* (2004) demonstrated that skeletal overexpression of IGF-1 protects dystrophic myofibers from muscle breakdown and necrosis.[73] Similarity, Li and colleagues have reported IGF-1 overexpression can attenuate myocyte necrosis in an animal model of coronary artery narrowing.[90] There is also accumulating evidence that circulating levels of IGF-1 and tumour necrosis factor-α (TNF-α) are inversely related in patients with heart failure[91] and that the IGF-1R can protect against TNF toxicity *in vitro*.[92] Thus it follows that application of IGF-1 in the critical period following myocardial infarction may provide cardiac protection by reducing entry of reversibly damaged cells into the necroptotic pathway.

1.2.6 IGF-1 delivery and stem cell recruitment

Ellison and colleagues have also been interested in IGF-1, but rather for its ability to recruit endogenous CSCs for myocardial repair. Using a swine model of chronic myocardial infarction, recombinant IGF-1 and hepatocyte growth factor (HGF) protein were co-administered one month post-MI.[93] The co-administration of IGF-1/HGF increased the recruitment of CSCs within infarct and border zones and led to the formation of new cardiomyocytes, capillaries and improved cardiac function.[93] More recently, this group combined IGF-1/HGF with a hydrogel to sustain growth factor

release, which in turn increased the effectiveness of IGF-1/HGF therapy.[94] These results would suggest that prolonged growth factor release might have salutatory effects on the cardiac repair process.

Although the addition of hydrogel to IGF-1/HGF improved functional outcomes, protein degradation was still observed (4 days-post delivery) suggesting alternative approaches to sustain IGF-1 delivery are warranted.[94] Haider *et al.* (2008) have also investigated IGF-1 actions on endogenous stem cell recruitment but using virally modified IGF-1-overexpressing mesenchymal stem cells as a means of delivering IGF-1 to infarcted rat myocardium.[36] Here, IGF-1 was detected 7 days post cell injection with similar repots of IGF-1 acting through a mechanism of stem cell recruitment to infarct regions with improvements in vascularization, infarct scarring and cardiac performance.[36] Therefore IGF-1 may provide cardiac repair through recruitment of endogenous stem cells and techniques aimed toward improving IGF-1 release may enhance therapy efficacy.

1.2.7 Systemic IGF-1 and concerns of oncogenicity

IGF-1 has the ability to act in both endocrine as well as paracrine/autocrine fashion. When acting as an endocrine hormone, growth hormone (GH) stimulates IGF-1 production by the liver, which then enters the circulation. In circulation, IGF-1 is bound to protein carriers (IGFBPs) which mediate IGF-1 transport as well as modulating the proteins half-life, tissue specificity, neutralizing or strengthening its actions.[95] The IGF-1/GH axis has been heavily studied, with deficiencies associated with ageing, and cardiovascular risk factors.[95] At this time there are no ongoing clinical trials using direct

IGF-1 supplementation in patients with heart failure. However, a synthetic form of IGF-1 (Increlex) is currently being used for the treatment of IGF-1 deficiencies in children. Due to potential oncogenicity associated with high circulating IGF-1 levels, this therapy is not recommended for children with cancer or adults.[96]

1.3 HUMAN CSCS AS A PLATFORM FOR IGF-1 OVER-EXPRESSION

Although application of IGF-1 alone is an attractive solution to improve cardiac performance and myocardial viability, the limited half-life of the protein as well as variability/safety concerns using time-release formations limit clinical translation. The rationale for genetically engineering *ex vivo* proliferated CSCs as a reservoir for IGF-1 over expression can be justified by the observation that these cells home to sites of injury.[97] Furthermore the expression of IGF-1R on the c-Kit+ sub-population within CSCs opens the possibility that IGF-1 over-expression may provide autocrine support to transplanted cells.[75] Other stem cell sources such as endothelial progenitor cells (EPCs) [35] and bone marrow mesenchymal stem cells (MSCs) [36] have previously demonstrated the feasibility of using cell products for IGF-1 delivery with hints of therapeutic benefit on cardiac repair following myocardial infarction in rodent models. In the next sections, the previous attempts of IGF-1 overexpression within MSCs and EPCs will be discussed as well as the novelty of the current study.

1.3.1 Genetic enhancement of MSCs and EPCs with IGF-1

The enhancement of bone marrow MSCs and peripheral EPCs with IGF-1 overexpressing adenoviral vectors have similarly demonstrated improvements in left ventricle function, reduction in apoptosis and improvements in vascularization when injected at the time of experimental myocardial infarction.[35, 36] More specifically, the transduction of MSCs with IGF-1 improved MSC engraftment with corresponding up-regulation of IGF-1 and phospho-Akt expression within the infarcted rat tissue 7 days after cell transplantation.[36] The mechanism by which the IGF-1 transduced MSCs led to improvements in myocardial function were through reductions in apoptosis and enhancements in stem cell mobilization via stromal derived factor-1 (SDF-1).[36]

In a similar study, early EPCs were *ex vivo* expanded and transduced with IGF-1 adenoviral vector prior to autologous transplantation in rats at time of experimental myocardial infarction. In culture, this group was able to demonstrate EPCs overexpressing IGF-1 have enhanced proliferative and anti-apoptotic actions under oxidative stress (H_2O_2) when compared to lentiviral controls. Furthermore, the injection of genetically modified EPCs with IGF-1 translated into sustained functional improvements in fractional shortening 12 weeks after myocardial infarction with reductions cardiac apoptosis, enhancements in myocyte proliferation and increases in vascularization within infarcted regions.[35] However the mechanism by which the observed IGF-1 over-expressing EPCs improved cardiac function was not fully addressed, although authors speculated the enhanced secretion of IGF-1 stimulated the paracrine mechanism of cell-mediated repair.

1.3.2 Novelty of this research study

Although the two previously discussed studies suggest IGF-1 plays an important role in transplant cell survival, this study is the first to evaluate the therapeutic potential of IGF-1 over-expression within CSCs. Here, we evaluate the responsiveness of both transplanted CSC and host myocardium to IGF-1 and delay cell transplantation to a time that reflects the period required to culture cells and to a period where the myocardium remains responsive to IGF-1 stimulation. Finally, we demonstrate the fundamental mechanism underlying IGF-1 mediated effects on myocardial apoptosis and necroptosis.

2.0 STUDY AIMS, HYPOTHESES AND OBJECTIVES

Although CSCs have shown to improve cardiac function and repair, these improvements are largely limited by impaired cell survival and engraftment.[33] IGF-1 has potent anti-apoptotic and pro-survival actions with positive outcomes on cardiac performance after myocardial injury. [35, 36, 47, 48, 49, 50, 72, 73] Given that CSC do not naturally secrete high levels of IGF-1,[28] this study will genetically enhance human IGF-1 secretion by *ex vivo* proliferated CSCs and evaluate how the enhancement of this cytokine affects CSC-mediated cardiac repair.

General hypothesis: Human CSCs genetically engineered to overexpress IGF-1 will boost cell survival and enhance myocardial repair in an immunodeficient mouse model of myocardial ischemia.

General study aims:

1. To investigate if lv-IGF-1 CSCs promote cell survival by investigating the degree of cell proliferation, cell apoptosis and cell necroptosis under culture conditions that mimic the ischemic myocardium.
2. To understand the mechanisms by which transplanted lv-IGF-1 CSCs promote cell survival and myocardial repair by investigating cell engraftment and myocardial apoptosis.

3.0 MATERIAL AND METHODS

3.1 Effects of LAD ligation on IGF-1 receptor expression

To rationalize the delivery of IGF-1 in the post infarct model, twenty-seven C57/BL6 mice (Charles River) were randomized to undergo thoracotomy with left anterior descending artery (LAD) ligation (n=24) or a control sham thoracotomy with no LAD ligation (n=3). Mice were sacrificed 1, 7, 14, and 21 days after LAD ligation and the heart was dissected into sections of interest (infarct, infarct border zone and off-target (LV posterior wall) regions). RNA was isolated using TRIzol for quantitative PCR of IGF-1 receptor (IGF-1R) and insulin receptor (IR) expression using commercial PrimeTime qPCR Assays and Primers (Integrated DNA Technologies). IGF-1R and IR transcript expression was normalized to GAPDH and presented as fold change over sham operated animals. Immunohistochemistry was used to confirm IGF-1R and IR expression using commercial antibodies for the IGF-1R (Abcam, ab131476), IR (Abcam, ab137747), and cardiac troponin T (TnT; ab10214, Abcam).

3.2 Cell Culture

Human cardiac stem cells (CSCs) were cultured from atrial appendage specimens obtained during clinically-indicated procedures after informed consent under a protocol approved by the University of Ottawa Heart Institute Research Ethics Board. Atrial appendages were processed as previously described.[27,28] Briefly; tissue was cut into fragments, partially digested with collagenase IV (1mg/ml; GIBCO) and plated within cardiac explant culture media (CEM; Iscove's Modified Dulbecco's Medium (GIBCO), 20% FBS (GIBCO), 100U/ml penicillin G (GIBCO), 100ug/ml streptomycin (GIBCO),

2mmol/l L-glutamine (GIBCO), and 0.1mmol/l 2-mercaptoethanol (GIBCO)). During the first week of culture, a layer of loosely-adherent cells emerged from the plated cardiac tissue and were harvested using mild enzymatic digestion (0.05% trypsin; GIBCO). Each specimen underwent cell harvest up to four times. The heterogeneous CSC product was characterized for progenitor (c-Kit; FAB332A, RD Systems) and mesenchymal (CD90; 555596, BD Biosciences), markers using flow cytometry (Guava easyCyte 8HT, EMD Millipore).

The colorimetric WST-8 assay (Cell counting kit 8, Dojindo Molecular Technologies, Inc. Gaithersburg, MD) was used to track cell viability and proliferation. To mirror the environment of infarcted myocardium, cell growth was challenged using low serum CEM media (1% FBS) and cultured in hypoxic conditions (1% oxygen). The number of CSC was determined by generating a standard curve of $3x10^4$ serially diluted CSCs (Supplemental Figure 1). Population doubling time was calculated using standard techniques [Doubling time= $(t_2-t_1)\log 2/(\log N_2-\log N_1)$, where (t) is the difference in time from starting (1) and final (2) cell counts (N)].[27, 98]

3.3 Flow cytometry for IGF-1 receptors and CSC phenotype

The surface expression of the IGF-1R and IR on representative CSC sub-fractions was determined using flow cytometry. Monoclonal antibodies and isotype control monoclonal antibodies for CD90 (555596, BD Biosciences), c-Kit (FAB332A, RD Systems), IGF-1R (Abcam, ab131476) and IR (Abcam, ab137747) were used to label human CSCs. In a similar manner, the phenotypic profile of the CSCs transplanted into NOD SCID mice was confirmed using flow cytometry for c-Kit and CD90 expression.

A minimum of 40,000 events was collected with fluorescent compensation performed using single labelled controls. The percentage of positive cells was defined as the percent of the population falling above the 99th percentile of the isotype control. All measures were performed using Flow-Jo (v. 7.2.2 Treestar).

3.4 Lentiviral transduction of the mesenchymal sub-population within CSCs to over-express IGF-1

A third generation lentiviral vector system was used to over-express: 1) mature IGF-1 in concert with a fluorescent reporter (green fluorescent protein, GFP; LV-IGF1) or 2) a GFP reporter only (null transduction control; LV-GFP) (Figure 4). Commercially sourced Human IGF-1 cDNA (NM_001111283; GenScript Inc., USA) was PCR amplified (TopTaq, Qiagen; Supplemental Table 1) and inserted into a third generation lentivirus vector backbone with transgene expression under control by the constitutive cytomegalovirus (CMV) promoter.[99] Protein BLAST indicated 90% homology between human and murine IGF-1 obviating concerns regarding the use of xenogeneic model. Lentivirus was generated using HEK293 co-transfection followed by viral suspension filter column concentration (Centricon Plus-70; Millipore). Viral titers were verified using quantitative PCR for lentiviral particles.[100]

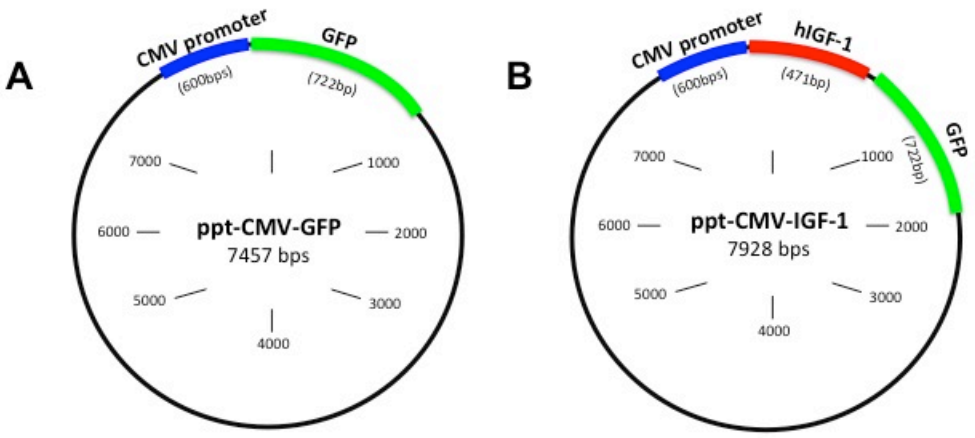


Figure 4. Third generation lentiviral vectors used for (A) lv-GFP and (B) lv-IGF-1 virus propagation. CMV: cytomegalovirus; GFP: green fluorescence protein; hIGF-1: human insulin-like growth factor-1.

This mesenchymal CD90+ sub-population within CSCs was selected as the platform for IGF-1 over-expression as: 1) it provides a reliable population of easily transduced cells that likely contributes very little to CSC-mediated myocardial repair,[13, 19, 101] and 2) emerging evidence that direct transduction of the entire CSC admixture may disrupt paracrine production while impairing cardiac repair.[34] As demonstrated in Figure 5, the CD90+ sub-population within CSCs was immunomagnetically sorted (123-21D; DynaMag; Life Technologies) using a human-specific CD90+ antibody (555596; BD Biosciences) and cultured in parallel from the CD90- depleted fraction under identical conditions (CEM). CD90+ cells were transduced with LV-IGF1 or lv-GFP and both sub-populations were recombined after 48 hours. Successful expression of the GFP reporter was confirmed in all cultures using fluorescent microscopy (Axio Observer, Zeiss) and flow cytometry (Guava easyCyte 8HT, EMD Millipore).

