Second Generation Cardiac Cell Therapy: Combining Cardiac Stem Cells and Circulating Angiogenic Cells for the Treatment of Ischemic Heart Disease.

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Abstract

Blood-derived circulatory angiogenic cells (CACs) and resident cardiac stem cells (CSCs) have both been shown to improve cardiac function after myocardial infarction (MI) but the superiority of either cell type has long been an area of speculation with no definitive head-to-head trial. In this study, we compared the paracrine profile of human CACs and CSCs, alone or in combination. We characterized the therapeutic ability of these cells to salvage myocardial function in an immunodeficient mouse model of MI by transplanting these cells as both single and dual cell therapies seven days after experimental anterior wall MI. CACs and CSCs demonstrated unique paracrine repertoires with equivalent effects on angiogenesis, stem cell migration and myocardial repair. Combination therapy with both cell types synergistically improves post infarct myocardial function greater than either therapy alone. This synergy is likely mediated by the complementary paracrine signatures that promote revascularization and the growth of new myocardium.

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αSMA	alpha smooth muscle actin
ACEI	angiotensin-converting enzyme inhibitors
ARB	angiotensin receptor blockers
BMI	body mass index
cTnT	cardiac troponin T
CAC	circulating angiogenic cell
CCS	Canadian Cardiovascular Society
CGM	cardiogenic media
CSC	cardiac stem cell
EGF	epidermal growth factor
GFR	glomerular filtration rate
HGF	hepatocyte growth factor
HUVEC	human umbilical vein endothelial cell
IL-6	interleukin 6
LV	left ventricle
LVEF	left ventricular ejection fraction
MI	myocardial infarction
NHDF	normal human dermal fibroblast
NYHA	New York Heart Association
SDF-1	stromal cell-derived factor 1
VEGF	vascular endothelial growth factor
vWF	von Willebrand factor

1. Introduction

1.1 The need for stem cell therapy in patients with heart failure

Modern device, drug, lifestyle and surgical advances in cardiac care have dramatically improved patient survival after cardiac injury. As a result, the health care system is experiencing a growing number of patients living with chronic heart failure (HF). Current estimates would suggest that HF afflicts over 71 million adults (43 million under age 65) in North America, resulting in over 71 000 Canadian deaths per year with an ongoing cost of over 22 billion dollars to the Canadian economy.[1] This burden is forecast to increase in coming years with corresponding increases in deaths and hospitalizations.

The strategy of transplanting stem cells into damaged myocardium has since emerged as a novel means of treating patients with ongoing HF. Ideal graft cells should be autologous, easy to expand *in vitro*, able to engraft and differentiate into functional cardiac myocytes that couple electromechanically with the surrounding myocardium.[2] Most importantly, transplantation of cells should improve cardiac function and prevent ventricular remodeling. To date, a number of different cell types have been transplanted in experimental models, including fetal myocytes, embryonic stem cell derived myocytes, skeletal myoblasts, mesenchymal stem cells and several cell types derived from the bone marrow.[3-8] Most recently, CSC therapy has shown great promise at restoring cardiac function given they are autologous and capable of differentiating into working myocardium without evidence for non-cardiac transformation.

1.2 The disputed existence of CSCs

1.2.1 Dogma challenged: Stem cell candidates discovered within the adult heart

At the end of the twentieth century, dogma prevailed that the mammalian heart was a terminally differentiated organ with a set number of cardiomyocytes predetermined at birth.[9] It was thought that a stable population of cardiomyocytes slowly dwindled with advancing years and no means of myocyte renewal.[9-15] Under this paradigm, cardiomyocytes adapted to injury by dying or enlarging while cellular integrity was maintained through continuous replenishment of intracellular organelles.[14, 16]

Towards the turn of the century, several studies began to document the existence of a small population of cells within the adult heart that expressed characteristic stem cells markers and were capable of re-entering the cell cycle after cardiac injury.[17-20] The discovery of activated cyclins, cell cycle markers (e.g., KI67, MCM5, cdc6 and phosphoistone-H3) and incorporation of BrdU within diseased and normal adult hearts further hinted that a replenishing pool of cardiomyocytes existed.[17, 18, 20-22]

1.2.2 Evidence for myocardial turnover

In 2009, *Bergmann et al.* demonstrated direct evidence that the human heart undergoes myocardial turnover by retrospectively dating the age of existing cardiomyocytes.[23] The basis for this study was founded upon the spike in carbon-14 (14C) levels resulting from 1960's cold war above ground nuclear testing. Given that 14C diffuses from the atmosphere and into the food chain with subsequent incorporation into the molecular framework of both plants and animals, the authors were able to compare cardiomyocyte DNA ^{14}C content to

known atmospheric 14C levels. The stability of post-mitotic DNA 14C content provided the opportunity to retrospectively date the age of cardiac Troponin I (cTnI) selected myocytes to the atmospheric 14C as it reflects when that cell underwent division. Using this strategy, the authors estimated 55% of the original cardiomyocyte population remains after 50 years of life with an average turnover between 0.5-1.0%/year.

The degree of myocyte turnover remains a hotly debated subject with several divergent independent measures.[24-26] Kajstura and colleagues examined the post-mortem hearts of 8 cancer patients who had received therapeutic infusions of a thymidine analog which is incorporated into cycling cells.[24] Using this technique, the authors found myocardial turnover approached 22% per year with an average lifespan of eight years. The authors were able to demonstrate that these results were not confounded by DNA repair, nuclear ploidy formation or cell fusion. This rate of turnover is significantly higher than what was described in the 14C study by Bergman et al.- which may be explained by the modeling assumption that the number of myocytes and their turnover remained constant throughout life. This may not be a valid given evidence that myocytes are formed after birth [27] and the overall number of myocytes progressively decline with age.[28] Furthermore, the rates of myocyte turnover may change with the presence of clinical modifiers such as aging, hypertension and MI. Back of the envelope calculations suggest that if these variables were included in the calculations, the annual myocyte turnover approaches 18%.[29]

1.2.3 Evidence to support the existence of a resident population of cardiac stem cells

As evidence of myocardial turnover was unfolding, researchers in parallel fields began to uncover stem cell populations within other adult organ systems capable of regenerating

multiple cell types including neurons, adipocytes, hepatocytes, pancreatic cells, skeletal myoblasts and skin.[30-35] Given the discovery of cycling cardiomyocytes, the possibility of a resident cardiac stem cell precursor was acknowledged with the search beginning to identify and isolate cells capable of creating *de novo* cardiomyocytes.

Side population cells in the myocardium

Isolation of the first post-natal resident cardiac stem cells came through application of skeletal myoblast culture techniques to the adult heart.[36] In this study, mouse hearts were enzymatically digested and treated with Hoechst dye for flow cytometry isolation of a subpopulation of cells that effluxed the dye (side population (SP) cells). These cells had reduced or absence of lineage markers indicative of cardiac identity and differentiated into functional cardiomyocytes when co-cultured on a feeder layer of purified mature cardiomyocytes. Interestingly, the authors compared SP cells from transgenic mice harboring a dominant negative form of the cardiac transcription factor MEF2C to those isolated from wild-type mice. This study is pertinent as mice deficient in MEF2C exhibited hypoplastic ventricles with impaired *in situ* repair as demonstrated by the inability to mount pathological responses (i.e., fibrosis or immune cell infiltration) to cardiac stress.[37] Consistent with this notion, the pool of SP cells was reduced in adult MEF2C deficient mice implying that the resident SP had undergone a substantial depletion as they were being recruited and/or activated as a result of the increased physiological demand. This theory was further supported by an increase in cardiomyocyte counts within MEF2C deficient hearts that paralleled the depletion of SP cells. The authors also noted that the numbers of SP cells declined with age suggesting these cells were recruited in response to normal physiological growth demands in an aging heart.

Subsequent studies characterizing the phenotype of myocardial SP cells identified the ATP-binding cassette transporter ABCG2 as a marker of universal cardiac SP identity throughout embryogenesis that persists into adulthood.[38-40] A robust yet restricted expression of ABCG2+ cells at embryonic day 8.5 was identified within the developing heart that diminished to a subpopulation of cells throughout gestation.[38] In this study, the authors demonstrated these cells did not co-express the intermediate filament protein desmin, which is known to be expressed early during cardiac differentiation. In the adult heart, isolated SP cells proved capable of proliferation as well as cardiogenic differentiation were shown to be characterized by ABCG2 expression and co-expressed a number of other stem cell related surface antigens including Sca-1 and c-Kit to varying degrees.[38-40] While bone marrow SP cells express the surface antigen CD31 [41], it was noted that a sizable proportion of murine cardiac SP cells (~10%) expressed stem cell antigen 1 (Sca-1+) in the absence of CD31.[40] These murine Sca-1+/CD31- SP cells were suggested as a purified cardiomyogenic precursors capable of *in vitro* cardiogenic differentiation.