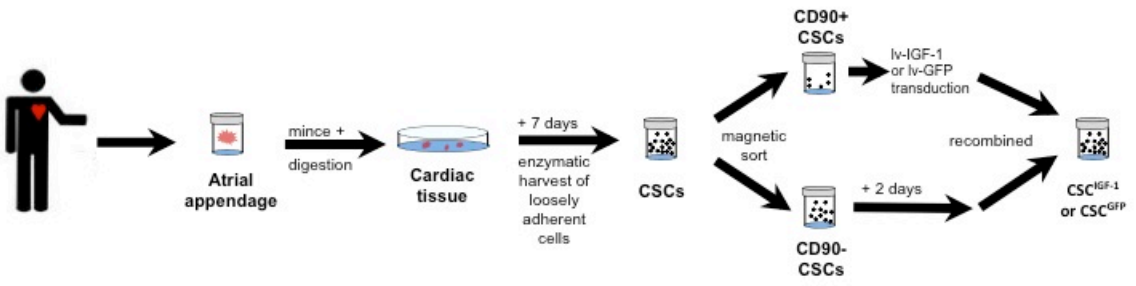


Figure 5. Schemata of the study design highlighting cell source, CD90 guided separation and lv-GFP or lv-IGF-1 transduction prior to recombination for further experimentation.

IGF-1 transgene expression was assessed using PCR profiling (4 x 10^5 CSCs (RNeasy Mini, Qiagen; TaqMan Reverse Transcription Reagents, Applied Biosystems)) and custom primers (Supplement Table 1). The ability of engineered CSCs to over-express IGF-1 was evaluated in conditioned media using enzyme-linked immunosorbent assay (ELISA; DG100, R&D Systems). Conditioned media was obtained from transduced and non-transduced CSCs after 48 hours of culture in hypoxic (1% oxygen) low serum (1% FBS) conditions to simulate the environment of the infarcted myocardium. CSCs were seeded at 70% confluence ($5x10^4$) in in low serum basal media (1% FBS) and placed in hypoxic (1% oxygen) incubation with media collection at 48 hours after plating. All immunosorbent measures were normalized to media volume and protein content obtained from cell lysates. The influence IGF-1 over-expression on the paracrine profile of CSC conditioned media was evaluated using a custom protein array (Human Cytokine Antibody Array G Series kit; RayBiotech, USA) according to the manufacturer's directions. All immunosorbent measures were normalized to the protein content and media volume.

3.5 Effects of IGF-1 engineering on cell death

The capacity to IGF-1 engineered CSCs to withstand cell death was assessed after culture in hypoxic (1% oxygen) low serum (1% FBS) conditions by examining:

1) Colorimetric assessment of proliferation using the WST-8 assay (CCK8, Dojindo). Baseline CSC measurements were performed 8 hours after cell plating with sequential measurements 24 and 48 hours after initial baseline reading. CSC numbers were extrapolated using a standard curve using $4X10^4$ serially diluted CSCs .
2) Expression of early apoptosis using annexin V apoptosis kit (559763; BD Biosciences). CSCs were stained with annexin V and 7-AAD according to manufacturers instructions. Apoptotic signal was detected by flow cytometry and analysis performed in Flow-Jo (v. 7.2.2 Treestar). A minimum of 40,000 events was collected in duplicate and fluorescent compensation was performed using single labelled controls.
3) Expression of Bcl-2, Fos and Jun pro-survival transcripts using relative PCR. RNA was isolated from CSCs using TRIzol and transcript expression was assessed using commercial PrimeTime qPCR Assays and Primers (Integrated DNA Technologies). Expression was normalized to GAPDH and presented as a fold change in transcript expression relative to normoxic CSC transcript expression (5%O2 and 20% FBS).
4) Expression of 35 apoptosis related and stress activated proteins using a commercial human apoptosis array (ARY009; R&D Systems). Protein lysates (400ug) from lv-GFP or lv-IGF-1 CSCs were collected 48h after hypoxic and low serum exposure. Proteins were spotted in duplicate on nitrocellulose membranes and exposed on X-ray

film for 1-10min according to manufacturers directions. Relative densitometry analysis was performed using ImageJ software and normalized to loading controls.

5) Expression of necroptosis markers using western blots with antibodies against RIP1(ab106393;Abcam), RIP3 (ab152130;Abcam), Caspase 8 (ab25901;Abcam) and FADD (ab24533;Abcam).The expression of necroptosis proteins was normalized to GAPDH and quantified using densitometry.

The potential pro-survival effects of IGF-1 engineered CSCs on neighboring myocardium were explored using direct and indirect (transwell) co-culture systems with neonatal rat ventricular myocytes (NRVM; R-CM-561, Lonza). CSC and NRVM co-cultures were evaluated for cell survival after 48h of culture in hypoxic (1% oxygen) low serum (1% FBS) media. CSCs were distinguished from NRVMs in direct co-culture systems using DiO Vybrant Cell-Labeling Solutions (Molecular Probes). NRVMs and CSC co-cultures underwent analysis of:

1) Cell viability using colorimetric WST-8 assay.

2) Degree of apoptosis (flow cytometry for annexin V and DiO).

3) Expression of the anti-apoptotic protein Bcl-2 (Abcam, ab692).

3.6 Myocardial infarction, cell injection and effects of cell therapy

Figure 6 outlines the experimental design that was utilized to evaluate the effects of lv-IGF-1 CSC therapy in a mouse model of myocardial ischemia. One week after LAD ligation, NOD SCID mice (Charles River; 4-6 weeks old) were randomized to receive intramyocardial injection of lv-GFP transduced CSCs (n=9), lv-IGF-1-transduced CSCs

(n=8) or inactive vehicle (PBS; n=4).[28] Echocardiographic guidance (VisualSonics V1.3.8, Toronto, Canada) was used to inject 100,000 CSCs or PBS as one injection at the apex and another at the lateral infarct border zone. All mice underwent echocardiographic imaging at 14 and 21 days after cell transplantation with left ventricular chamber dimensions and ejection fraction calculated from the parasternal images using standard techniques.[28]

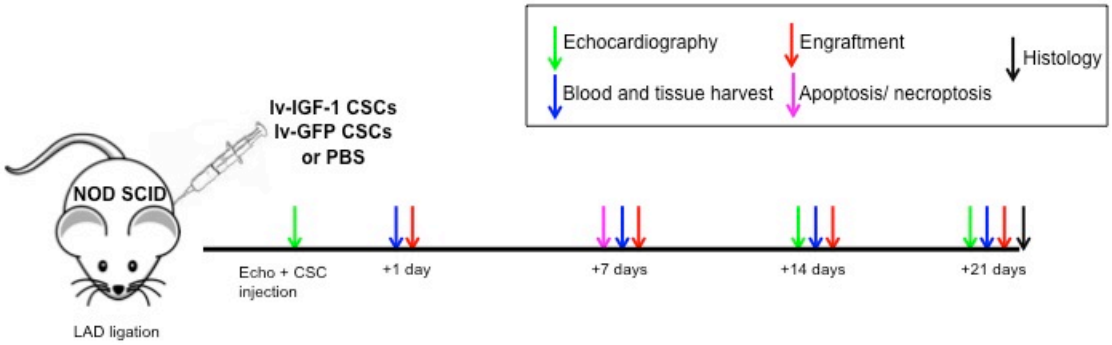


Figure 6. *In vivo* experimental design for lv-IGF-1 CSC therapy evaluations using an immunodeficient mouse model of myocardial ischemia. NOD SCID (non-obese diabetic severely compromised immunodificient mouse), LAD (left anterior descending artery).

Quantitative morphometry and scar burden was assessed 21 days after cell injection using Masson's trichrome staining (Invitrogen, Canada). The spatiotemporal progression of human and mouse IGF-1 expression was characterized in a separate series of NOD SCID mice after transplantation of lv-GFP and lv-IGF-1 transduced CSCs. These mice were sacrificed 1, 7 and 14 days after cell injection (n=3/group). Peripheral blood was obtained prior to euthanasia to evaluate circulating levels of human IGF-1 after cell therapy. The ventricles were sectioned into three regions (infarct, infarct border

zone and off-target (LV posterior wall)) for simultaneous DNA, protein and mRNA extraction using TRIzol (Life Technologies). Transcript expression of human-specific IGF-1 and mouse IGF-1 content was assessed using commercial PrimeTime qPCR Assays and Primers (Integrated DNA Technologies) with confirmed species specificity (Supplemental Figure 2). Transcript expression was normalized to GAPDH and analysis was performed using the $2^{-\Delta\Delta CT}$ method.[102]

Ventricular human cell engraftment of injected human CSCs was verified using qPCR for non-coding human DNA Alu sequences (Supplemental Figure 3)(Munoz et al, 2005; Latham et al, 2013). To evaluate the influence of enhanced IGF-1 expression on cardiac apoptosis, relative PCR expression of Bcl-2, Bax and p53 transcripts 7 days post CSC injection was evaluated using commercially purchased PrimeTime qPCR Assays and Primers (Integrated DNA Technologies). Expression was normalized to GAPDH and presented as a fold change in transcript expression relative to sham-operated mice.

3.7 Statistical analysis

All data is presented as mean ± SEM. To determine if differences existed within groups, data was analyzed by a one-way or repeated measures ANOVA; if such differences existed, Bonferroni's corrected t-test was used to determine the group(s) with the difference(s) (SPSS v20.0.0). In all cases, variances were assumed to be equal and normality was confirmed prior to further post-hoc testing. Differences in categorical measures were analyzed using a Chi Square test. A final value of $P \leq 0.05$ was considered significant for all analyses. All probability values reported are 2-sided.

4.0 RESULTS

4.1 Evaluation of the IGF-1 receptor following myocardial infarction

To rationalize the timing of IGF-1 delivery following myocardial infarction (MI), an experimental MI was performed in mice and the spatiotemporal expression of the mouse IGF-1 receptor and insulin receptor (IR) was evaluated using quantitative PCR. One, 7, 14 and 21 days post-MI, the infarcted left ventricle was sectioned into infarct, border zone and off target regions (posterior wall). An additional subset of mice that did not receive MI (thoracotomy operation only) served as the baseline sham-operated group for receptor normalization.

As early as 24 hours following experimental MI, the IGF-1R expression became significantly up-regulated by 1.4±0.7 fold within the infarct border zone when compared to sham-operated mice ($p=0.02$). IGF-1R transcript expression remained elevated thought the 21 days sampling period, with peak expression detected between 7 and 14 days after LAD ligation within the infarct zone (3.0±0.8 and 4.1±1.7 fold increase, respectively; $p<0.05$ vs. sham-operated mice). In contrast, the expression of IR following MI was not markedly altered after MI as compared to sham-operated mice (Figure 7a) (Supplemental Table 2 for quantitative PCR Cq values).

Immunohistochemical imaging of post-infarcted sections confirmed similar degrees of widespread strong expression of IGF-1R within ischemic border zone and infarct regions (Figure 7b; single stained sections Supplemental Figure 4). In contrast, IR was rarely observed and did not significantly change in response to myocardial injury. Taken together, this data demonstrates that IGF-1R expression is enhanced for 21 days after MI and rationalizes the timing of IGF-1 delivery to the infarcted myocardium.

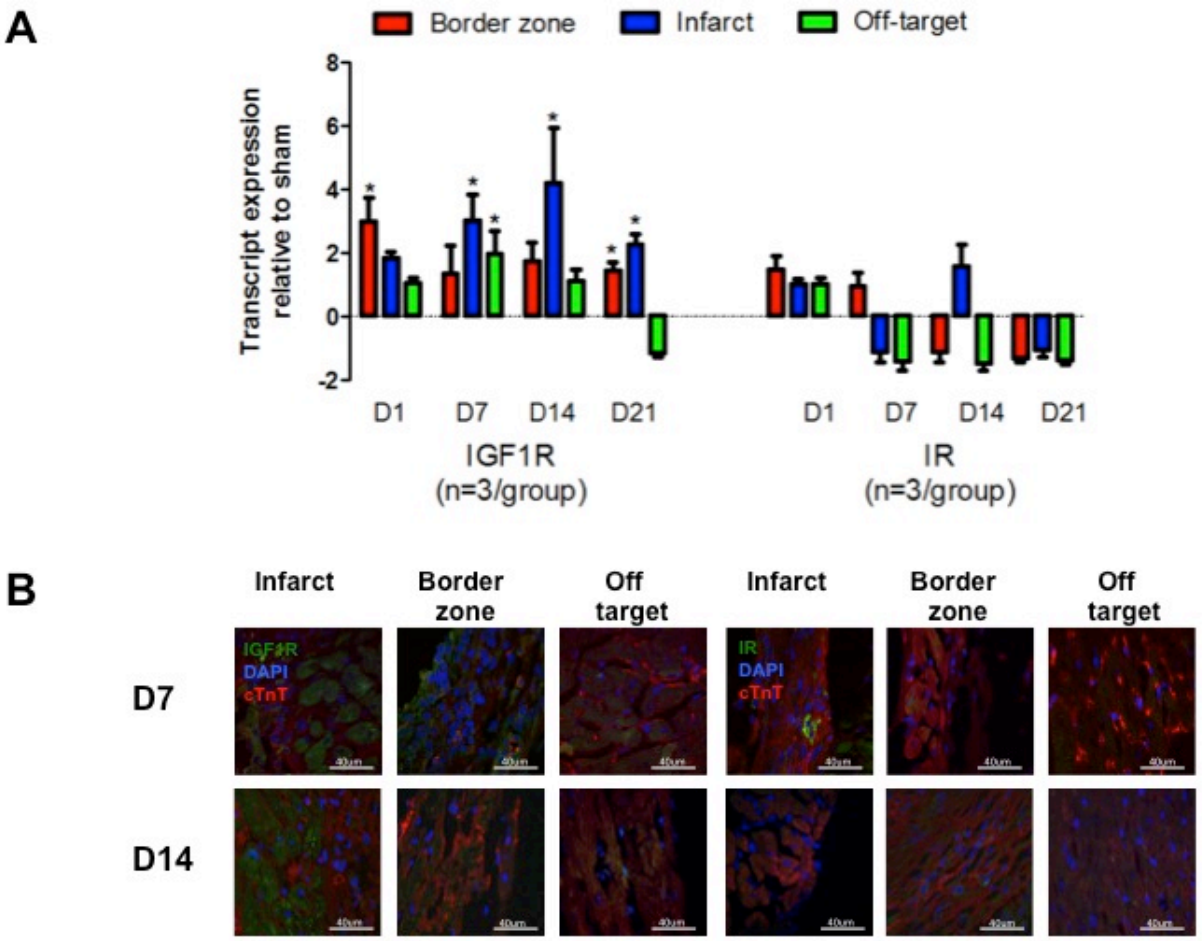


Figure 7. Spatiotemporal expression of IGF1-R rationalizes the delivery of CSCs genetically engineered to over-express IGF-1 soon after experimental myocardial infarction. A. Quantitative RT-PCR of IGF-1R expression and IR expression at 1, 7, 14 and 21 days post MI in mice (n=3 mice analyzed per time period) with mRNA transcript expression normalized to the sham operation.*$p<0.05$ vs. Sham-operated mice.

B. Representative immunohistochemical images of infarct, border zone and off target myocardium 7 and14 days post-MI in mice (n=3 mice analyzed per time period). IGF-1R: IGF-1 receptor; IR: Insulin receptor.

4.2 Patient demographics

Ten patients participated in this study (age 66±3 years; BMI 29±2 kg/m^2, Table 3). Patient atrial appendages were collected at time of surgery and immediately processed for CSC culture. CSCs obtained from all 10 patients were used for *in vitro* experimentation, with 6 patients also contributing to *in vivo* experiments. The majority of enrolled patients were male (90%) with a history of stable cardiac disease with numerous cardiovascular risk factors, including diabetes (50%), hypertension (70%) and dyslipidemia (80%). A high percentage of patients had a history of coronary artery disease (90%), MI (50%), valvular heart disease (50%) and a small percentage with congestive heart failure (10%). The majority of patients underwent elective cardiac surgery for coronary bypass alone (60%), valve repair/replacement alone (10%) or coronary bypass with valve repair/replacement (30%). Patients were on stable cardiac medications, including anti-platelet therapy (90%), beta-blockers (90%), statins (90%) and angiotensin-converting enzyme inhibitors and/or angiotensin receptor blockers (30%). Patients included for *in vivo* and *in vitro* studies demonstrated similar baseline clinical characteristics with no significant differences between groups. CSCs obtained from patients were characterized for cardiac (c-Kit) and mesenchymal (CD90) progenitors and were representative of previous publications (27±4% CD90+/c-Kit-, 2.6±0.5% c-Kit+/CD90- and 71±4% CD90-/c-Kit-; Supplemental Figure 5a).[28]

	All patients (n=10)	*In vitro* studies (n=10)	*In vivo* studies (n=6)
Age(yrs)	66±3	66±3	64±5
BMI (kg/m2)	29±2	29±2	28±2
Gender (%male)	90%	90%	83%
Diabetes	50%	50%	50%
Hypertension	70%	70%	67%
Dyslipidemia	80%	80%	83%
Ongoing smoking	20%	20%	17%
Thyroid disease	0%	0%	0%
Peripheral vascular disease	20%	20%	17%
Coronary artery disease	90%	90%	100%
History of MI	50%	50%	50%
Vavular heart disease	50%	50%	33%
Congrestive heart failure	10%	10%	17%
NYHA class	1.8±0.5	1.8±0.5	1.5±0.5
LV ejection fraction	50±5	50±5	50±5
CCS class	3.1±0.3	3.1±0.3	3.4±.4
Creatine (umol/L)	90±10	90±10	92±13
Medications:			
Anti-platelet therapy	90%	90%	83%
Beta-blocker	90%	90%	83%
Statins	90%	90%	83%
ACEI or ARB	30%	30%	33%

Table 2. Clinical characteristics of atrial appendage donors. Body mass index (BMI), myocardial infarction (MI), New York Heart Association (NYHA), left ventricle (LV), Canadian Cardiovascular Society (CCS), angiotensin-converting enzyme inhibitors (ACEI), angiotensin receptor blockers (ARB).

4.3 IGF-1 receptor systems in human CSCs

While human CSCs naturally produce low levels of IGF-1 (149±16 pg/ml*mg, n=3 CSC lines), the exact source of this cytokine production within the heterogeneous

CSC population is unclear. Sub-culture of isolated cardiac progenitor (c-Kit+/CD90-), mesenchymal progenitor (c-Kit-/CD90+) and double negative cells (c-Kit-/CD90-) demonstrated that IGF-1 production was equivalent in all 3 cell populations (Supplemental Figure 5c). Flow cytometry demonstrated that the IGF-1R or IR was expressed by 79±3% and 61±5 of CSCs, respectively (Supplemental Figure 5b). Of these, the cardiac progenitor (c-Kit+/CD90-) and mesenchymal progenitor (c-Kit-/CD90+) sub-populations had the greatest receptor expression (Chi square value 17.2 and 12.1, respectively; $p<0.01$ vs. the expected frequency of IGF-1R or IR in the c-Kit-/CD90- sub-population; Figure 8). This data demonstrates that CSCs produce modest amounts of IGF-1 and possess the capacity for IGF-1 mediated pre-conditioning to enhance survival.

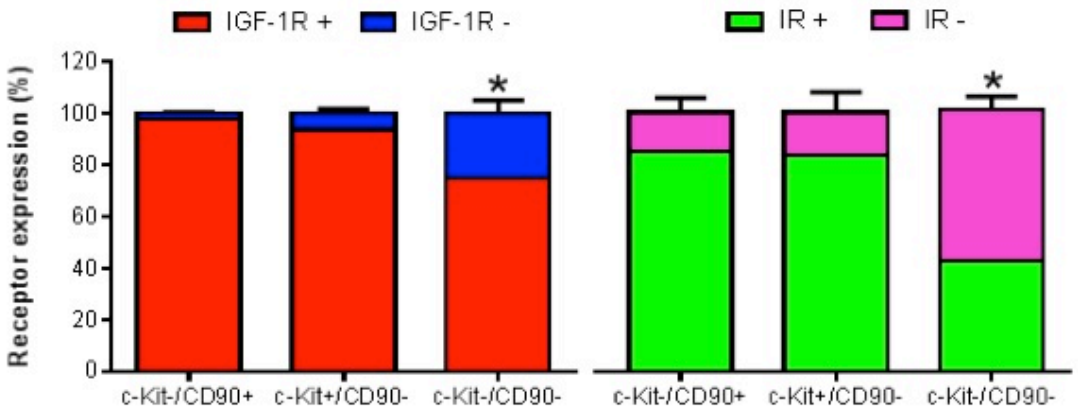


Figure 8. Over-expression of IGF-1 from the CD90+ CSC population may provide autocrine and paracrine CSC signaling. Flow cytometric analysis of IGF-1 receptor (IGF-1R) and insulin receptor (IR) co-expression on CSCs subpopulations (n=3). *$p<0.01$ vs. the expected frequency of IGF-1R or IR in the c-Kit-/CD90- sub-population.

4.4 Somatic gene transfer increases IGF-1 secretion without blunting the paracrine signature of CSCs

CSCs were genetically engineered to over-express IGF-1 by transducing isolated CD90+ cells with lv-IGF-1 and then recombined with the CD90- depleted fraction (Figure 4). To control for effects attributable to lentiviral transduction, a separate portion of each CSC patient line also underwent transduction of the CD90+ with the lv-GFP vector prior to recombination. As demonstrated in Figure 9a, somatic gene transfer provided discreet GFP and IGF-1 transcripts without evidence for a fused GFP-IGF-1 product. Flow cytometry demonstrated that 83±2% of the transduced CD90+ cells expressed the lv-IGF-1 GFP reporter 48 hours after transduction. The IGF-1 RNA transcript content within recombined lv-IGF-1 transduced CSCs was increased 3848±2565 fold as compared to baseline expression within lv-GFP treated control cells ($p=0.001$). Conditioned media from recombined lv-IGF-1 transduced CSCs demonstrated an MOI dependent increase in IGF-1 protein secretion (Figure 9b). Furthermore, the transduction of CSCs with lv-IGF-1 provided a peak 6.3±0.2 fold increase in IGF-1 content within conditioned media at an MOI of 20 (1206±290 vs. 149±39 pg/ml*mg in non-transduced CSC conditioned media; $p\leq0.05$) which was sustained for over 3 weeks in culture (1028±160 vs. 700.5±133 pg/ml*mg at 2 and 3 weeks, respectively; $p=0.15$).

Lentiviral transduction with lv-GFP or lv-IGF-1 at an MOI of 20 did not impair proliferation when compared to non-transduced CSCs (population doubling time: 41±10 or 45±8 hours vs. 43±18 hours, respectively; p=ns; Supplemental Figure 6). In contrast to lentiviral mediated over-expression of HIF-1a,[34] transduction of CSCs with lv-IGF-1 did not impair cytokine production (Chi square value 2.00, $p=0.55$ vs. the expected frequency

of cytokines in lv-GFP transduced CSCs; Figure 9c). Instead, all profiled cytokines trended towards an enhanced secretion with marked transforming growth factor beta (TGFβ1) production alone showing statistical significance (Chi square value 8.00; p=0.005; vs. the expected frequency of TGFβ1 in lv-GFP CSCs). This data confirms the notion that transduction of the mesenchymal sub-population within CSCs with lv-IGF-1 provides a reliable reservoir to enhance IGF-1 content while not harming the overall cytokine profile of CSCs.

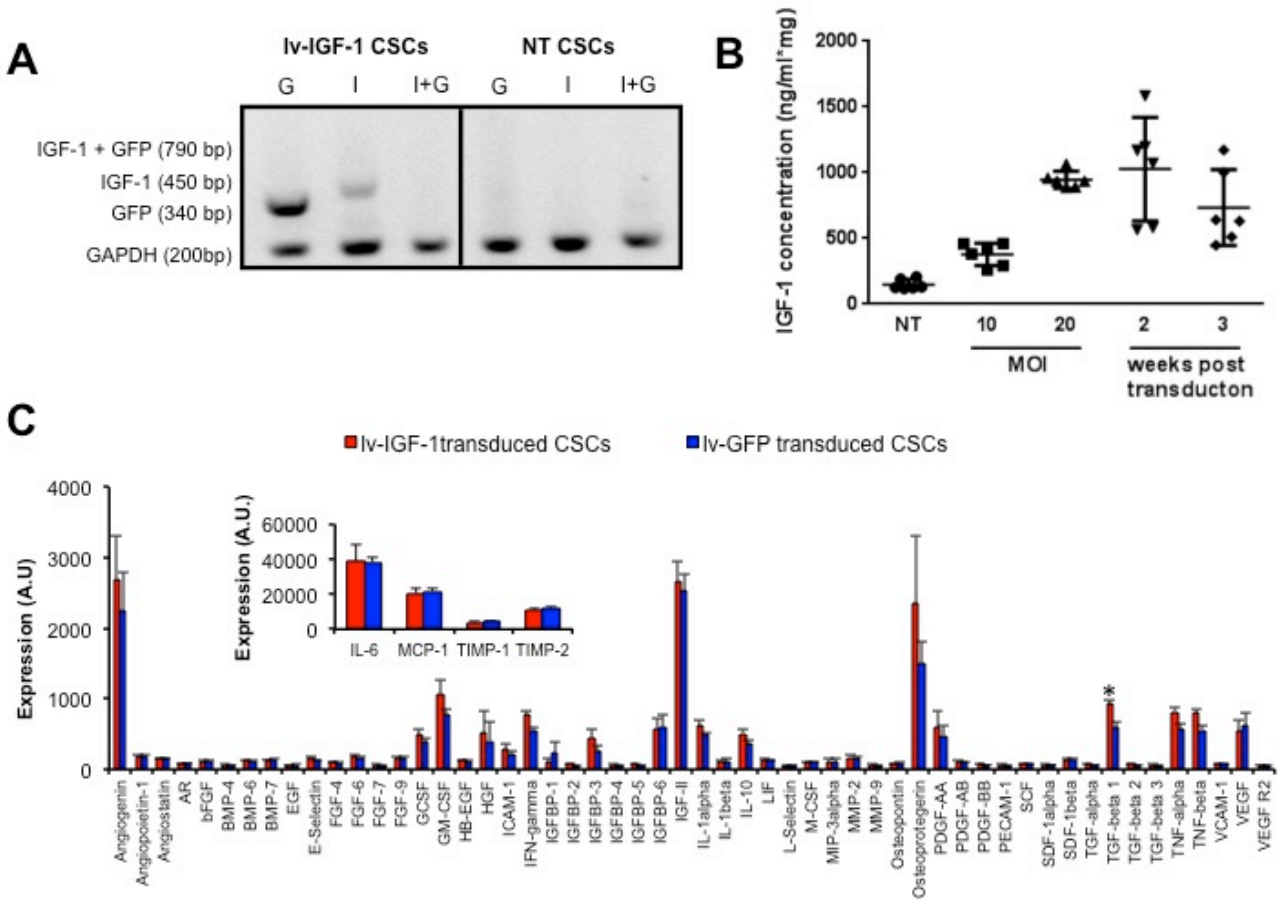


Figure 9. Lentiviral–mediated over-expression of human IGF-1 in CSCs. A. RT PCR of LV-IGF-1 and non-transduced CSCs demonstrating that somatic gene transfer provided discreet transcripts for GFP and IGF-1 without production of a GFP-IGF-1 fusion

product. B. MOI dependent secretion of human IGF-1 protein within media conditioned by CSCs 48 hours after recombination of the LV-IGF-1 transduced CD90+ sub-population with the non-transduced CD90- sub-fraction (n=3). * $p \leq 0.05$ vs. non-transduced CSCs. C. Densitometry analysis of growth factors produced by lv-IGF-1 transduced CSCs as compared to lv-GFP transduced CSCs (n=4). * $p \leq 0.05$ vs. lv-GFP CSCs.

4.5 Over-expression of IGF-1 by CSCs promotes CSC and cardiomyocyte survival

To evaluate the pro-survival and proliferative effects of IGF-1 within stress conditions, CSCs were cultured in hypoxic (1% O2) reduced serum (1%) media. CSCs genetically engineered to over-expressed IGF-1 demonstrated enhanced proliferation compared to lv-GFP CSCs and non-transduced CSCs (population doubling: 46±8 vs. 206±224 and -19±38 hours, respectively; $p \leq 0.01$; Figure 10a and Supplemental Figure 6). To study the pro-survival paracrine effects lv-IGF-1 CSCs may have on neighboring myocardium, neonatal rat ventricular myocytes (NRVMS) were cultured in CSC conditioned media. NRVMs subjected to 1% hypoxia in the presence of lv-IGF-1 CSC conditioned media demonstrated enhanced viability when compared to NRVMs cultured in lv-GFP or non-transduced CSC conditioned media (-15.2±4.7 vs. -20.4±2.9 and -24.1±2.2% viable NRVMs compared to baseline, respectively; $p \leq 0.01$; Figure 10a).

To assess the anti-apoptotic effects of lv-IGF-1 CSCs within CSCs as well as neighboring myocardium, both cell types were evaluated for the apoptotic marker annexin V using co-culture systems where (1) CSCs pre-labeled with DiO dye and NRVMs were allowed direct contact (direct co-culture) or (2) CSCs and NRVMs were separated by a porous membrane using a transwell insert (indirect co-culture). Two days

after 1% hypoxia, lv-IGF-1 CSCs exhibited lower expression of annexin V within both co-cultures compared to lv-GFP or non-transduced CSCs, respectively ($p \leq 0.05$; Figure 10b). Similarly, direct and indirect co-culture of lv-IGF-1 CSCs with NRVMs reduced expression of annexin V within both myocytes and co-cultured CSCs (Figure 10c).