Sca-1+/CD31- cells have been shown to migrate to areas of ischemic damage after an acute MI in mice.[42] Unsurprisingly, Sca-1 knockout transgenic mice have impaired myocardial and progenitor cell function.[43] Application of the murine antibody for Sca-1 to human cardiac derived cells identifies a population with characteristics suggestive of a cardiac precursor [44]. However these Sca-1+ human cells also significantly co-segregate with the c-Kit antigen suggesting that both epitopes may indicate the same population of cells.[45] This trenchant finding is well taken given the observation that the human epitope of Sca-1 has yet to be identified.

Tyrosine receptor kinase (c-Kit) as a marker of resident cardiac progenitor cells

Twenty years of experience with hematological stem cells provided the rationale to explore the heart for resident cells expressing the tyrosine receptor kinase (c-Kit) in the hopes of identifying a population of cells capable of providing endogenous repair.[22] These studies demonstrated clusters of cells expressing c-Kit+ cells confined to areas of low cardiac stress within the atrial appendage and ventricular apex/base. Since then, clusters of c-Kit+ cells have been identified in animal models and human autopsy specimens throughout the entire lifespan of the organ.[26, 46-51]

While cardiac c-Kit+ cells do not co-express lineage associated markers (bone marrow, cardiac, neuronal, mast cells, or skeletal muscle) or transcriptional factors,[47] these cells often co-segregate with MDR1 and Sca-1.[46, 48] Experiments with transgenic mice expressing GFP labelled c-Kit cells demonstrate that these cells are mobilized to sites of acute ischemic damage where they proliferate and differentiate into new cardiomyocytes within two weeks of initial injury.[52] Emerging evidence has demonstrated that hypoxia plays a key role in mediating this physiological response.[53] Although the function of the c-Kit receptor remains unclear, it has been shown to play a pivotal role in maintaining *in vivo* differentiation of cardiomyocytes within the adult myocardium.[54] This was suggested by the use of transgenic mice heterozygous for a deletion of the transmembrane domain of the c-Kit receptor and missense mutation that reduced the overall tyrosine kinase activity by >95%. Prolonged pressure overload caused by aortic constriction reduced the hypertophic response presumably by eliminating the ability of c-Kit+ cells to differentiate and respond to physiological challenges.

Recently, Ferreira-Martins and colleagues demonstrated that c-Kit^{+} cells are the predominant stem cell marker present in the developing fetal heart.[55] These cells were found to undergo asymmetrical cellular divisions after stimulation by spontaneous calcium ion oscillations within the developing mouse heart. After division, these cells progressively differentiated into mature cardiomyocytes, gradually losing molecular stem cell markers and the capacity for replication. The authors hypothesize that an identical hierarchy model can be applied towards c-Kit+ cells in the adult myocardium with participation in ongoing myocyte turnover and preservation of organ function.

Based on the further separation of c-Kit+ cell niches nestled in the coronary circulation from clusters residing in the interstitium between cardiomyocytes, two distinct classes of c-Kit+ CSCs have been proposed.[51] The first CSC resides within niches in the adult myocardium and was suggested to contribute towards myocyte turnover. These typical environments are surrounded by supporting fibroblasts and contain c-Kit+ cell clusters capable of both symmetrical and asymmetrical cellular divisions.[46, 50] The other class of CSCs was proposed as a source of vascular cells (endothelial and smooth muscle lineage) with a peri-vascular distribution throughout the coronary circulation.[56] Finally, *ex vivo* proliferated sub-fractions of both c-Kit+ cell types were found to express typical cellular and molecular markers indicative of myogenic and vascular progenitor.

The capacity of the c-Kit marker to identify multipotent adult progenitor cells has not gone unchallenged with frequent difficulty identifying c-Kit+ cells using routine human autopsy specimens.[57] This difficulty has lead to the proposal that the c-Kit+ marker may represent proliferation of cardiac mast cells rather than genuine progenitor cells. While cellular and molecular profiling of resident c-Kit+ cells refutes this notion, studies using conditionally

labelled c-Kit+ cells have suggested that these adult resident c-Kit+ cells possess vasculogenic potential.[58] In this study, neonatal and adult transgenic mice expressing GFP under the influence of the c-Kit+ promoter underwent surgical MIs. Cells expressing GFP were found in the infarct area of both cohorts however only in neonatal mice were blood vessels and cardiomyocytes of unambiguous c-Kit+ origin identified. In the adult hearts, only vascular differentiation of c-Kit+ origin was observed but differences in the durability of GFP expression during myogenic differentiation and the relative migratory capacity of adult and neonatal c-Kit+ cells opens questions regarding the overall generalizability of these findings.

1.2.4 Additional markers of resident cardiac stem cells

SSEA-1+ is a carbohydrate adhesion molecule first demonstrated on embryonic stem cells.[59] Since then, this antigen has been found on the surface of other adult organ stem cell populations and rodent CSCs. [60, 61] In CSCs, SSEA-1+ cells co-express with cardiac transcription factors (i.e., Nkx2.5, GATA-4) and other CSC associated makers (c-Kit, Sca-1). These cells have been shown to differentiate into cells of cardiac lineage and provide myocardial repair when transplanted after myocardial infarction.

The cardiac transcription factor Isl-1 is a specific embryological marker of cardiac identity that transcriptionally activates cardiogenic differentiation through myocyte associated transcription factor MEF2C in conjunction with GATA-4.[62] Homozygous deletion of Isl-1 in transgenic animal models leads to defects in cardiac development and the speculation that Isl-1 expression denotes a cardiac progenitor population.[63, 64] Conflicting reports however have debated the existence of this cell population within the adult myocardium as their presence is rare and appears to be unaffected by acute myocardial insults.[65] Emerging

evidence would suggest that cells expressing surface markers of CSC-lineage often co-segregate with ISL-1 among other primitive cardiac transcription factors (e.g., NKX2.5, Gata-4).[66]

As outlined, a number of surface antigens identify CSC candidates within the adult myocardium. Despite efforts characterizing the phenotype of these various populations, little is known about the ultimate origin of each cell type. Based on the prevailing hematological literature, these stem cell populations likely represent instantaneous snapshots along a continuum of stem cell maturity as resident CSCs proliferate and differentiate into new cardiomyocytes. However proof is lacking and additional work is needed to precisely define what distinguishes a resident CSC.

1.2.5 Extra-cardiac stem cell sources also participate in cardiac repair

Post mortem studies of sex-mismatch cardiac transplant patients have provided a unique opportunity to determine the origin of human cardiomyocytes by identifying the infiltration of male cells (i.e., Y-chromosome positive cells) from male recipients into transplanted female hearts.[22] This study documented chimeric organs with a significant number of recipient Y-chromosome positive cardiomyocytes and endothelial cells within the female donor heart suggestive that extra-cardiac stem cells may seed the transplanted heart to provide low grade repair. While the degree of cardiac chimerism is debatable,[22, 67-69] this study does provide direct evidence that the transplanted heart undergoes self-renewal from a non-cardiac source that migrates and colonizes the donor heart.

1.2.6 Resident CSC response to cardiac insult

Attempts to characterize the response of resident CSC to cardiac insult have yielded unique insights into the function of CSCs during myocardial repair. One of the first attempts describing myocyte renewal demonstrated clusters of cells along the infarct border zone that positively stained for Ki-67 (a nuclear antigen denoting cell division) in human hearts from patients who had died within twelve days of an acute MI.[17] This observation was confirmed by Hsieh and colleagues who, using transgenic cre-lox technology, demonstrated significant cardiomyocyte renewal along the infarct border zone following an MI.[70] Although the authors could only speculate that stem cells had contributed to the myocardial renewal observed, Fransioli and colleagues further supported the notion that c-Kit+ progenitor cells participate in myocardial repair through the demonstration that these cells rapidly migrate to areas of ischemic injury.[52] Furthermore, the authors demonstrated that c-Kit+ progenitor cells were capable of proliferating within these regions and differentiating to a cardiomyocyte phenotypes as they gradually lost c-Kit/GFP expression with the simultaneously onset of committed MEF2C expression. Given evidence that resident Sca-1+/CD133- also migrate to sites of ischemic injury,[42] the absolute ontogeny of cells mediating myocardial repair remains unclear but accumulating evidence strongly supports the conclusion that a pool of multipotent stem cells exist within the adult heart and provides low grade repair after myocardial injury.