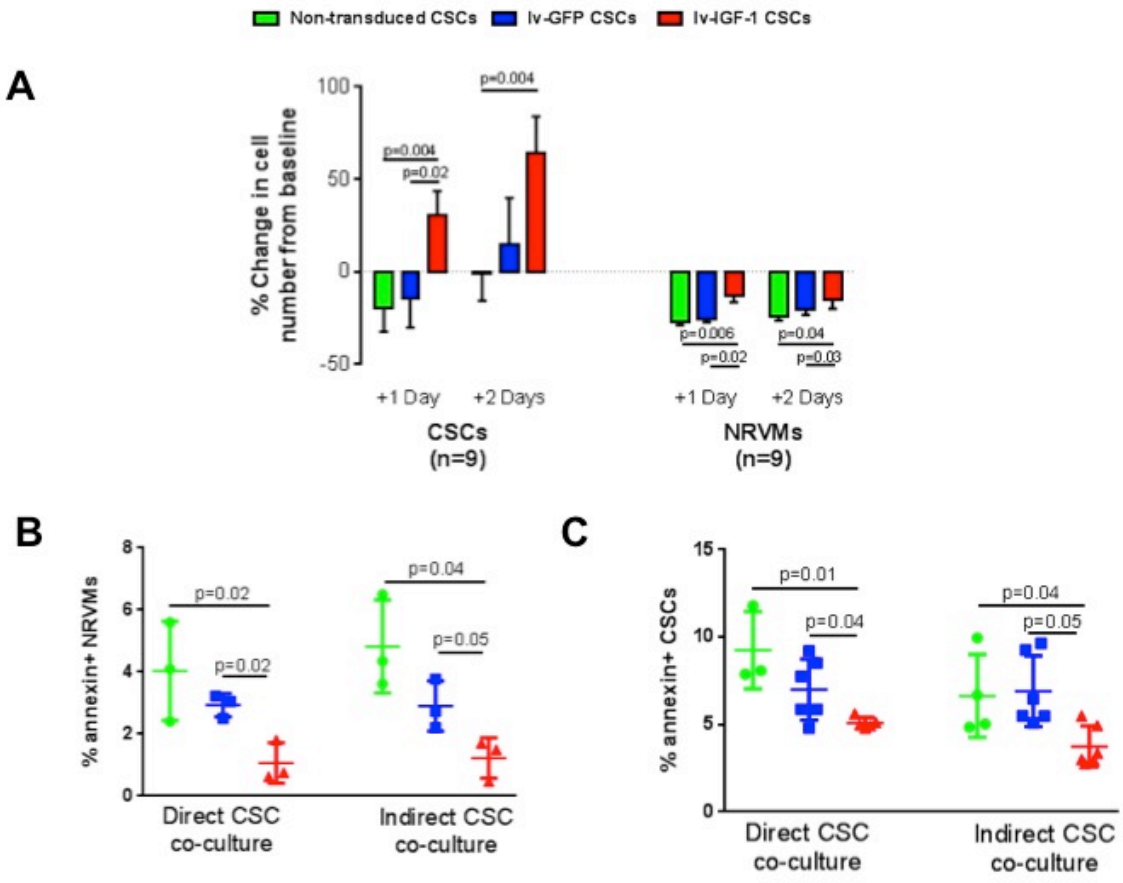


Figure 10. Over-expression of IGF-1 protects CSCs and NRVMs against hypoxic (1%) and low serum (1%) culture conditions. A. Change in the number of lv-GFP, lv-IGF-1 and non-transduced CSCs from the initial baseline count (+8 hours post plating) or NRVMs when cultured with conditioned media from lv-GFP, lv-IGF-1 and non-transduced CSCs. B. Analysis of flow cytometry demonstrating the effect of IGF-1 over-

expression on early markers of apoptosis (annexin V) on CSCs when directly co-cultured or indirectly co-cultured with NRVMs. C. Analysis of annexin V on NRVMs when directly co-cultured or indirectly co-cultured. NRVM: neonatal rat ventricular myocytes.

This pro-survival effect is likely mediated in part by the ability of lv-IGF-1 CSCs to stimulate pro-survival signalling within surrounding myocytes as demonstrated by the increased in BCL2+ NRVMs as compared to culture within lv-GFP or non-transduced CSC conditioned media (44.6±3 vs.30±3 and 25±3% respectively, $p \leq 0.005$; Figure 11a). In a manner similar, the role of necroptotic cell death was evaluated in lv-IGF-1 CSC cultures. As demonstrated in Figure 11b and 11c, the expression of proteins involved necroptosis (RIP1 and RIP3) were not influenced by lv-IGF-1. However, the apoptotic proteins involved in the decision of apoptosis rather than necroptosis (Caspase-8 and FADD) were significantly down-regulated by 2.9±1.3 and 2.8±0.8 fold within lv-IGF-1 compared to non-transduced CSC cultures, respectively ($p<0.05$). This data would suggest lv-IGF-1 CSCs plays a vital role in protecting CSC from apoptotic with little influence on necroptotic cell death.

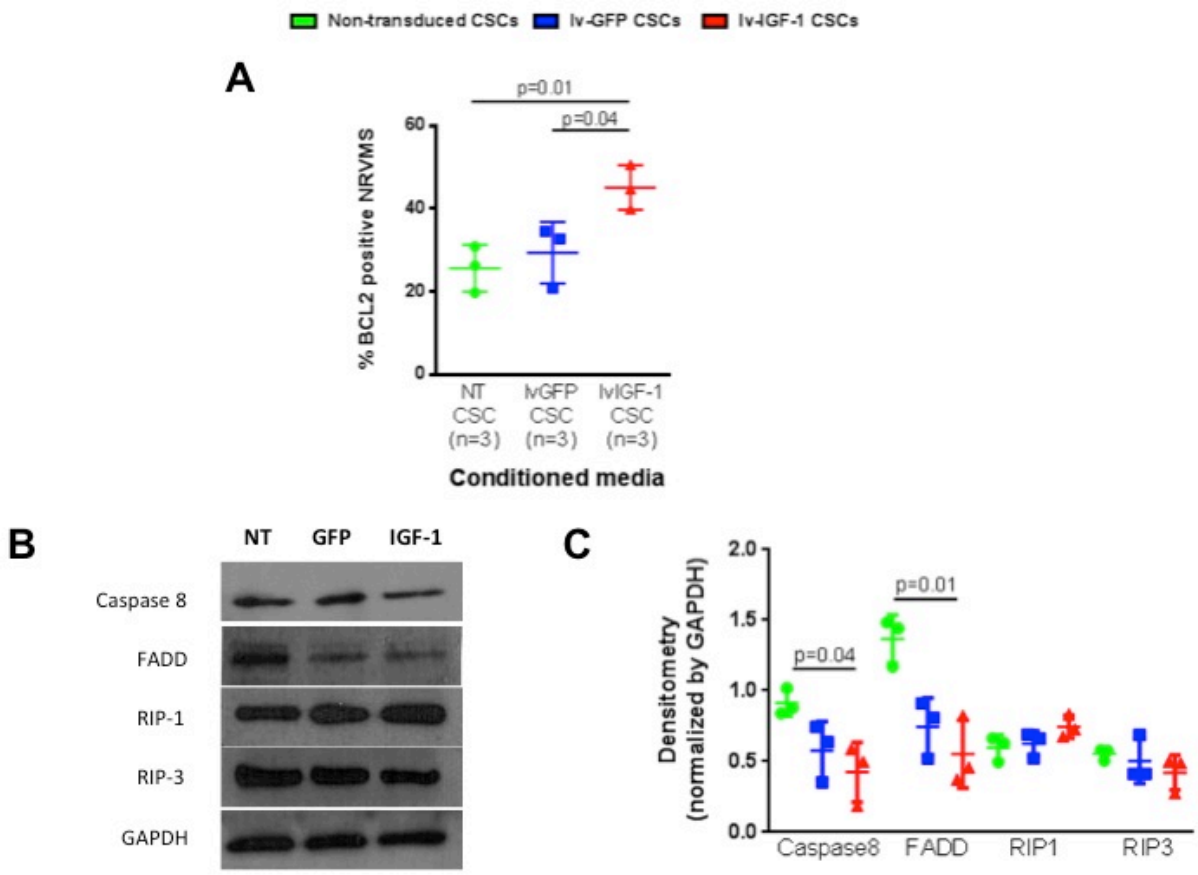


Figure 11. Evaluating the role of apoptosis and necroptosis in hypoxic (1%) low serum (1% FBS) culture conditions. A. Immunofluorescent analysis of BCL2 co-expression with NRVMs cultured with conditioned media from lv-GFP, lv-IGF-1 or non-transduced CSC conditioned media (n=3). B. Representative western blot images of proteins involved in necroptosis (Caspase 8, FADD, RIP1 and RIP3). C. Densitometry analysis of proteins involved in necroptosis (expression normalized to GAPDH) (n=3). FADD: Fas-associated death domain; RIP1: Receptor-interacting protein 1; RIP3: Receptor-interacting protein 3.

4.6 Mechanisms behind IGF-1 proliferative and anti-apoptotic actions in CSCs

To evaluate the molecular effects of IGF-1 enrichment on CSC proliferation and cell survival, the expression of Jun, Fos and Bcl-2 were quantified using relative PCR. CSCs were exposed to hypoxic (1% O2) low serum (1% FBS) and relative transcript expression was normalized to normoxic CSC (5% O_2 and 20% FBS). As demonstrated in Figure 12a, Stressed CSCs showed significant increases in Jun and Fos transcript profiles when compared to normoxic CSCs ($p<0.05$) suggesting ERK and MAPK pathway up-regulation may play a vital role in sustaining the viability of CSCs in hypoxia. In contrast, Bcl-2 expression was significantly reduced 1.5±0.2 fold ($p=0.01$) after hypoxic culture exposure suggesting the removal of serum and oxygen may be impairing Akt stimulation. When specifically evaluating the actions of lv-IGF-1 overexpression on CSC hypoxic cultures, there is a significant enhancement of all three pro-survival transcripts when compared to lv-GFP or non-transduced CSCs ($p<0.05$). This data suggests the enhanced stimulation of ERK, MAPK and Akt pathways within lv-IGF-1 CSC cultures may explain the sustained proliferative and survival actions under oxygen and serum deprivation.

To further understand the mechanisms of IGF-1 overexpression within CSCs on programmed cell death, the expression of 35 apoptosis-related proteins were analyzed using a commercial apoptosis proteome profiler array. CSC protein lysates were collected 48h after hypoxic reduced serum culture with lv-IGF-1 CSC protein expression presented as a fold change relative to lv-GFP CSCs. As demonstrated in Figure 12b (Supplemental Figure 7), the protein profile of important anti-apoptotic proteins such as Bcl-2, Bcl-x, HSP70 and survivin were elevated in lv-IGF-1 CSCs when compared to lv-GFP CSCs

($p<0.05$). This data further suggests IGF-1 may act as a pro-survival agent by enhancing anti-apoptotic proteins to withstand cellular stressors such as low oxygen and nutrient deprivation.

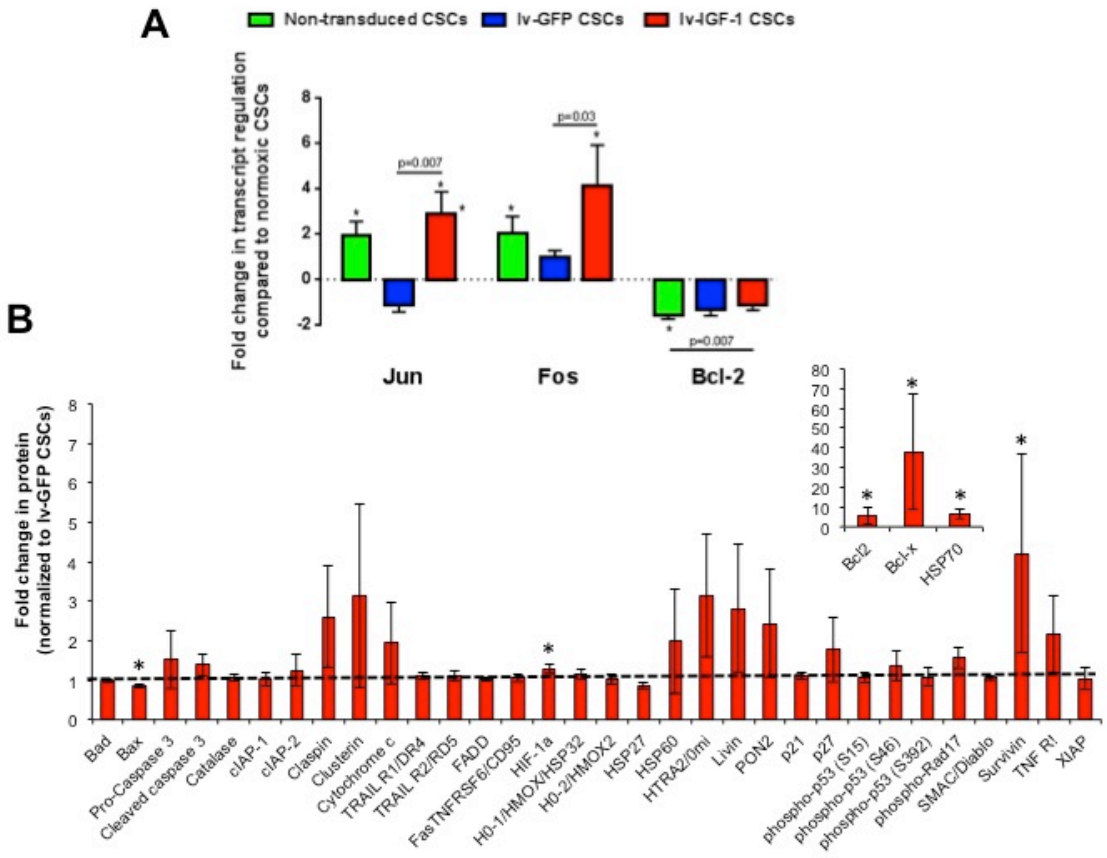


Figure 12. Over-expression of IGF-1 within CSCs enhances cell survival under hypoxia by up-regulating pro-survival transcripts and anti-apoptotic proteins. A. Quantitative PCR analysis of MAPK/ERK and Akt signaling pathway activation of lv-GFP, lv-IGF-1 and non-transduced CSCs after 48h of hypoxic (1%) and low serum (1%) exposure (transcript expression normalized to normoxic non-transduced CSCs) (n=4). *$p\leq0.05$ vs. normoxic CSCs B. The relative expression of 35 apoptosis related and stress activated proteins in lv-IGF-1 CSCs using a commercial human apoptosis proteome profiler array. Expression normalized to lv-GFP CSCs (n=4). *$p\leq0.05$ vs. lv-GFP transduced CSCs.