1.3 Therapeutic application of CSCs to restore ventricular function

With the emerging evidence that resident CSCs reside in adult myocardium and participate in myocardial repair, two distinct isolation techniques were designed to isolate and expand a population of CSCs for transplantation.[47, 71, 72] The goal of these techniques was to provide

a clinically applicable cell product capable of myocardial regeneration after an acute myocardial insult. Due to the limited numbers of CSCs in adult tissue (1 in 30,000 cells)[47] and reasonable clinical restriction in tissue availability, culture protocols were required to isolate and expand CSCs to enhance the overall stem-ness of the cell product before transplanting them into areas of ischemic damage.

1.3.1 Antigenic selection and expansion of candidate cells

Mechanical/enzymatic dissociation of tissue followed by antigenic selection of candidate cells (c-Kit+, sca-1+, SSEA-1+) formed the initial effort to isolate a population of progenitors for cardiac transplantation. The first effort was described by Beltrami and colleagues with a focus upon creating a purified population of c-Kit+ progenitor cells from whole rat hearts.[47] After isolation and expansion within defined media, the authors showed that these c-Kit+ cells did not express markers of erthroid, fibroblast, lymphoid, myeloid or skeletal muscle origin. Interestingly, a modest portion of these cells (7-10%) expressed transcriptional factors associated with early cardiac commitment including Nkx2.5, GATA-4 and MEF2C. The overall variance of transcriptional factors being expressed by this purified population of c-Kit+ cells suggests that these progenitor cells were isolated at various levels of commitment. C-kit+ CSCs were shown to be clonogenic, self-replicative and capable of differentiating into all three major cardiac lineages (smooth muscle, vascular and cardiomyocyte) *in vitro* and after delivery into animal models of myocardial ischemia.[47, 50]

Subsequent studies translated this technique to clinical biopsy samples by first culturing a mixed population of progenitor cells directly from plated myocardial tissue followed by antigenic selection.[47, 50] These antigenically purified c-Kit+ cells represented 1.1±1.0% of all

collected cells and were successfully expanded using a defined media to a clinically applicable population. Transplanted human c-Kit+ cells regenerated myocardium in rodents, partially restored ventricular contractile function (left ventricular ejection fraction; LVEF), attenuated chamber dilation and improved overall ventricular performance. Most importantly, the authors were able to show that these cells were not fusing with the myocytes of the recipient heart and they were capable of electromechanically coupling with the surrounding myocardium.[47, 50]

Using a similar purification technique other groups have demonstrated that Sca-1+ can be isolated from adult mouse whole heart digestion.[42, 45] Freshly isolated Sca-1^{+} cells were found to co-express c-Kit to a modest degree (≈20%) and lack expression of CD34, C45, Flk-1 and lineage associated markers.[45] Furthermore, Sca-1+ cells have been shown to express early cardiac-associated transcriptional factors including GATA4, Mef2C and Tef-1.[13] Transplantation of Sca-1+/CD31- cells at the time of murine myocardial infarction demonstrated significant improvement of LVEF when compared to Sca-1-/CD31- and vehicle controls while demonstrating the capacity to differentiate into all three cardiac lineages.[42]

1.3.2 Culture guided isolation of resident CSCs

While antigenic selection provides an initial homogenous population of CSCs, the clinical application of these cells requires an extended period of culture to generate a therapeutically relevant dose.[51] This creates a number of limitations including complexity, cost, phenotypic drift and the possibility of malignant transformation.[73] As a result, several groups have refined a culture guided outgrowth technique that ultimately utilizes a

heterogeneous population of cells that spontaneously emigrates from plated tissue fragments in culture.[48, 50, 72, 74] When samples of minced cardiac tissue are cultured, a lawn of flat cells emigrates spontaneously from the plated tissue. Within that lawn, clusters of CSCs emerge and proliferate. Using mild enzymatic dissociation, loosely-adherent cells surrounding the explant (termed cardiac outgrowth) can be serially harvested. This CSC outgrowth contains sub-populations of cells expressing cardiac progenitor (c-Kit+), endothelial progenitor (CD31+, CD34+), mesenchymal progenitor (CD-90^{+}) related antigens. Most importantly, the cardiac progenitor subpopulation (i.e., c-Kit+ cells) have been shown to express transcription factors indicative of multipotent capacity (Nkx2.5, GATA4, MEF2c and isl-1).[29, 47, 61, 72, 75-78] In keeping with this finding, we and others have shown that these cells are self-renewing, clonogenic, and multipotent.[71, 72, 74, 77, 79-81] Unfortunately, direct application of the initial cell product to larger-scale models, or the clinical setting, is limited by a constant output return to the scale of production with the amount of outgrowth collected changing in proportion to the amount of tissue plated.

As such, this technique has undergone extensive refinement to progress towards clinical translation. Messina et al. incorporated the advancements in neural stem cell culture to demonstrate that three-dimensional sub-culture within sphere aggregates could significantly enhance the proportion of c-Kit^{+} cells within collected cardiac outgrowth.[32] These aptly named “cardiospheres” were shown to create a niche like environment that enhanced stem-ness through cell-cell interactions resulting in a product with boosted regenerative potency.[71, 82-84] Recent studies have demonstrated that ERK/Sp1 signaling, downstream from E-selectin-matrix interactions, plays a role in sphere growth by enhancing VEGF production and leading to auto/paracrine stimulation of sphere maturation.[85] Although application of cardiospheres has been shown to provide a dose-dependent improvement in

myocardial function,[86] the size of the end product cardiosphere approaches 70-100 microns effectively precluding intra-coronary delivery due to concerns regarding coronary occlusion-thus requiring direct intra-myocardial application. Direct injection was thought to reduce the wide-spread application of this therapy to transplantation at the time of surgical procedures or needing specialized guided intra-myocardial catheter delivery. Expansion of cardiosphere "enriched" cells to single cells for intra-coronary delivery was therefore validated to provide population of CSC for widespread clinical application.[72, 82] These cardiosphere-derived cells have since been shown to improve myocardial function after application in models of ischemic and non-ischemic injury.[87, 88]

While several laboratories have worked toward refining and validating methods to extract and expand CSCs *ex-vivo*, the validity of the guided culture method has been called into question by studies with variations in culture techniques.[89-91] These studies and others demonstrate the impact culture techniques upon the end cell product phenotype and the caution that should be taken when applying these results to established cell products.[82, 89] Over the last 5 years, the reproducibility, safety and efficacy of *ex vivo* CSC products have become well established in the literature with these cells representing a strong candidate for autologous cellular cardiomyoplasty.[45, 50, 61, 71, 72] In light of this work, transplantation of *ex vivo* proliferated CSCs has moved toward phase one and phase two clinical trials.[92-94]

1.3.3 Mechanisms governing myocardial repair by *ex vivo* proliferated CSCs

The mechanism driving the functional improvements after CSC transplantation remains poorly understood.[95, 96] Initial work examining CSCs as an autologous cell therapy showed that these cells were capable of differentiating into cells with mechanical and calcium

transient characteristics of functional cardiomyocytes *in vivo*. Improvements in ventricular performance were attributed to the creation of new cardiomyocytes that were able to electromechanically couple with the surrounding myocardium through the formation of gap junctions.[47, 50] Additional studies supported the idea of direct differentiation leading to enhanced functional performance by showing these cells were capable of differentiating into all three major cardiac lineages and pointed to increases in vascular density within infarcted regions after CSCs were introduced.[47, 50, 72, 77]

These straightforward conclusions were challenged by Terrovitis et al. who demonstrated very modest short (17% at 1 hour) and long (<1% at three weeks) term engraftment of injected cells despite evidence for marked functional improvements.[97] Interestingly, mechanical measures to enhance the acute retention were associated with improved long term persistence and greater functional benefits. Further work by Shen et al. echoed these findings by demonstrating a dose-dependent delivery of cardiosphere cell products functional benefit from CSC transplantation after acute myocardial infarction.[86] Presumably, the majority of injected CSCs are acutely lost through vascular clearance (venous and lymphatic) with a minority undergoing mechanical extravagation or intra-cavitary delivery.[98]

Malliaras et al. recently showed that CSCs transplanted from an allogeneic donor can exert robust functional improvements that persist long after these cells are cleared by the host's immune system.[99] Given that other cell products, such as mesenchymal [100] and endothelial progenitor cells [101], have been shown to exert their effects through paracrine mediated signalling, it is likely that cardioprotective cytokines produced by transplanted CSCs improve cardiac function by stimulating the endogenous repair or salvage mechanisms. Recently Chimenti et al. showed that these CSCs are capable of releasing cardioprotective and

immunomodulatory cytokines that stimulate cardiac and vascular growth while recruiting endogenous stem cells.[77] In an attempt to quantify the humoral effects of human CSC transplantation in mouse myocardial infarctions, Chimenti et al. compared regions of increased vascular and myocyte density between treated and sham groups after 28 days and noted that while CSC treated mice had enhanced vascular and myogenic growth, only a small portion of these cells were derived of human origin.

On balance, the majority of benefits after transplantation of first generation CSC products are likely derived through paracrine mediated repair with occasional evidence for persistence and transdifferentiation into working myocardium. The capacity for producing real working myocardium represents the hope for this therapy in the future but only after surmounting the obstacles to acute and long term CSC retention.

1.3.4 Large animal pre-clinical studies of CSC therapy

Despite an incomplete understanding of the mechanisms underlying CSC-mediated benefits, reproducible small animal studies prompted a number of groups to refine both cell culture and delivery techniques for pre-clinical large animal studies. CSCs cultured using GLP techniques from large animal ventricular biopsies provided cell product with quality consistent with those obtained from small-animal models.[49, 102] Early proof supporting the transition of delivery from invasive intra-myocardial injection to intra-coronary came from a small animal study demonstrating intra-cavitary left ventricular injection of CSCs 30 days after MI delivered an effective “dose” of CSCs via the coronary arteries and was associated with significant treatment benefits.[103] Finally, Takehara and colleagues demonstrated that cardiospheres embedded within a synthetic hydro-gel sheet controlling the slow release of

the growth factor bFGF provided enhanced functional benefits when injected 4 weeks after myocardial infarction; providing a logical platform for clinical translation.[102]

1.3.5 Clinical potential of CSCs

Recent primary percutaneous intervention trials suggest that 42% of patients present with anterior wall infarcts and, despite leaving the lab with an open artery, 21% of all patients leave hospital with a LVEF less than 40%.[104] Historically, several studies have identified these patients as a sub-group requiring intensive follow-up given a 5-fold increased mortality within one year after MI.[105] Despite improvements in post MI care, patients with low LVEF (<45%) prior to discharge still remain at high risk for major adverse cardiac event with 40% incidence during an eight year follow up.[106] Thus a number of pre-clinical stem cell studies designed to deliver cell products recently after MI have focused on patients with LVEF less than 40-45%.[92-94, 107-109]

Chronic heart failure itself is a progressive disease with acute decline in function secondary to cardiac events and unremitting maladaptive remodelling in undamaged areas.[110] Despite the profound effects of chronic heart failure on morbidity and mortality, this patient sub-group has been enrolled in very few studies which likely reflects the limited preclinical evidence supporting the use of current cell products.

1.3.6 Phase one clinical trials examining CSC therapy

To date, one phase one clinical trial has been completed (CADUCEUS) with two more underway (SCIPIO, ALCADIA). These trials are designed to examine the effects of CSC

transplantation in different populations with different cell products with sufficient power to establish product safety (see Table 1.1).

CADUCEUS

This phase one clinical trial titled “CArdiosphere-Derived aUtologous stem CElls to reverse ventricUlar dySfunction (CADUCEUS)” enrolled patients with a recent myocardial infarction and MRI LVEF less than 45%.[82, 92] Upon informed consent, these patients underwent a ventricular biopsy and CDCs were injected after four to eight weeks of culture down the infarct related artery. Patients were randomized to receive three different weight-based doses of CDCs and were compared to a usual treatment group. Given concerns relating to a second intervention, no placebo/vehicle group was permitted by the FDA over-sight committee. CADUCEUS demonstrated that CDC injection was a safe autologous cell therapy with no significant adverse events associated with the cell therapy. Administration of CDCs significantly reduced scar size 12 months after cell transplantation with a trend towards enhanced ventricular function. This data formed the platform for a recently proposed phase 2 trial examining the capacity of allogeneic CDCs to improve post MI function.[94]

SCIPIO

The “cardiac Stem Cells In Patients with Ischaemic cardiOmyopathy (SCIPIO)” trial remains uncompleted with the first interim results published in November 2011.[93] This randomized, open-label, single-centre trial targeted patients requiring coronary artery bypass grafting with a LVEF of less than 40%. At the time of surgery, left atrial appendages were harvested

and the c-Kit+ cells were isolated for expansion over 113±4 days. CSCs were administered down a patent coronary artery or graft supplying the infarcted area. As with the CADUCEUS study, a placebo group was not performed and a single dose of CSCs was compared to a usual treatment group. Preliminary results did not demonstrate an increase in adverse events associated with stem cell transplantation. Twelve months after cell transplant, administration of c-Kit+ cells was associated with significant improvements in ventricular performance with a corresponding decrease infarct size (Figure 1.1).

ALCADIA

A third phase one clinical trial titled AutoLogous Human CArdiac-Derived Stem Cell to Treat Ischemic cArdiomyopathy (ALCADIA) began in April 2010 and was expected to be completed by March 2013.[111] This study differs from the two previous clinical trials as the aim is to evaluate the safety of intracoronary injections of human cardiospheres in conjunction with the controlled release of bFGF using a surgically implanted gelatin sheet. This study enrolled patients with chronic ischemic cardiomyopathy (15% ≤ LVEF ≤ 35%) scheduled for routine CABG procedures. bFGF gelatin sheets were implanted at the time of surgery and endomyocardial biopsies provide CSC populations through the use of standard cardiosphere culture techniques. The primary end-point of this study will conclude after a 12-month follow up period, in which the safety and efficacy of this combination therapy will be evaluated.

Table 1.1. Summary of human CSC phase one clinical trials.

	CADUCEUS	SCIPIO	ALCADIA
Type of study	Phase one		
Cell type	CDCs	c-Kit$^+$ cells only	CDCs after bFGF hydro-gel
Number injected	15-25 million	500,000 - 1 million	0.5 million per kg body weight
Route of administration	Intra-coronary injection		Surgical + intra-coronary injection
Population	Post STEMI	Stable CAD with heart failure	Heart failure patients with chronic ischemic cardiomyopathy
Time from enrolment to injection	Cells were infused 4-8 weeks after biopsies were harvested	Cells were isolated from right atrial appendages and cultured for 113±4 days prior to infusion	Study in progress
Safety	No increased adverse events		Study in progress
Benefit	MRI evidence for regeneration with trends for improved EF	Improved EF and reduced infarct size	Study in progress
Critique	Open-label, highly selected patient subgroup with SCIPIO still unfinished		Study in progress

Three phase one clinical trials are in progress or have been completed evaluating the safety of first generation human CSC products in patients with heart failure or recent MI. Early results suggest that CSC transplantation provides modest improvements in ventricular ejection fraction and have established CSC transplantation as a safe and viable cell therapy for future phase 2/3 studies.