4.7 Over-expression of IGF-1 by CSCs enhances cardiac repair and engraftment of transplanted CSCs

The effect of lv-IGF-1 CSCs on cardiac repair was assessed in an immunodeficient mouse model of myocardial ischemia (Figure 5). As shown in Figure 13a, transplant of both lv-IGF-1 and lv-GFP CSCs increased human IGF-1 transcript in the infarct and border zone. Superior and sustained expression of IGF-1 within the infarcted myocardium was seen in animals treated with lv-IGF-1 CSCs until the end of follow-up (+ 21 days post intra-myocardial injection) (Supplemental Table 4 for PCR Cq values). Off target screening for human IGF-1 within serum collected 1, 7 and 14 days after CSC transplantation demonstrated that IGF-1 protein remained confined to the area of injection (Supplemental Figure 8a); obviating concerns regarding systemic teratogenicity. [103,104]

As shown in Figure 13b and Supplemental Table 3, mice treated with lv-IGF-1 or lv-GFP CSCs demonstrated greater left ventricular repair than PBS treated controls (41±1% or. 37±1% vs. 23±1%, respectively; $p \leq 0.05$) with superior benefits seen in lv-IGF-1 CSC treated mice ($p=0.01$ vs. lv-GFP CSC treated mice). Histology performed 21 days after CSC transplantation confirmed these results with lv-IGF-1 CSC treated mice having a 2.2±0.4 fold reduction in scar burden when compared to lv-GFP CSC treated mice (6±1% vs. 11±1% scar, respectively; $p=0.005$; Supplemental Figure 8b). While transplant of lv-IGF-1 CSCs did not markedly influence acute retention of transplanted cells, animals that received lv-IGF-1 CSCs demonstrated a progressive increase in long-term CSC retention such that CSC retention was increased 9.1±3.6 fold 21 days after intra-myocardial injection ($p=0.05$) as compared to injection of lv-GFP transduced CSCs

(Figure 13c; Supplemental Figure 8c). Immunohistochemical analysis of the human nuclear antigen (HNA) 21 days after CSC transplantation confirmed lv-IGF-1 CSC therapy boosted CSC engraftment when compared to lv-GFP CSC transplantation (8.5±2.2 vs. 3.2±0.7 HNA+ cells, respectively; p=0.05; Figure 13d) without altering CSC ability to differentiate into cardiomyocyte, smooth muscle or endothelial cell fate (Supplemental Figure 9).

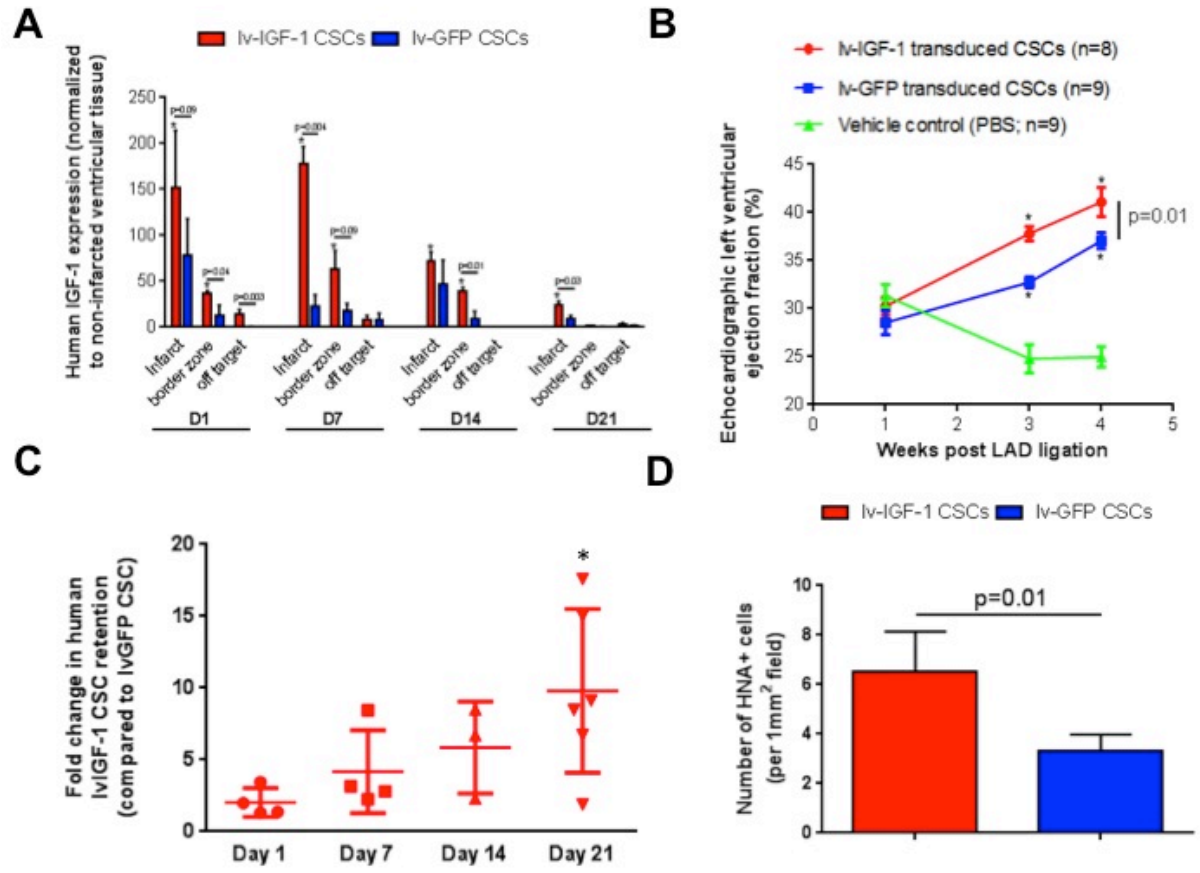


Figure 13. Transplantation of human CSCs genetically engineered to over-express IGF-1 enhances post-infarct cardiac repair. A. Spatiotemporal expression of human IGF-1 transcript within infarct, border zone and off target ventricular tissue in mice following lv-IGF-1 or lv-GFP CSC transplantation. *p<0.05 vs. non-infarcted (sham-operated mice). B. Effect of lv-GFP transduced CSCs, lv-IGF-1 transduced CSCs and vehicle intra-myocardial injection on NOD SCID mouse echocardiographic left ventricular ejection fraction. *p<0.05 vs. PBS injected. C. Relative PCR analysis of human lv-IGF-1 CSC retention following cell transplantation (normalized to lv-GFP CSCs). *p=0.05 vs. lv-GFP transduced CSCs. D. Immunohistochemical quantification of human nuclear antigen (HNA) 21 days post cell injection. *p<0.05 vs. lv-GFP transduced CSCs.

4.8 Transplantation of CSCs that over-express IGF-1 reduces myocardial apoptosis

To evaluate if lv-IGF-1 CSC therapy reduces myocardial cell death, ventricular infarct and infarct border zone regions were investigated for the degree of apoptotic pathways stimulation 7 days after CSC transplantation. Relative PCR using commercial primers against mouse Bcl-2, Bax and p53 was used to evaluate the degree of myocardial apoptosis (with transcript expression normalized to non-infarcted (sham-operated) ventricular tissue). As demonstrated in figure 14a, myocardial infarction significantly up-regulated the expression profile of Bcl-2, Bax and p53 within infarcted tissue when compared to non-infarcted tissue suggesting apoptosis may be participating in tissue injury at this time. Here, the transplantation of lv-IGF-1 CSCs significantly reduced infarct-related apoptosis as demonstrated by a 3.4±0.5 and 2.6±0.9 fold reduction in Bax and p53 expression ($p<0.05$) and a trend towards enhanced anti-apoptotic expression of Bcl-2 when compared to lv-GFP CSC transplantation (6.2±1.8 vs. 3.1±1.1 fold; $p=0.1$)(Supplemental Table 5 for PCR Cq values). This data is in support of lv-IGF-1 CSC therapy to reduce myocardial apoptosis and may explain reductions in infarct size as apoptosis has been linked to degree of myocardial damage.[6]

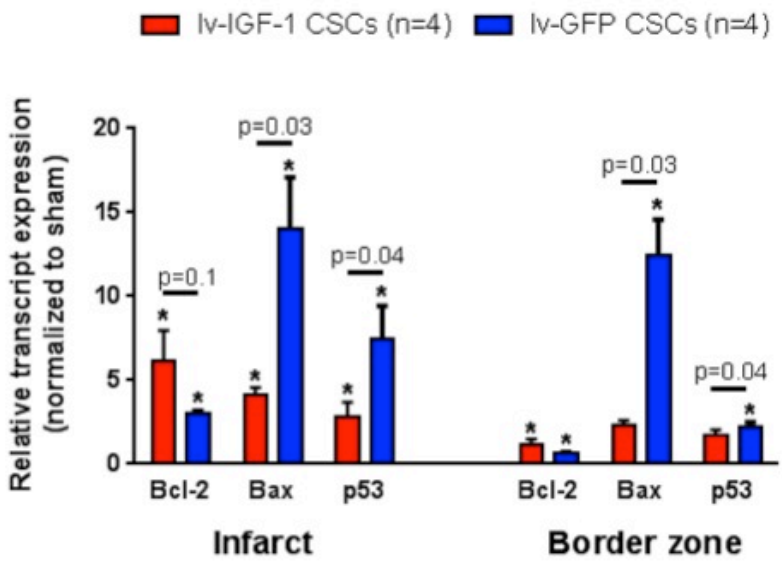


Figure 14. Transplantation of IGF-1 over-expressing CSCs reduces myocardial cell death after myocardial infarction in mice. Relative PCR analysis of apoptosis-related transcripts within infarct and border zones 7 days after experimental MI. Relative transcript expression is presented as fold change relative to sham-operated mice. *$p<0.05$ vs. sham-operated mice.

5.0 DISCUSSION

First generation CSC therapy has shown to improve cardiac function and reduce scarring after myocardial injury.[13, 14, 28, 29] However, these benefits are observed in the absence of robust cell engraftment suggesting CSC repair is largely driven by the paracrine release of growth factors and their trophic actions on damaged host myocardial tissue.[26] Apoptosis is thought to play a key role in myocardial cell loss and scarring following myocardial damage.[6] Given the pro-survival and anti-apoptotic role of IGF-1, this cytokine was enriched within our CSC population prior to cell transplantation in light of improving the endogenous repair process. Here, we have demonstrated that the genetic enhancement or "priming" CSCs with IGF-1 enables these cells to sustain proliferation and survival by the up-regulation of pro-survival genes when introduced into culture conditions that mimic the ischemic myocardium. In the mouse model of myocardial ischemia, the transplantation of IGF-1 enriched CSCs boosted transplant cell engraftment, reduced myocardial apoptosis and enhanced cardiac performance. This data suggests lv-IGF-1 CSC therapy may provide superior cardiac repair by promoting transplant cell survival thereby prolonging the release of IGF-1 to reduce myocardial apoptosis and promoting myocardial salvage.

5.1 Rational for selecting IGF-1 as a therapeutic target for overexpression

IGF-1 was selected for genetic enhancement within CSCs as is it has potent pro-survival and cardio-protective effects. [35, 36, 47, 48, 49, 50, 72, 73] Since IGF-1 is not robustly expressed by our CSCs, the aim of this study was to enhance its expression to promote transplanted cell survival and subsequently sustain the paracrine actions of CSCs on

endogenous repair and/or direct differentiation into working myocardium. IGF-1 has well documented actions on promoting cellular survival in hypoxic environments[105] and its receptor becomes up-regulated within myocardial tissue after myocardial infarction,[106] further rationalizing the use of IGF-1 as a therapeutic target for cardiac repair.

Other IGF family members such as IGF-2 and insulin were considered as they have growth-regulating actions via the IGF-1R. However, IGF-2 is thought to be involved in CSC maturation, death and myocyte differentiation rather than cell survival[75] and insulin has shown to negatively influence cardiac function.[107] Therefore, this study specifically targeted IGF-1 overexpression as this IGF family member has shown to consistently promote cell survival and cardiac repair in preclinical animal models.[92,75,108] However, a combinational approach of overexpressing more than one cytokine would be the next steps as it may provide synergistic actions on the repair process. For example, the combination of IGF-1 (pro-survival cytokine) with a cell recruitment cytokine (SDF-1) may show great therapeutic promise. Although the combination of different cytokines does not fall within the scope of this project, it warrants future investigation.

5.2 Evaluating the responsiveness of the infarcted myocardium and CSCs to IGF-1

The results of the present study indicate that the paracrine engineering of CSCs to overexpress IGF-1 enhances cardiac repair when transplanted at a time in which the IGF-1R is most elevated. Investigations that evaluate the responsiveness of both transplanted cell and its host myocardium to the IGF-1 ligand are limited. This is an important feature to examine, as IGF-1 requires its receptor (IGF-1R) to mediate beneficial effects. Therefore, this study also sought to evaluate when they myocardium is most responsive

to IGF-1 and if CSCs are adequately equipped with the IGF-1R for autocrine/paracrine signaling.

Previous work by Cheng and colleagues have demonstrated IGF-1R becomes up-regulated with myocardial infarction and that receptor expression is elevated 7 days post infarction.[106] Here, we wanted to further explore the spatiotemporal profile of IGF-1R and to determine if IGF-1R expression remains elevated 7 days after infarction.[106] Like Cheng and colleagues, we have found the IGF-1R becomes up-regulated following myocardial infarction. Further IGF-1R profiling revealed peak expression between day 7 and day 14 suggesting maximal IGF-1/IGF-1R axis stimulation may occur at this time. From this data we were able to select the 7 days post-MI time point for IGF-1 delivery.

Like the myocardium, CSCs are equipped with the IGF-1R suggesting they may participate in autocrine/paracrine IGF-1 signaling. However, the exact distribution of the IGF-1R within the heterogeneous CSC populations has not been well studied. Previous studies by D'Amario and colleagues have focused on the c-Kit+/IGF-1R+ population and have found that c-Kit+ CSCs co-express the IGF-1R.[75] However, the question if other CSC antigens co-localize with IGF-1R has not been addressed. Here, we expand our knowledge on the IGF-1R co-localization within the progenitor (cKit+), mesenchymal (CD90+) and lineage negative (c-Kit-/CD90-) CSC populations. We also investigated the degree of IR co-localization since higher concentrations IGF-1 can also activate the insulin receptor.[109] By flow cytometric analysis, we found that the majority of CSCs express the IGF-1R and IR, with enhanced receptor expression within the c-Kit+ and the CD90+ subpopulations suggesting these populations have substantial potential to engage in IGF-1 signaling. From this data, we can appreciate the potential of stimulating the

IGF-1 system within infarcted myocardium as the receptor remains elevated and transplanted cells have the ability to respond and amplify the pro-survival actions of IGF-1.