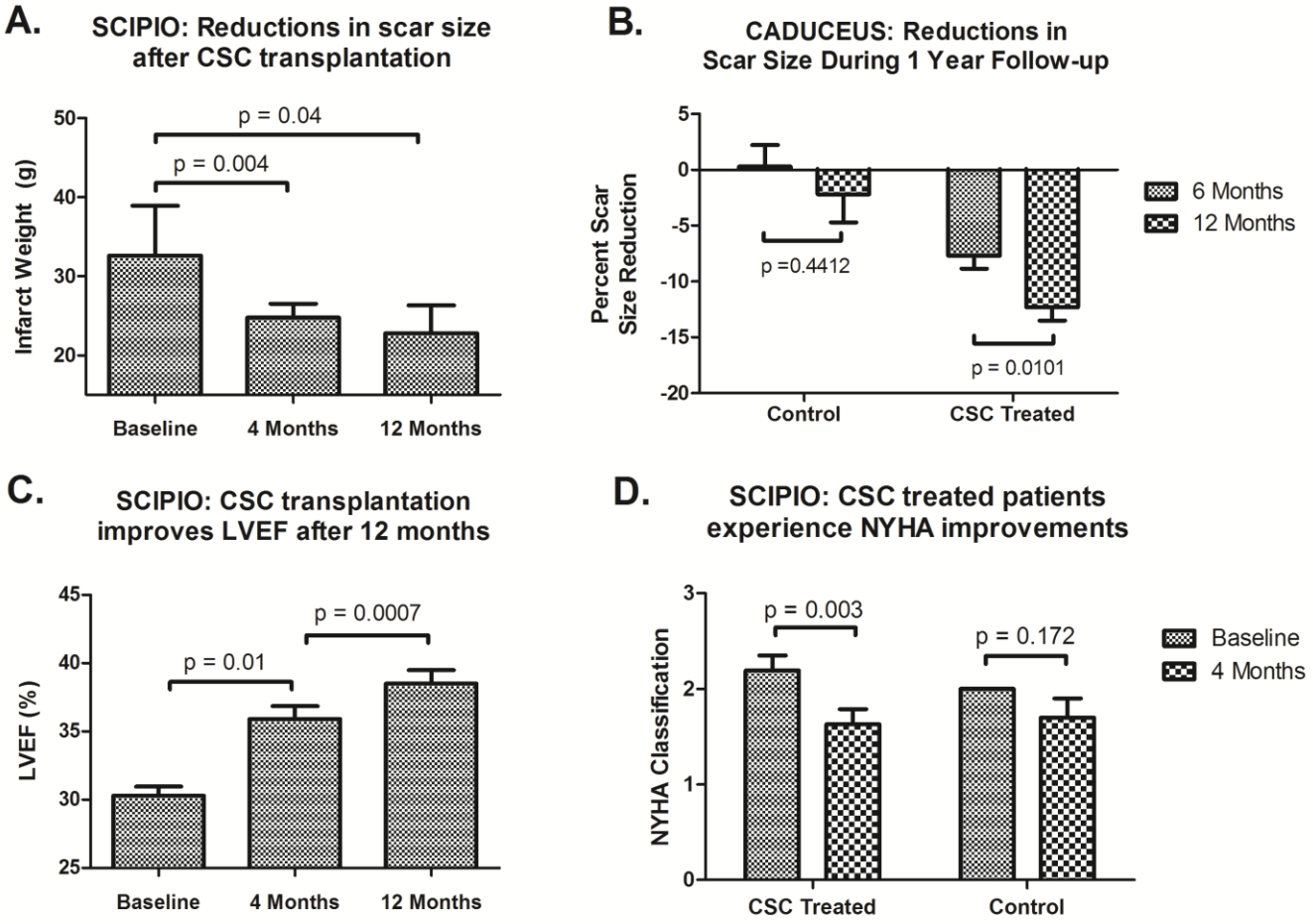


Figure 1.1. Summary data from CSC phase 1 clinical trials. Early data from two phase one clinical trials demonstrates reduction in infarct size (A and B) in parallel with improvements in myocardial function (C) and measures of heart failure severity (D) [92, 93].

1.4 Future directions for CSC therapy

Despite promising early results from the first phase one clinical trials the full potential of CSC therapy likely has yet to be realized because of limited acute retention, poor chronic engraftment and modest efficacy in cells cultured from patients with co-morbidities.[97, 98] The promise of these cells lies in the potential for true long term engraftment with generation of new working myocardium. Based on early data exploring the fundamental mechanisms underpinning CSC-mediated benefits, it appears that CSCs posses a potent cytokine profile that has fueled the early clinical results but it is uncertain if these will translate to long term benefits. As a result, a number of research initiatives have focused upon enhancing CSCs to develop next generation therapies to capitalize on the potential for long term regeneration and true functional recovery.

1.4.1 Effect of patient co-morbidities on CSC regenerative potential

Despite the promise of autologous CSCs, significant hurdles remain before this technology can be effectively translated to the clinic. One of the most significant barriers to this technology surrounds the regenerative capacity of these *ex vivo* stem cells cultured directly from patient tissue specimens – the very same individuals who will likely require this therapy in the future. In other organ stem cells, increasing chronological age and co-morbidities have been shown to inhibit performance but the degree to which this translates to first generation CSC products is not known (see Table 1.2).

The initial publication describing this technology focused primarily on cells cultured from the tissue donated by post-transplant patients.[72] Cells from the right ventricular apex of these immunosuppressed patients did not differ significantly in crude measures of cell growth and

myocardial repair/salvage. However, since then several publications from non-transplant patients have hinted that the regenerative capacity of *ex vivo* proliferated CSCs may be impaired by patient co-morbidities.[74, 78-80, 112] These studies indicate that patient variables such as greater age and male gender are predictive of reduced CSC yields. Unfortunately, these studies examined only crude surrogate end points (i.e., cell culture numbers and proliferation) without reference to actual myocardial repair or the fundamental mechanisms underlying cell-mediated cardiac repair.

One approach to avoid this potential limitation may be provided by recent evidence that, akin to mesenchymal stem cells, the CDC product demonstrates a tendency toward being immune-priviledged.[99] *In vitro* studies demonstrated that CDCs do not activate a humoral immune memory response as a result of limited MHC class 2/B7 expression and limited inflammatory cytokine expression. Importantly, CDCs from Brown Norway rats transplanted into genetically dissimilar Wistar Kyoto rats (allogeneic transplant) improved post infarct cardiac function to the same degree as cells transplanted within the same inbred strain of rat (i.e., Wistar Kyoto into genetically identical Wistar Kyoto; autologous transplant). This data implies that the CDC product may represent an “off the shelf” cell therapy that could be provided from healthy donors free of limitations imposed by patient co-morbidities. Given that long term engraftment was shown to be negligible, this data underscores the importance of paracrine mediated repair using this first generation stem cell product.

Table 1.2. Patient co-morbidities alter stem cell function.

Cardiovascular risk factor	Effect on EPC biology	Effect on CSC biology
Age	↓EPC numbers,[113, 113-115] ↓EPC proliferation,[116] ↓EPC migration,[116] ↓EPC survival,[116] ↓EPC oxidative stress resistance[117]	↓CSC numbers,[80, 112, 118] ↓CSC proliferation,[112] ↑CSC senescence[118, 119]
Female Gender	↑EPC numbers,[120, 121] ↑EPC CFU,[120, 122] ↑EPC migration,[122] ↑EPC adhesion,[120] ↓EPC senescence,[123] ↑reendothelialization[120]	↑CPC numbers[78]
Diabetes	↓EPC numbers,[124] ↓EPC proliferation,[125, 126] ↓EPC migration,[127] ↓EPC oxidative stress resistance,[127] ↓ vasculogenic potential,[125, 126]	↓CSC numbers,[128, 129] ↑CSC senescence[129]
Congestive Heart Failure	↓↑EPC numbers[130-133]	↓↑CSC numbers,[29, 76, 134, 135] ↓↑CSC proliferation[29, 76, 134, 135]
Idiopathic pulmonary arterial hypertension	↓↑EPC numbers,[136, 137] ↓EPC proliferation,[138] ↓EPC migration,[138] ↓ vasculogenic potential,[137, 139] ↑EPC senescence[139]	↑CPC numbers[140]
Hypertension	↓↑EPC numbers,[141, 142] ↓↑EPC CFU,[141, 142] ↑EPC senescence[143]	
Body weight	↓EPC numbers,[144-147] ↓EPC CFU,[144] ↓EPC proliferation,[145] ↑EPC apoptosis[148]	
Smoking	↓EPC numbers,[114, 149] ↓EPC proliferation,[114, 149, 150] ↓EPC adhesion,[150] ↓EPC migration,[150] ↓vasculogenic potential[150]	
Second hand smoke inhalation	↑EPC numbers,[151] ↓EPC migration[151]	
Homocystein	↓↔EPC numbers,[152, 153] ↓EPC CFU,[154] ↓EPC proliferation,[154] ↑EPC senescence[154]	
Cholesterol - oxLDL	↓EPC migration,[114] ↓EPC survival,[155] ↓ vasculogenic potential[155]	
Cholesterol - HDL	↑EPC numbers,[156] ↑EPC CFU,[156, 157] ↑EPC adhesion,[156] ↓EPC apoptosis,[156, 157] ↑reendothelialization[156]	
Adiponectin	↑EPC numbers,[158] ↑EPC proliferation,[158] ↑EPC differentiation,[158, 159] ↑vasculogenic potential[158]	
High risk cardiovascular profile	↓EPC numbers,[114, 160] ↓EPC CFU[113]	
Nicotine	↑EPC numbers,[161] ↑EPC proliferation,[161] ↑EPC adhesion,[161] ↑EPC migration,[161] ↑vasculogenic potential[161], ↓EPC apoptosis[162]	

A variety of patient co-morbidities have been shown to modulate blood derived stem cell function and number. The relationship between patient phenotype and CSC function still

needs to be adequately defined and could significantly impact the development of next generation CSC products.

1.4.2 Enhancing CSC cell products by refining culture techniques

Several unfortunate studies have illustrated the ability of variances in CSC culture practice to result in phenotypic deviation and limited functional repair.[82, 84] It follows that similar modifications in the culture milieu may provide the opportunity to enhance the stem-ness and regenerative potential of cells. This notion is supported by a number of non-CSC studies demonstrating the ability of targeted manipulation to enhance stem cell efficacy using AVE-9488[163], PPAR agonists[164, 165], statins[166, 167] and TGF-B[168]. Further, a recent study demonstrated that *ex vivo* proliferation of CSCs in physiological levels of oxygen (5% oxygen) may significantly improve CSC performance when compared to culture conditions at atmospheric oxygen (20% oxygen) levels.[169, 170] This is likely a consequence of increased oxidative stress created by atmospheric oxygen concentrations leading to genomic instability and impaired CSC function. Thus refined culture techniques may provide a new direction to engineer the next generation of CSC therapy.

1.4.3 CSC enhancement using *ex vivo* genetic modification

Direct genetic modification of stem cells prior to cell transplantation has been explored as a means to enhance cardiac repair. Direct genetic engineering of non-cardiac stem cells has also been used to improve cell survival (β-Akt,[171] SDF-1,[172, 173] Bcl-2,[174] PDGF[175], Pim-1[176]), electrical integration (Cx43)[177], differentiation (TGF-β,[178] TNF-α), homing/migration (CD18,[179] β1-integrin,[180] CXCR4,[181] CCR2,[182] eNOS[183]) and vasculogenesis (HGF,[184] HIF-1,[185] VGEF,[186] SDF-1,[187] bFGF[188]). Comparing these different approaches is problematic given variations in cell type (fetal myocytes, embryonic stem cells, skeletal myoblasts,

mesenchymal stem cells and several cell types derived from the bone marrow) and strategies for gene transfer to cells (viral, plasmid).

To date, very few studies have been performed to genetically enhance CSCs prior to transplantation. In one study, genetic modification of CSCs to over express Pim-1 kinase, a key player in Akt signaling, showed very promising pre-clinical evidence for benefit.[189-191] This approach is intended to decrease cell clearance via apoptosis, which is thought to be a major challenge faced by CSCs once they are introduced into the harsh environment of post-infarcted myocardial tissue. As expected, over-expression of Pim-1 kinase has been shown to improve acute engraftment and long-term retention in pre-clinical models.[190, 192] Although modulation of Pim-1 kinase provides very good proof-of-principle evidence supporting this approach, clinical translation is expected to be problematic due to the oncogenic potential of this vector.

1.4.4 Biomaterial approaches to enhance CSC therapy

The most significant improvements in CSC therapy have been recently come through biomaterial approaches to enhance acute engraftment with a view towards improving engraftment downstream.[97, 102, 193-196] Low acute retention of injected cells is thought to reflect a combination of mechanical extrusion, off-target disbursement and clearance from the heart through lymphatic or venous drainage. Initial attempts to improve acute retention have used biomaterials to anchor transplanted cells within the myocardium upon injection. These scaffold materials provide additional trophic support to cells by providing intrinsic adhesion stimuli that increase differentiation potential and paracrine secretion of

cardioprotective cytokines as well as reductions in early cell loss due to contact initiated apoptosis.[197-200]

These biomaterial approaches have been rapidly translated to pre-clinical CSC studies with some degree of success. Terrovitis and colleagues demonstrated that application of fibrin glue at the site of intra-myocardial injection marked increased acute CSC retention with significant improvement in functional recovery.[97] Using a more complex approach, Cheng and colleagues demonstrated that CSCs could be safely labeled with iron microspheres prior to transplantation.[193] Retention of injected cells was improved by applying a magnet to the chest wall at the time of intracardiac injection with salutary effect on post MI recovery. Finally two studies have examined the use of platelet gels that naturally contain a rich cocktail of cytokines capable of preserving reversibly damaged and preventing stem cell apoptosis. Injected alone, platelet gels stimulated endogenous CSC recruitment, increased capillary density within infarcted tissue and decreased compensatory myocyte hypertrophy in rat MI models.[102, 195] Synergistic benefits were observed when CSCs co-administered with synthetic platelet gels suggesting that combination therapy enhanced the paracrine profile that could be delivered to the healing myocardium.[194]

1.5 Circulating angiogenic cells as a therapeutic candidate for myocardial repair

The search for cell products capable of myocardial repair has yielded a variety of candidates. In the last 10 years, circulating angiogenic cells (CACs or early outgrowth endothelial progenitor cells) have emerged as the most promising subtype of blood-derived stem cells for myocardial repair given their capacity to form new blood vessels (vasculogenesis) while stimulating existing blood vessels to expand (angiogenesis) through

paracrine stimulation.[101] As a result, this promising cell candidate is under investigation in a variety of clinical trials.[201, 202]

1.5.1 Characterization of CACs

CACs are an active subtype of bone marrow derived progenitor cells that express a variety of endothelial markers and are incorporated into the developing vessels in ischemic tissues.[203] Phenotypically, immature CACs express CD133 and vascular endothelial growth factor receptor-2 (VEGFR-2) while further differentiated CACs are thought to co-express CD34 and VEGFR-2. Ischemic conditions induce an up-regulation of CD133+ cells released from the bone marrow while circulating bone marrow derived progenitor cells are attracted to damaged tissues where they contribute tissue repair/formation.[204] CACs simultaneously promote vascular regeneration by creating new blood vessels *de novo* (vasculogenesis) and by stimulating existing blood vessels to expand (angiogenesis) through a variety of different cell signalling mechanisms including autocrine and paracrine stimulation.[101] Unfortunately the capacity of endogenous CACs to hone to sites of damage is modest which may limit their capacity to provide meaningful endogenous vascular support after a myocardial infarction. Strategies aimed at introducing or recruiting circulating CACs to ischemic tissue could lead to improvements in cardiac function as these cells have been shown to be effective at mediating tissue repair.

1.5.2 Isolation and expansion of CACs in culture

Immature CAC populations can be isolated from peripheral blood samples using density-gradient centrifugation to separate total peripheral blood mononuclear cells (PMNCs). Once

separated, PMNCs can be cultured on a fibronectin-coated dish in an enriched base media. After four days, the non-adherent cells are removed and the culture is grown for an additional week. At this point, the resulting cellular population is considered the early growth CAC population, otherwise known as the early outgrowth endothelial progenitor cells.[205]

1.5.3 CACs modulate cardiac repair through revascularization and stimulation of host repair mechanisms

Considerable evidence suggests that transplantation of CACs enhances neovascularisation[206, 207] and may improve heart function when injected into ischemic heart models.[108] *In vitro* studies have shown that CD133+VEGFR-2+ cells co-cultured with neonatal cardiomyocytes adopted biochemical and functional features of cardiac tissue such as calcium transients and gap junction mediated dye transfers.[208] However, recent evidence suggests that the direct transdifferentiation of transplanted CACs into cardiomyocytes and endothelial cells is modest *in vitro* and observed improvements in cardiac function *in vivo* are likely not due to transdifferentiation into cardiomyocytes in lieu of insufficient evidence to support this notion.[209, 210] CACs readily promote angiogenesis through paracrine signalling that likely underlies the majority of the observed functional benefits.[211] Thus the observed improvements in cardiac function after CACs injection can be attributed to one of two mechanisms. Firstly, enhanced tissue environment promotes an increase in the recruitment of endogenous progenitor cells and/or stem cells. Secondly, by releasing cell signalling molecules, angiogenesis is stimulated. Regardless of the mechanism(s) involved, significant functional improvements are lost in the absence of adequate CAC engraftment, consistent with the improvements seen as a result of CSCs.[211]

1.6 Combining cell therapies to stimulate cell synergy

In light of the number of stem cell candidates for myocardial repair of dissimilar ontology, the notion that treatment with complimentary cell types may provide synergistic enhancements fits given the variety of mechanisms through which these cells exert functional improvements. This hypothesis is based on the premise that cells supporting the surrounding host tissue may increase the retention and proliferative capacity of cells capable of forming true contractile elements. Further, the ability of these cells to modulate host repair/protective responses through paracrine stimulation may be enhanced through overlapping or complimentary cytokine signatures from the separate cell sources. Despite this rationale, limited published work supports this notion, while treatment with non-clinical cell types (i.e., angiogenic cells + skeletal myoblasts or epicardium-derived cells + cardiac progenitor cells) demonstrates superior effects when combination cell products are injected.[212-214] Although this work supports the idea of cell synergy, this enhanced therapeutic capacity of these cells holds little clinical relevance as these cells are not conducive as a human cell therapy. Thus a need for investigation exists within the literature to directly explore combining the two leading pre-clinical agents for myocardial repair in order to determine if synergistic benefits result when applied after myocardial infarction.

1.7 Examining the potential of CACs and CSCs alone and in combination as candidates for myocardial repair

While both CAC and CSC transplantation into ischemic myocardium provides individual benefits, speculation remains as to which cell source is superior. Recently, Li and

colleagues demonstrated that an expanded primary culture CSC product provides greater functional benefits than treatment with a variety of mesenchymal stem cell products cultured from healthy individuals.[57] This finding may reflect the balance of paracrine factors produced by the different cell products, although the greater persistence and differentiation of transplanted CSCs likely contributes in part to the observed benefits. To provide a "real world" context, we designed this study to compare the effect of primary cultured human CACs and CSCs on myocardial function after delayed (7 day) injection into an immunodeficient mouse model of myocardial ischemia. Importantly, both cell sources were cultured from cardiac patients undergoing clinically indicated procedures and reflect the target population that may need future cell therapy. The fundamental mechanisms underlying cell mediated benefits was contrasted using angiogenic, cardiogenic and paracrine profiling of the two cell candidates. Given the disparate ontogeny and possible mechanism of benefit, the effects of combination therapy were explored to discover if co-administration of both cell types is detrimental, ineffectual or synergistic.