5.3 Lentiviral approach to enhance IGF-1 gene expression in CSCs

CSC cytokine balance plays a pivotal role in functional outcomes on cardiac repair and great care is required to successfully improve the CSC secretome.[34, 110] This is apparent by initial attempts to broaden the CSC paracrine profile where viral overexpression of HIF-1a did not translate to improvements in CSC-mediated repair.[34] These initial attempts were surprising, as HIF-1a overexpressing transgenic mice have shown great promise in attenuating infarct size and improving cardiac function after myocardial infarction.[111] HIF-1a is also known to play an important role in the cellular response to hypoxia and can regulated genes for angiogenesis suggesting transgene selection may have not been the sole reason for study shortcomings. Rather, the viral modification of entire CSC population may have disrupted the overall paracrine balance. In a similar manner, the isolation and injection of c-Kit purified CDC populations into infarcted mice was found to reduce cardiac function when compared to the transplantation of the heterogeneous CDC population further suggesting paracrine balance plays a pivotal role in cell-mediated cardiac repair process.[110]

As such, the present study selectively targeted the mesenchymal CSC fraction for GFP or IGF-1 lentiviral-mediated gene transfer with heterogeneous population restoration by recombining with the mesnchymal depleted population. In culture, the viral application did not negatively impact CSC viability, proliferation or blunt the secretion of

cytokines from CSCs; rather we observed an enhanced secretion of TGF-β1. Furthermore, the transplantation of lv-IGF-1 CSCs into infarcted mice demonstrated a superior therapeutic outcome suggesting that targeting of mesenchymal CSC subpopulation may alleviate such concerns associated with the initial attempts by Bonios *et al.* (2011). The current study findings are consistent with unpublished work from our lab that similarly transduced the mesenchymal CSC subpopulation with lv-SDF-1α and has demonstrated function improvements in a mouse model of myocardial ischemia.

5.4 Understanding proliferative actions of IGF-1 over-expressing CSCs

In culture, we assessed CSCs proliferative abilities under hypoxic and low serum conditions in order to mimic the environment CSCs would encounter when injected into the ischemic myocardium. The overexpression of IGF-1 within CSCs enhanced cell proliferation when compared to lv-GFP and non-transduced CSCs under low oxygen and serum conditions. This is consistent with literature reports of IGF-1 stimulating cell cycle progression.[112, 113]

In the present study, the enhanced proliferative potential by lv-IGF-1 CSC can be explained by their enhanced MAPK/ERK pathway stimulation as both Jun and Fos transcription factors were elevated. These results are expected as Jun and Fos can form heterodimers (forming the AP-1 early transcription factor) to influence gene expression of the cell cycle.[114] More specifically, Jun has been shown to directly control the transcription of cyclin D1, which is required for the G_1 to S phase progression in the cell cycle.[115] Work by Schreiber et al. (1999) have also demonstrated Jun overexpression is associated with accelerated proliferation in fibroblast cells whereas Jun knockouts have

severe defects in cell proliferation.[116] Therefore the association of elevated expression of both Jun and Fos with IGF-1 enriched CSCs may explain the enhanced rates of proliferation observed after culturing CSC in low oxygen and serum media. It should also be noted that the transduction of CSCs with lv-IGF-1 also elevated the expression of TGF-β1, which has also been know to stimulate cell proliferation via the TGF beta receptor.[117] Taken together, the preconditioning of CSC with lv-IGF-1 faithfully enhanced proliferation with corresponding increases in both Jun and Fos expression.

In this study, we also investigated the proliferative potential of lv-IGF-1 CSCs under normal serum and oxygen media conditions (5% O_2, 20% FBS). Interestingly, normoxic lv-IGF-1 CSCs had no significant change in cell proliferation between lv-GFP or non-transduced CSCs. Similarly there was no change in the expression of Jun or Fos transcription factors. Perhaps the overexpression of IGF-1 within CSCs acts more to sustain proliferation as population doubling times between lv-IGF-1 hypoxic and lv-IGF-1 normoxic were found to be nearly identical (45.2 vs. 46.1 hours). This data would suggest that at the "priming" of CSC with IGF-1 enhances their ability to withstand proliferative declines associated with oxygen-nutrient depravation.

5.5 Understanding how IGF-1 over-expressing CSCs boost cell survival

To evaluate the ability of IGF-1 over-expressing CSCs to withstand cell death, both apoptosis and necroptosis were evaluated following hypoxic and low serum culture conditions. Although direct evaluation of IGF-1 on necroptosis is limited, IGF-1 has been shown to mediate protection of dystrophic myofibers from necrosis.[73] Unlike Shavlakadze *et al.*, (2004) we did not see any influence of IGF-1 overexpression on

necroptosis suggesting IGF-1 might primarily promote cell survival through a mechanism of reducing apoptosis via the up-regulation of anti-apoptotic factors or pro-survival factors. This was first evident by western blot analysis of caspase 8, FADD and RIP proteins. Although there is crosstalk between the initiation of the necroptotic and apoptotic pathway, necroptosis has been found to prevail in the absence of caspases.[80] Here, our analysis demonstrated lv-IGF-1 CSCs had reduced FADD and caspase-8 expression with no change in RIP proteins suggesting CSC survival may be mediated through reducing apoptotic cell death rather than necroptotic cell death.

The idea that lv-IGF-1 CSCs can reduce apoptotic cell death was further confirmed in the apoptosis proteome profiler array in which anti-apoptosis proteins (Bcl-2, Bcl-x survivin and HIF-1a) were found to be up-regulated while the pro-apoptotic protein Bax was found to be down-regulated. The observed up-regulation of Bcl-2 and Bcl-x is in accordance with a known mechanism by which growth factors such as Epo, IL-2, VEGF promote cell survival.[118] There is also molecular evidence linking IGF-1 signaling and Bcl-2 expression via Akt phosphorylation, CREB activation and c-AMP-response element (CRE) binding to a site within the Bcl-2 promoter.[119] In accordance with literature, Bcl-2 was found to be elevated at the RNA and protein level within lv-IGF-1 CSCs. Bcl-2 up-regulation was also observed in myocytes co-cultured with lv-IGF-1 as well as infarcted mouse myocardium following lv-IGF-1 CSC injection. Therefore, the observed up-regulation of Bcl-2 appears to play an important role by with lv-IGF-1 CSC promote cell survival and elicit functional benefits following injection into ischemic myocardium.

5.6 Evaluating the mechanisms by which IGF-1 over-expressing CSCs promote myocardial repair: promoting transplant cell survival and persistence

Although the use of cell therapy has shown promise in the clinical arena, one major limitation is that the transplanted cells do not persist. As such, the main mechanism behind the positive outcomes is mediated by the paracrines secreted by the transplanted cells.[26] The second and less operative mechanism to explain the benefits from cell therapy is that the cells engraft, differentiate and form functional myocardium. Firbrin glue experiments to prevent cell leakage and enhance cell retention in the post-MI setting have shown to improve cardiac repair.[33] In the present study, we used an alternative approach to promote transplanted cell survival and engraftment through the genetic enhancement of IGF-1. In keeping with the Terrovitis study, our enhanced cell engraftment was associated with improved cardiac function and reduced myocardial damage after lv-IGF-1 CSC transplantation. Although deciphering the exact mechanism by which lv-IGF-1 CSCs can promote myocardial repair is challenging, the present study suggests the underlying principle is sustained cell persistence as more cells are capable of 1) participating in the paracrine release of growth factors to promote endogenous repair (e.g. sustained release of IGF-1 to reduce myocardial apoptosis) and 2) participating in the differentiation of CSC into working myocardium.

Myocardial infarction has been shown to induce apoptotic myocyte death and consequently determine the degree of infarct damage and myocardial scarring.[6] As IGF-1 has known actions of blunting the apoptotic pathway, it seems logical that the sustained release of IGF-1 within ischemic regions would have positive outcomes on the degree of infarct damage. Furthermore, approaches that can sustain IGF-1 protein release have

shown to have salutatory effects on cardiac repair and regeneration process.[94] Therefore, one aim of this study was to use CSCs as a reservoir for constitutive expression of IGF-1. Results from the present study demonstrate the delivery of lv-IGF-1 CSCs enhances and sustains human IGF-1 expression, with little off target detection (posterior ventricular wall and blood circulation). In accordance with enhanced IGF-1 secretion, there was also reduced myocardial scarring and apoptosis (Bax and p53) after lv-IGF-1 CSC transplantation. This data suggests lv-IGF-1 CSCs may promote myocardial repair by reducing the degree myocardial apoptosis.

The second mechanism to explain the observed functional benefits of lv-IGF-1 CSC transplantation is more cells are being retained and capable of differentiating into myocardium. In this study, the histological examination of infarcted mouse myocardium 21 days following lv-IGF-1 or lv-GFP CSC delivery demonstrate more human cells are detected within infarct regions, without altering cell fate into cardiomyocyte, endothelial or smooth muscle. This data is consistent with in culture and *in vivo* demonstration of CSCs having the ability to differentiate into functional myocardium.[18, 19]

5.7 Study Limitations

In the present thesis, CSCs were obtained from aged patients that were undergoing bypass or valve repair with numerous cardiovascular risk factors such as diabetes, hypertension, coronary artery disease, vavlular heart disease and/or heart failure. Although CSCs were obtained from a population representative of potential future recipients of autologous CSC therapy, “young or healthy” atrial tissue did not contribute to this study. Although literature is limited, certain comorbidities may alter the

efficacy of the CSC product obtained from sick or diseased patients. Work by Cesselli and colleagues have shown CSCs obtained from aged or heart failure patients have dysfunctional telomeres and decreased function.[120] Similarity, unpublished data from our lab suggests CSCs obtained from patients with high LTS scores (LTS: long term risk stratification model for survivors of an acute coronary syndrome) or patients with diabetes negatively correlate with CSC function. It should also be noted that aging is associated with a reduced fraction of IGF-1 responsive CSCs (IGF-1R+).[75] Although the effects of age and disease on CSC function are still debated [121] the absence of "young/healthy" CSC contributions may have influenced the results in this study. CSCs generated from aged/diseased tissue may have a greater restorative potential compared to CSCs obtained from healthy or young patients as these cells may already be adequately equipped with endogenous IGF-1 or functional mechanisms to sustain cellular survival.[122] It should also be noted that the majority of CSC donors were male (90%). Although this probably did not influence study outcomes as sex has not been found to alter the CSC function, secretion of IGF-1 or expression of IGF-1R within CSCs.[75]

A second limitation in this thesis is the use of a human immunodeficiency virus (HIV) based lentiviral system to overexpress IGF-1 under the control of a constitutively active CMV promoter. The use of lentivirus was selected for this basic framework study to evaluate the feasibility and therapeutic benefits offered by lv-IGF-1 CSC transplantation. Clinically speaking, lentivirus may offer concerns of oncogenicity as this virus can randomly integrate into its host's genome.[123] Constitutive CMV expression of IGF-1 also possesses concerns for oncogenicity;[103, 104] yet no mice were found to have tumors or systemic elevations of IGF-1. Nonetheless, the use of an adeno-associated virus

(AAV) would provide a safe alternative as this virus specifically integrates within a non-coding region on chromosome 19.[124] Furthermore, the use of cardiac-specific AAV serotypes, cardiac-specific promoters or inducible promoters may be required for clinical translation as they would provide gene control to areas within the heart and reduce undesirable off-target tissue expression and tumor development.[125, 126]

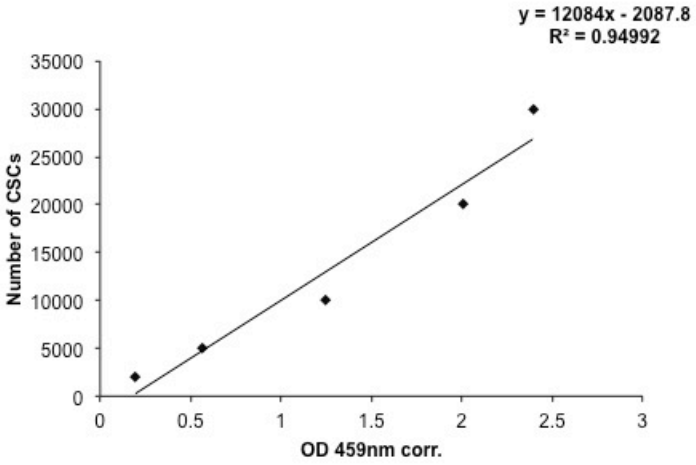
In addition to the mentioned limitations, questions still remain as to what cellular pathways are responsible for lv-IGF-1 CSC pro-survival and proliferative actions. In the present study we believe the preserved proliferative function of lv-IGF-1 CSCs is mediated by the ERK/MAPK pathway stimulation as Jun and Fos transcripts were found to be elevated. For the anti-apoptotic actions of lv-IGF-1 CSCs we believe this is mediated through Akt stimulation and subsequent up-regulation of anti-apoptotic proteins (Bcl-2 Bcl-x, surivivin). However HIF-1α and TGF-β1 were also found to be up-regulated in lv-IGF-1 CSC cultures suggesting more than one pro-survival system may be contributing to the observed functional benefits. To make pathway identification even more difficult there is crosstalk among pathways. For example the roles of Bcl-2 family members is not limited to anti-apoptotic action as they have been shown to affect cell cycle via the mitotic kinase Polo-like kinase 3 (PLK2).[118] Similarity, Jun does not solely act on cell proliferation as it can also play a role in apoptosis.[127]

In summary, we strongly believe it is a combination of many pro-survival/anti-apoptotic proteins working together to sustain cell proliferative and cell survival. Further work that selectively inhibits MAKP/ERK or Akt mediators may provide further insight as to what pathways may be providing IGF-1 cytopotection and functional beneficial effects *in vivo*.

6.0 CONCLUSIONS

The genetic enhancement of IGF-1 in human CSCs improves cardiac repair when delivered 7 days after myocardial infarction in mice. The genetic "priming" of CSCs with IGF-1 promotes transplant cell survival, boosts long term CSC engraftment and delivers sustained and local IGF-1 to damaged myocardium. This increase in IGF-1 content was associated with the up-regulation of pro-survival factors and reduced myocardial apoptosis in mice 7 days after lv-IGF-1 CSC transplantation. In summary, IGF-1 enriched CSCs enhance cardiac repair by boosting transplanted cell survival and reducing myocardial apoptosis to improve myocardial function and salvage of damaged myocardium.