1.8 Preclinical design issues

To ensure proper methodology for clinical translation, a number of steps were taken in study design to ensure accuracy and reproducibility of the data. One area of concern surrounds investigator bias when evaluating key findings such as ejection fraction. Technicians injecting the cells into the animal, conducting the ECHO readings and measuring the dimensions were all blinded to the treatment received. Furthermore, two blinded reviewers (RS and NL) were used interchangeably throughout this study to conduct the ECHO measurements. With respect to the mice, patient selection bias was avoided by conducting an LVEF evaluation 7 days post-MI, prior to stem cell transplantation. Animals who did not

have sufficient heart function (LVEF <20) or who did not receive a large enough infarct (LVEF >40) were excluded from the study. Animals were then randomized prior to treatment groups.

2. Rationale, Research Aims, Hypotheses and Objectives

2.1 Rationale

1. CACs and CSCs are two leading preclinical stem cell candidates for myocardial repair, yet the relative therapeutic capacity of CACs versus CSCs has never been subjected to direct comparison in basic or clinical studies.
2. Previous work combining stem cells (angiopoietic progenitor cells and skeletal myoblasts as well as epicardium-derived cells and cardiac progenitor cells) for myocardial repair demonstrated superior effects using combination cell therapy yielding reduced scar size and improvements in ventricular function.[214-216]

2.2 Research Aims

1. To compare the effect of human CSCs and CACs on myocardial function after injection into an immunodeficient mouse model of myocardial ischemia.
2. To determine whether there are synergistic effects of human CSCs and CACs on myocardial function after injection of both cell types together into an immunodeficient mouse model of myocardial ischemia.

2.3 Hypotheses

1. Intra-cardiac injection of stem cells will improve post MI cardiac function, however a greater capacity to differentiate will be seen with CSCs as these cells have a greater capacity to differentiate into working myocardium.

2. Combination intra-cardiac injection of CSCs and CACs will improve post MI cardiac function greater than either therapy alone. This synergy is mediated by the greater ability of CACs to promote revascularization that better supports the growth and transdifferentiation of CSCs.

2.4 Specific Objectives

1. Characterize and compare the pro-angiogenic and cardiomyogenic cytokine profile of CSCs and CACs *in vitro* using conditioned media experiments.
2. Compare the functional effects of CACs and CSCs 21 days after transplantation into an immunodeficient mouse model of acute myocardial infarction.
3. Characterize the *in vivo* mechanisms through which CACs and CSCs provide functional improvements using histological and qPCR analysis.
4. Characterize the effects that culturing CACs and CSCs together *in vitro* has on the paracrine secretion and function of these stem cells.
5. Determine if a combinational cell product (CAC + CSC) will elicit a synergistic effect providing an enhanced cell therapy through functional and histological analysis.

3. Methods

3.1 Patients and cell culture

Human CACs and CSCs were cultured from tissue samples obtained from patients undergoing clinically-indicated heart surgery after informed consent. All protocols were approved by the University of Ottawa Heart Institution Research Ethics Board. Inclusion criteria for tissue donors selected patients between the ages of 18 and 80 who required cardiac surgery for coronary artery bypass grafting and/or valve surgery. Exclusion criteria included chronic infectious diseases (HIV, hepatitis), pregnant women or active sepsis.

CSCs were cultured as described previously.[72, 74] Cardiac tissue was minced, enzymatically digested with collagenase (1mg/mL; Invitrogen) and plated as cardiac explants on fibronectin coated dishes within cardiac explant media (CEM; Iscove's Modified Dulbecco's Medium (Invitrogen), 20% fetal bovine serum (FBS) (Invitrogen), 100 U/ml penicillin G, 100 ug/ml streptomycin (Invitrogen), 2 mmol/l L-glutamine (Invitrogen) and 0.1 mmol/l 2-mercaptoethanol (Invitrogen)). After seven days in culture, the heterogeneous population of cells (termed cardiac outgrowth) that spontaneously emigrated from the plated tissue was harvested using mild trypsinization (0.05% trypsin; Invitrogen). Three additional harvests were collected every 7-9 days. Only CSCs collected during the second harvest were used in this study.[74]

CACs (or early outgrowth endothelial progenitor cells) were isolated from peripheral blood samples as described previously.[201, 217-220] Mononuclear cells were isolated using density-gradient centrifugation (Histopaque 1077; Sigma-Aldrich, Canada) and placed in culture for

4-6 days in endothelial basal media (EBM-2; Clonetics, Canada) supplemented with EGM-2-MV-SingleQuots (Clonetics) that included 5% FBS, 50 ng/ml human vascular endothelial growth factor (VEGF), 50 ng/ml human insulin-like growth factor-1 (IGF-1) and 50 ng/mL human epidermal growth factor (EGF). CACs were harvested by mechanical dissociation and were used for experimentation within seven days of culture.

Normal human dermal fibroblasts (NHDFs) and human umbilical vein endothelial cells (HUVECs) were cultured according to the manufacturer's directions.

3.2 Conditioned media

Conditioned media was obtained from CSCs, CACs and NHDFs after 48 hours of culture in hypoxic conditions (1% oxygen) to simulate the environment of the infarcted myocardium. Cells were seeded at 90% confluency ($2.0x10^5$ CSCs, 3.0×10^6 CACs and $2.0x10^5$ NHDFs) in low serum basal media (Iscove's Modified Dulbecco's Medium, 1% fetal bovine serum (FBS), 100 U/ml penicillin G, 100 ug/ml streptomycin, 2 mmol/l L-glutamine and 0.1 mmol/l 2-mercaptoethanol) on 6-well plates (Corning).[77] To examine the relationship between CAC and CSC in co-culture conditions, conditioned media was collected from CSC/CAC co-cultures at different confluency ratios that corresponded to half the number of cells used in either single stem cell system (CSC^{low}/CAC^{high} $5.0 \times 10^4/1.5 \times 10^{6;}$; CSC^{high}/CAC^{low} $1.0 \times 10^5/7.5 \times 10^5$; CSC^{high}/CAC^{high} $1.0 \times 10^5/1.5 \times 10^6$). After 48 hours in culture, protein content of the cell lysate was determined using standard Bradford techniques (BioRad, Canada).

3.3 *In vitro* cytokine expression

Cytokine secretion in conditioned media was screened using a custom protein array (Human Cytokine Antibody Array G Series kit; RayBiotech, USA) according to the manufacturer's directions. The relative fluorescent signal was analyzed using a Genepix 4000B scanner with proprietary software (Molecular Devices Inc., USA). Median fluorescent values were normalized to the internal positive and negative controls. To detect growth factors that were secreted in excess to the negative cellular control (NHDF), the grouped NHDF densitometry signal was subtracted from individual CACs and CSCs values. Levels of cytokines of interest were confirmed using commercially available enzyme-linked immunosorbent (ELISA) assay kits (R&D Systems, USA; angiogenin (DAN00), epidermal growth factor (EGF; DEG00), hepatocyte growth factor (HGF; DHG00), interleukin-6 (IL-6; D6050), stromal cell-derived factor-1α (SDF-1α; DSA00) and vascular endothelial growth factor (VEGF; DVE00)). All immunosorbent measures were normalized to the protein content and media volume.

3.4 *In vitro* angiogenic differentiation and cell migration

The capacity of CACs and CSCs to stimulate angiogenic growth was assessed using a growth factor depleted matrigel assay (ECM625, Millipore) in accordance with the manufacturer's instructions, 2.0×10^4 human umbilical vein endothelial cells (HUVECs; Lonza) were seeded on matrigel with stem cell conditioned media (CSC-alone, CAC-alone, CSC/CAC co-cultures), NHDF conditioned media (negative cellular control) or serum free DMEM (negative media control). Random fields were analyzed using phase microscopy after 18 hours incubation in normoxic culture at 10x magnification. Cumulative tubular growth was determined using Image J software plug-in, NeuronJ (National Institutes of Health (NIH); http://rsb.info.nih.gov/ij).[74]

Similarly, the capacity of conditioned media to recruit CACs was assessed using fibronectin coated trans-well plates (24 wells, 3.0 µm pores; Corning) with 3.0 x 10^4 CACs plated in the upper well in serum-free DMEM while conditioned media (CSC-alone, CAC-alone, CSC/CAC co-cultures, NHDF) was placed in the bottom well. Serum free DMEM containing 100 ng VEGF was used as an unbiased control to normalize individual variations in CAC migration. After 24 hours of normoxic incubation, the inserts and the remaining upper compartment CACs were removed. CACs that had successfully migrated through the polycarbonate membrane were fixed (4% paraformaldehyde) and stained with DAPI (Sigma-Aldrich). Fluorescent microscopy (10x magnification, 6 random fields) was used to determine the average number of cells per random field (ImageJ, ICTN plugin, Center for Bio-Image Informatics, USA).