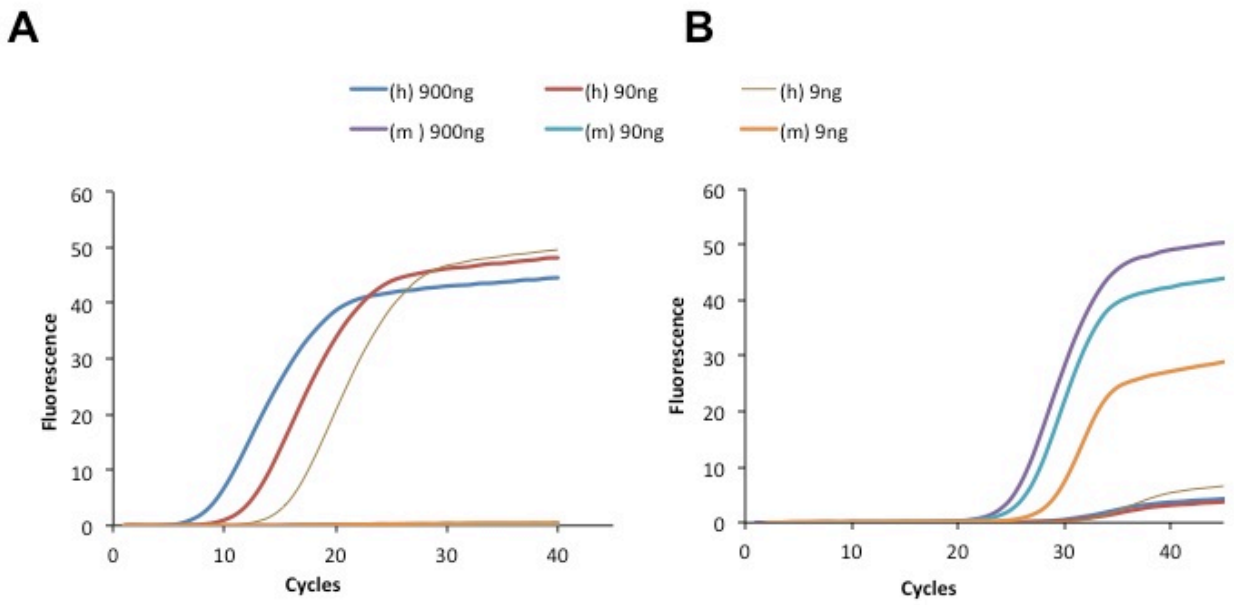
7.0 SUPPLEMENTAL FIGURES



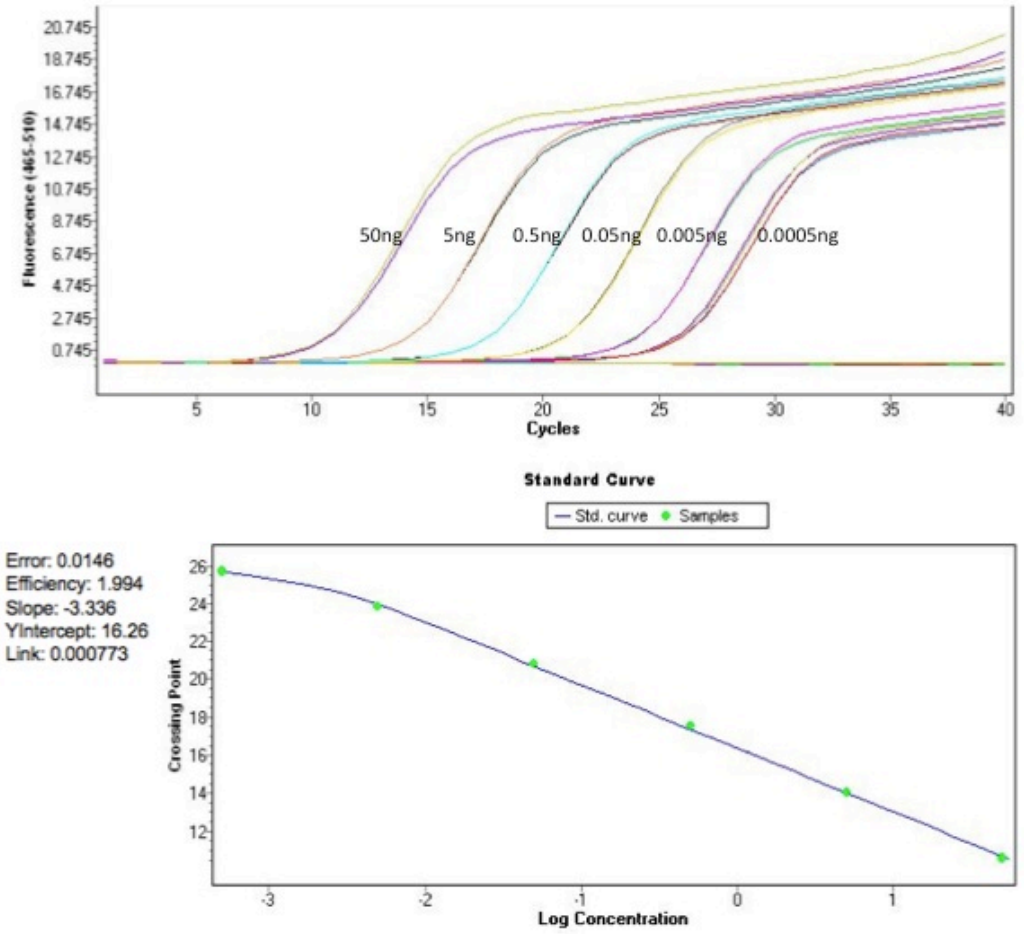
Supplemental Figure 1. Standard curve of human CSCs using the colorimetric WST-8 assay.

	Forward	Reverse	Probe
IGF1-cDNA	CGCCCCGGGATGGGAAAAATCAGC AGTCTTC	CGCGTCGACCTACTTGCGTTCTTCA AATGTA	NA
Human IGF-1	GAAGGTGAAGATGCACACCA	TTGGTAGATGGGGGCTGATA	NA
Human IGF-1	GCC TCCTTAGATCACAGCTC	GAT GCTCTTCAGTTCGTGTGT	ACTGCTGGAGCCATA CCCTGTG
Mouse IGF-1	ATCCACAATGCCTGTCTGAG	TGGATGCTCTTCAGTTCGTG	TGTTGAAGTAAAAGCCCCTCGGTCC
Pan GAPDH	ATGCCAGTGAGCTTCCCGTTCAGC	ACTGCCACCCAGAAGACTGTGGAT	TGCCAAGGCTGTGGGCAAGGTCAT

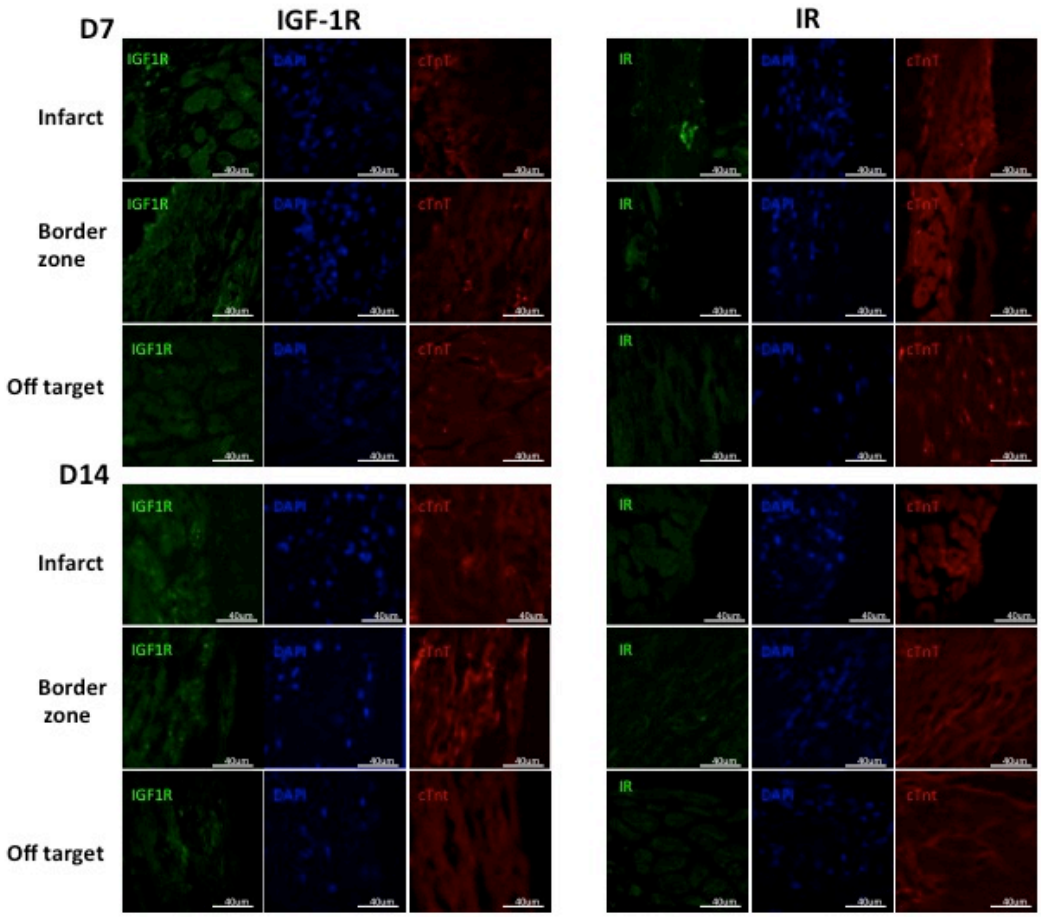
Supplemental Table 1. Primers for RT PCR and qPCR. NA=not applicable.



Supplemental Figure 2. Species-specific amplification of RNA transcripts. A. Detection of human-specific IGF-1 transcript after serial dilution of human RNA template into mouse RNA using a custom human specific IGF-1 PCR primers. B. Detection of mouse-specific IGF-1 transcript after serial dilution of mouse RNA template into human RNA using a custom mouse specific IGF-1 PCR primers.



Supplemental Figure 3. Human-specific Alu qPCR amplification. Alu standard curve generated with 50ng of human DNA that was serially diluted into mouse DNA for a total loading amount of 50ng of DNA.

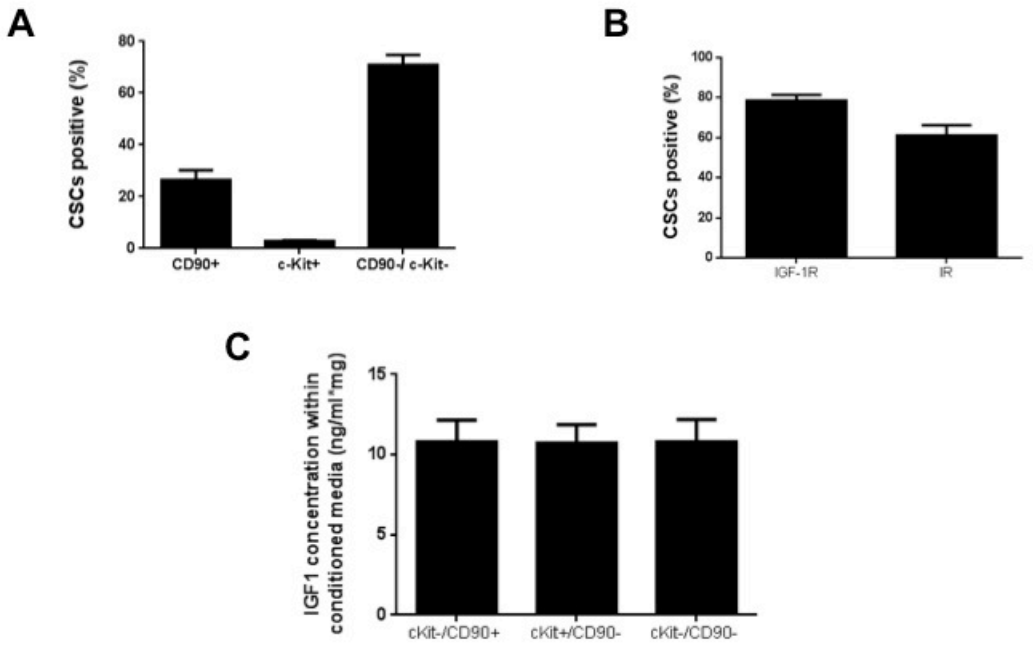


Supplemental Figure 4. Immunohistochemical staining of IGF-1R and IR 7 and 14 days post myocardial infarction in mice. IGF-1R: insulin-like growth factor-1 receptor; IR: insulin receptor; DAPI: nuclear stain; cTnT: cardiac troponin.

	IGF-1R $Cq_{(IGF-1R)-(GAPDH)}$	IR $Cq_{(IR)-(GAPDH)}$
Day 1		
Infarct	6.3±0.05	4.9±0.1
Border zone	6.2±0.2	5.0±0.06
Off target	6.9±0.2	4.8±0.05
Day 7		
Infarct	5.6±0.3	5.1±0.0-6
Border zone	6.2±0.4	5.0±0.2
Off target	6.8±0.06	5.2±0.1
Day 14		
Infarct	4.9±0.3	4.2±0.4
Border zone	6.2±0.2	5.0±0.1
Off target	6.8±0.2	5.4±0.2
Day 21		
Infarct	6.0±0.05	5.0±0.1
Border zone	6.6±0.3	5.2±0.3
Off target	7.2±0.1	5.2±0.1
Sham-operated		
Infarct	7.16±0.06	5.0±0.3
Border zone	7.1±0.05	4.8±0.07
Off target	7.0±0.03	4.8±0.05

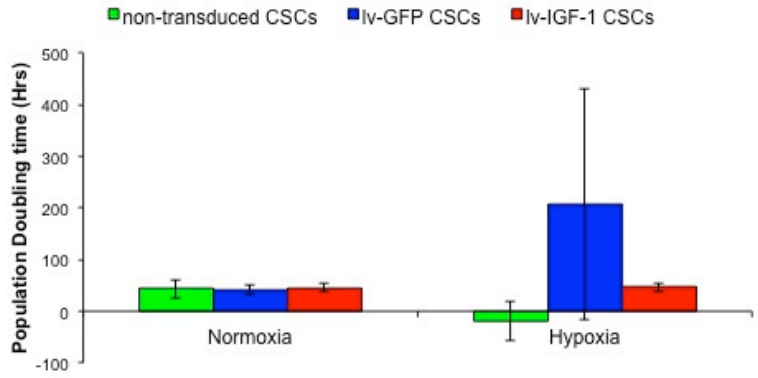
*p≤0.05 vs. sham operated
†p≤0.05 vs. lv-GFP CSCs

Supplemental Table 2. Relative PCR Cq values for IGF-1R and IR expression post myocardial infarction in mice.



Supplemental Figure 5.Characterization of CSC populations used for experimentation. A. Characterization of human CSCs for mesenchymal (CD90+), stem (c-Kit+) or lineage negative (CD90-/c-Kit-) markers. B. Flow cytometric analysis of total IGF-1R and IR expression on human CSCs. C. ELISA detection of human IGF-1 protein secretion from mesenchymal (CD90+), stem (c-Kit+) or lineage negative (CD90-/c-Kit-) CSC sub-populations.

A



B

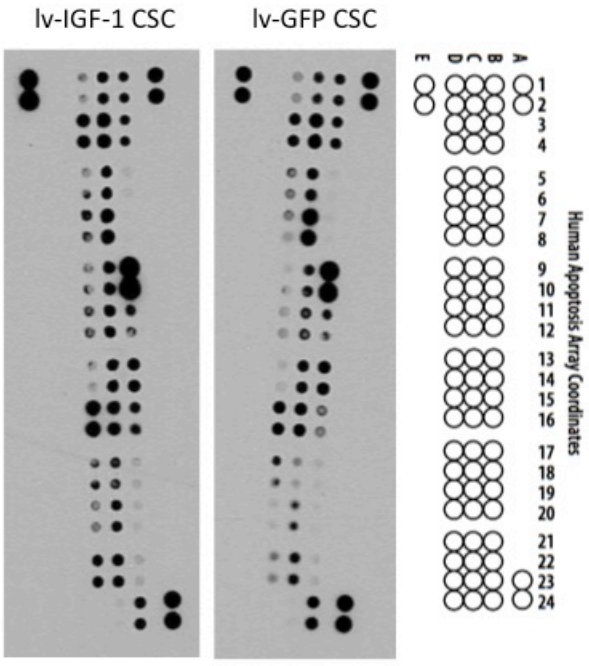
	Jun $Cq_{(Jun)-(GAPDH)}$	Fos $Cq_{(Fos)-(GAPDH)}$	Bcl-2 $Cq_{(Bcl-2)-(GAPDH)}$
Normoxic conditions ($5\%O_2$, 20% FBS)			
Non-transduced CSCs	2.26±0.17	9.61±0.17	10.5±0.12
lv-GFP transduced CSCs	1.97±0.36	9.776±0.18	9.89±0.16*
lv-IGF-1 transduced CSCs	2.45±0.15	10.0±0.20	10.11±0.04*

C

	Jun $Cq_{(Jun)-(GAPDH)}$	Fos $Cq_{(Fos)-(GAPDH)}$	Bcl-2 $Cq_{(Bcl-2)-(GAPDH)}$
Hypoxic conditions ($1\%O_2$, 1% FBS)			
Non-transduced CSCs	2.18±0.07	9.86±0.34	12.14±0.34
lv-GFP transduced CSCs	1.94±0.07	9.90±0.53	10.3±0.30*
lv-IGF-1 transduced CSCs	1.58±0.11*†	7.70±0.88*†	10.5±0.26*

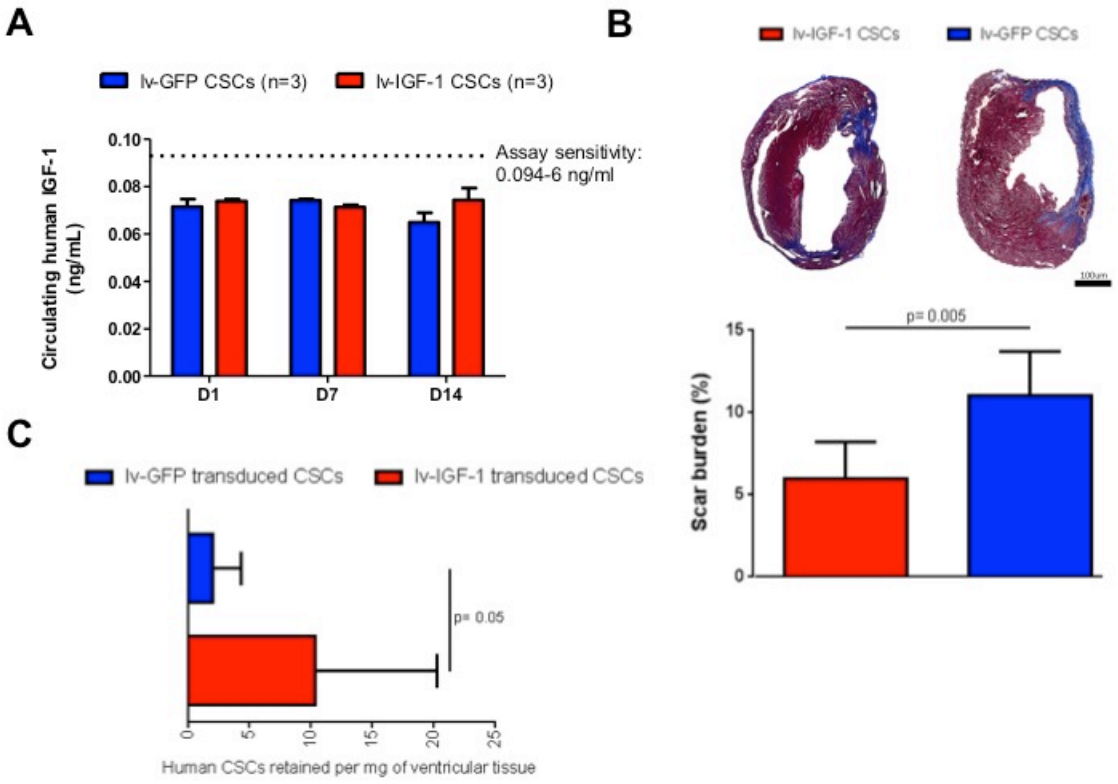
*p≤0.05 vs. Non-transduced
†p≤0.05 vs. lv-GFP CSCs

Supplemental Figure 6. Profiling CSC growth and survival under normoxic and hypoxic culture conditions. A. Population doubling time of non-transduced, lv-GFP transduced and lv-IGF-1 transduced CSCs under normoxic and hypoxic conditions. B. Normoxic pro-survival transcript expression (raw Cq values). C. Hypoxic pro-survival transcript expression (raw Cq values). *p≤0.05 vs. Non-transduced; †p≤0.05 vs. lv-GFP CSCs.



Coordinate	Target/Control
A1, A2	Reference Spots
A23, A24	Reference Spots
B1, B2	Bad
B3, B4	Bax
B5, B6	Bcl-2
B7, B8	Bcl-x
B9, B10	Pro-Caspase-3
B11, B12	Cleaved Caspase-3
B13, B14	Catalase
B15, B16	cIAP-1
B17, B18	cIAP-2
B19, B20	Claspin
B21, B22	Clusterin
B23, B24	Cytochrome c
C1, C2	TRAIL R1/DR4
C3, C4	TRAIL R2/DR5
C5, C6	FADD
C7, C8	Fas/TNFRSF6/CD95
C9, C10	HIF-1α
C11, C12	HO-1/HMOX1/HSP32
C13, C14	HO-2/HMOX2
C15, C16	HSP27
C17, C18	HSP60
C19, C20	HSP70
C21, C22	HTRA2/Omi
C23, C24	Livin
D1, D2	PON2
D3, D4	p21/CIP1/CDKN1A
D5, D6	p27/Kip1
D7, D8	Phospho-p53 (S15)
D9, D10	Phospho-p53 (S46)
D11, D12	Phospho-p53 (S392)
D13, D14	Phospho-Rad17 (S635)
D15, D16	SMAC/Diablo
D17, D18	Survivin
D19, D20	TNF RI/TNFRSF1A
D21, D22	XIAP
D23, D24	PBS (Negative Control)
E1, E2	Reference Spots

Supplemental Figure 7. Human apoptosis proteome profiler array and coordinates. Representative images of lv-IGF-1 and lv-GFP CSC expression of 35 apoptosis-related proteins. Data shown from 2 minute exposure.



Supplemental Figure 8.lv-IGF-1 CSC transplantation reduces infarct scarring without elevating system levels of human IGF-1. A. Quantification of circulating human IGF-1 protein in mouse blood plasma following lv-GFP and lv-IGF-1 CSC therapy. B. Masson's Trichrome analysis of scar burden 28 days after myocardial infarction. C. Quantitate CSC engraftment 21 days post cell injection by qPCR detection of human Alu.

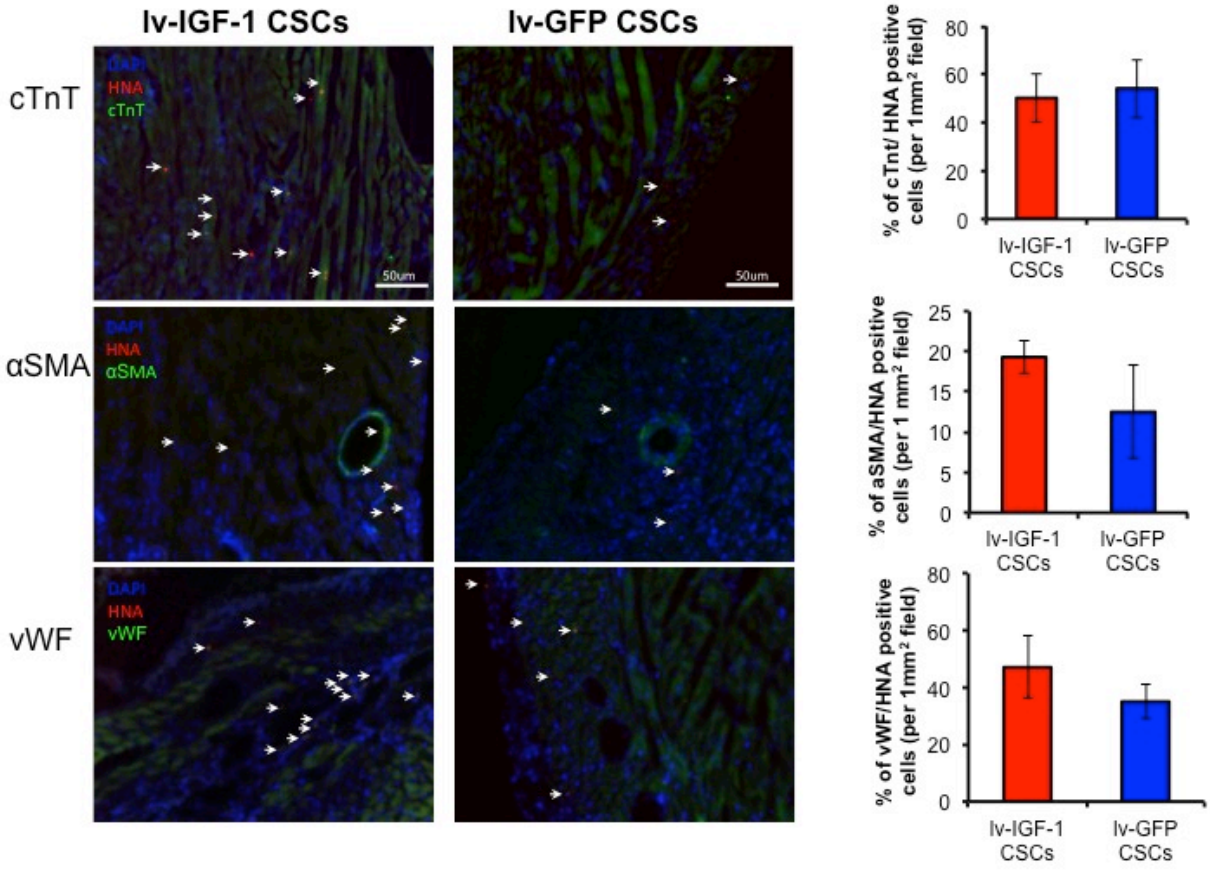
		End Diastolic Volume (μL)	End Systolic Volume (μL)	Stroke Volume (μL)	Ejection Fraction (%)
1 week post MI	vehicle	69.8±5.6	48.5±3.9	22.3±2.2	31.8±1.2
	lv-GFP transduced CSCs	73.1±4.1	52.3±3.1	20.8±1.3	28.5±1.2
	lv-IGF-1 transduced CSCs	70.3.7±2.3	49.1±1.6	21.2±1.0	30.2±0.9
3 weeks post MI	vehicle	79.8±11.2	41.5±9.7	18.3±1.8	24.0±1.6
	lv-GFP transduced CSCs	82.4±8.21	55.7±6.0	26.7±2.3	32.7±0.6
	lv-IGF-1 transduced CSCs	76.1±6.3	47.5±4.2	28.6±2.2	37.8±0.7*
4 weeks post MI	vehicle	85.6±10.0	66.4±8.7	19.1±1.8	23.1±1.3
	lv-GFP transduced CSCs	77.1±3.1	48.7±2.4	28.5±1.0	37.0.7±0.8
	lv-IGF-1 transduced CSCs	88.9±4.4*	52.9±3.7	36.0±1.1*	40.9±1.6*

Supplemental Table 3. Echocardiographic measurements of left ventricle over the 4 week follow-up period. *$p \leq 0.05$ vs. lv-GFP transduced CSCs.

	Human IGF-1 $Cq_{(hIGF\text{-}1)\text{-}(GAPDH)}$		Mouse IGF-1 $Cq_{(mIGF\text{-}1)\text{-}(GAPDH)}$	
	lv-IGF-1 CSCs	**lv-GFP CSCs**	**lv-IGF-1 CSCs**	**lv-GFP CSCs**
Day 1				
Infarct	6.8±0.6*	9.84±1.5	3.7±0.06*†	4.0±0.06*
Border zone	8.2±0.1*†	11.5±0.9	5.1±0.3*	5.7±0.2*
Off target	10.8±1.2†	14.4±0.4	6.7±0.07*†	7.6±0.4
Day 7				
Infarct	6.0±0.2*†	10.5±1.5	3.8±0.1*†	5.1±0.3*
Border zone	7.8±0.4*	11.1±1.6	6.4±0.08*	6.3±0.3*
Off target	11.9±1.4	13.9±1.6	7.4±0.1	8.0±0.3*
Day 14				
Infarct	7.3±0.2*	8.9±2.3	5.2±0.2*	5.1±0.3*
Border zone	8.1±0.2*†	11.27±2.1	6.4±0.2*	6.1±0.3*
Off target	14.4±1.2	13.3±2	7.6±0.05*	7.6±0.07
Day 21				
Infarct	8.9±0.2*†	10.7±0.7	6.8±0.4	6.4±0.5*
Border zone	13.6±0.8	12.0±1.1	6.4±0.1*	6.4±0.8
Off target	14.5±1.3	13.5±0.7	8.2±0.2*†	7.1±0.2
Sham-operated $Cq_{(hIGF\text{-}1)\text{-}(GAPDH)}$	**13.4±1.2**			
Sham-operated $Cq_{(mIGF\text{-}1)\text{-}(GAPDH)}$			**7.2±0.2**	

*p≤0.05 vs. sham operated
†p≤0.05 vs. lv-GFP CSCs

Supplemental Table 4. Relative PCR Cq values for human and mouse IGF-1 expression after myocardial infarction in mice.



Supplemental Figure 9. Immunohistochemical analysis of transplanted CSC fate into cardiomyocyte (cTnT), smooth muscle (αSMA) or endothelial (vWF) lineages 21 days after lv-IGF-1 or lv-GFP CSC intramyocardial injection.

	Bcl-2 $Cq_{(Bcl\text{-}2)\text{-}(GAPDH)}$	Bax $Cq_{(Bax)\text{-}(GAPDH)}$	p53 $Cq_{(p53)\text{-}(GAPDH)}$
lv-IGF-1 transplanted CSCs			
Infarct	11.6±0.5*	10.4±0.1*†	4.5±0.4*†
Border zone	12.6±0.5*	12.5±0.2†	5.9±0.5*†
lv-GFP transplanted CSCs			
Infarct	12.7±0.6*	8.4±2*	3.4±0.4*
Border zone	13.1±0.2*	11.5±0.5*	5.0±0.1*
Sham-operated	14.0±0.2	12.5±0.1	6.1±0.1

*p≤0.05 vs. sham operated
†p≤0.05 vs. lv-GFP CSCs

Supplemental Table 5. Relative PCR Cq values for mouse Bcl-2, Bax and p53 transcript expression 7 days after CSC injection into infarcted mice hearts.

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