3.5 Flow cytometry of transplanted cells

The phenotypic profile of the CACs and CSCs transplanted into NOD SCID mice was confirmed using flow cytometry (FACSAria I, BD Biosciences, USA). Cells were fixed with 4% paraformaldehyde and stored at 4°C. Monoclonal antibodies and similarly conjugated isotype-matched control monoclonal antibodies for CD34 (316401, eBioscience), CD90 (555596, BD Biosciences), c-Kit (FAB332A, RD Systems), CD133 (130-090-826, Miltenyi) and VEGFR2 (FAB357P, RD Systems) were used. A minimum of 100,000 events were collected after performing fluorescent compensation using single labelled controls. Positive cells were defined as the percent of the population falling above the 99th percentile of the isotype control (Cyflogic, v.1.2.1 CyFlo Ltd, USA).

3.6 Myocardial infarction, cell injection, and functional evaluation

Myocardial infarctions (MI) were performed in male NOD-SCID mice (8-9 weeks old) by permanent ligation of the left anterior descending (LAD) coronary artery.[74] Animals were injected with buprenorphine (0.05mg/kg; subcutaneous) one hour prior to surgery and twice daily thereafter for 3 days. During the ligation, mice were incubated and anesthetized using isoflurane (maintained at 2-3%) and upon closure, animals were injected with 0.5 cc of saline (subcutaneous).

Seven days after LAD ligation, 10-15 mice per group were injected with 1×10^5 cells (CSCs, CACs or NHDF) or a negative vehicle control (PBS) at the cardiac apex and lateral border zone. A fourth group received CAC and CSC co-administration at the cardiac apex and lateral border zone, with both CACs and CSCs (0.5×10^5 CSCs + 0.5×10^5 CACs) as a single cell therapy. Trans-thoracic intra-myocardial injection was performed using echocardiographic guidance to confirm cells were injected into the myocardium. Twenty one days, 28 days and 16 weeks (long-term cohort only) after LAD ligation, the effect of cell therapy was evaluated from the left ventricular ejection fraction (LVEF; VisualSonics V1.3.8, VisualSonics, Toronto, Canada). Animals were sedated using a ketamine/xylazine (100mg/ml / 20mg/mL) cocktail (10µL/g; intraperitoneal injection) during each ECHO procedure as well as during intramyocardial injections. To account for multiple comparisons made from the serial echocardiograms, this functional data was analyzed using a repeated measures mixed model with post-hoc testing done using t-tests, as appropriate, with Bonferonni's correction. Long-term effects of cell therapy were evaluated in a subset of mice from each group 16 weeks after MI (n=4). All functional evaluations were conducted and analyzed by investigators blinded to the animal's treatment group. After the last

assessment of myocardial function, the mice were euthanized and hearts excised for histology or quantitative PCR analysis.

3.7 Quantitative PCR (qPCR) analysis

Myocardial retention of transplanted cells was assessed in a subset of mice (n=3/group) using qPCR for non-coding human alu repeats (Table 3.1).[221] Left ventricular genomic DNA was extracted and qPCR was performed with transcript specific hydrolysis primer probes.

Table 3.1- qPCR primers for *in vivo* stem cell retention.

Transcript	Primer Sequence	Reference
Alu	*Fw* 5'-CAT GGT GAA ACC CCG TCT CTA-3' *Rv* 5'-GCC TCA GCC TCC CGA CTA G-3' *Pr* 5'-FAM – ATT AGC CGG GCG TGG TGG CG- TAMRA-3'	Munoz et al., 2005

Fw: Forward Primer
Rv: Reverse Primer
Pr: Probe

3.8 Histology

After the final assessment of myocardial function, the mice were sacrificed. The hearts were excised, fixed with 4% paraformaldehyde, embedded in OCT and sectioned. Tissue viability within the infarct zone was calculated from Masson's trichrome (Invitrogen, Canada) stained sections by tracing the infarct borders manually and then using ImageJ software to calculate the percent of viable myocardium within the overall infarcted area. To evaluate stem cell engraftment and differentiation, immunostaining for human nuclear antigen (HNA; SAB4500768, Sigma, Canada) was used to detect cells of human origin. Co-staining with non-specific α-SMA (ab125266; Abcam), cTnT (ab66133; Abcam) and vWF (11778-1-AP;

Proteintech Group, USA) was used to identify cells that differentiated into functional cardiomyocytes. Capillary density within the infarct border zone was assessed by staining for non-specific isolectin B4 expression (B-1205; Vector Laboratories, Canada) in conjunction with DAPI (Sigma, Canada). The total number of nuclei within one image field of the border zone were counted and assessed for isolectin B4 expression. For these measures, three sections were analyzed per animal and averaged with at least 3 animals per group.

3.9 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) (G-B Stat software). 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.

4. Results

4.1 Baseline demographics

Fifty three patients (69% male; age 68±2 years; BMI 29±1 kg/m^2, Table 4.1) were enrolled in the study. All patients had a history of stable cardiac disease with numerous cardiovascular risk factors, including diabetes (37%), hypertension (74%) and dyslipidemia (65%). The majority of patients had a history of coronary artery disease (75%), MI (22%), valvular heart disease (31%) and congestive heart failure (31%). The majority of patients underwent elective cardiac surgery for coronary bypass alone (65%) with the remainder undergoing valve repair/replacement alone (25%) or coronary bypass with valve repair/replacement (10%). No patient had experienced an acute coronary syndrome or admission for congestive heart failure for 6 months prior to sample collection. All patients were on stable cardiac medications including angiotensin-converting enzyme inhibitors and/or angiotensin receptor blockers (82%), anti-platelet therapy (75%), beta-blockers (69%) and statins (61%) for at least six months prior to surgery. While the baseline clinical characteristics of the patients were similar, notable exceptions included a tendency for better renal function (1.2±0.1 vs. 0.9± 0.1 ml/min; $p \leq 0.05$) and worse chronic stable angina (CCS class 1.2±0.1 vs. 0.3±0.2, $p \leq 0.05$) in the patients who donated samples for the *in vivo* experiments conducted in this study.

Atrial appendage specimens were collected at the time of cardiac surgery and began processing within one hour of harvest. To provide an unbiased comparison of CAC and CSC efficacy, blood samples for *in vitro* testing were collected at the time of cardiac surgery (Figure 4.1). In deference to a clinically translatable protocol and the different times required for stem cell culture (6 vs. 14 days), blood samples for *in vivo* testing were collected 8 days after cardiac

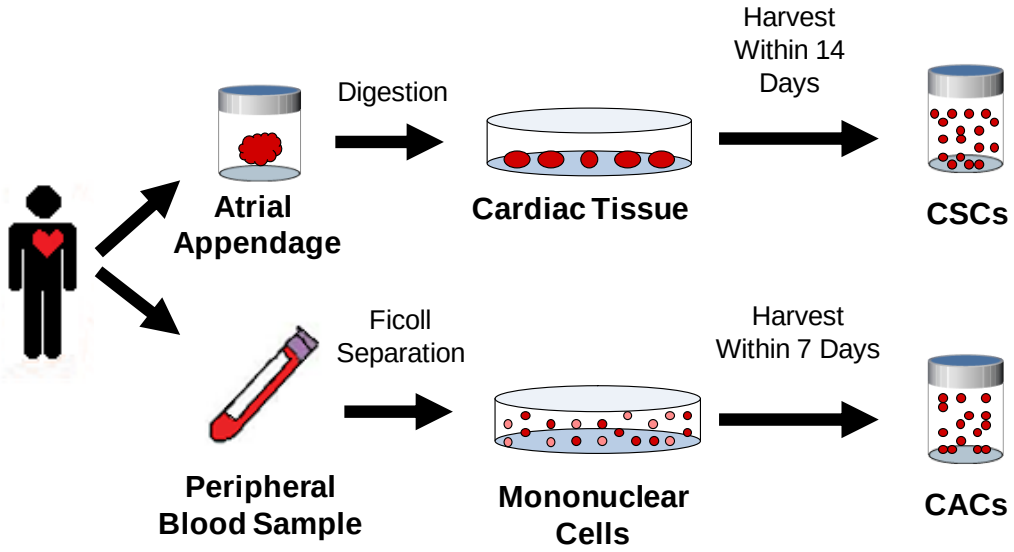
surgery. Flow cytometry of representative collections of both cell types demonstrated characteristic proportions of CAC and CSC identity markers (Figure 4.2). Age and other comorbidities were not found to influence overall culture yield.

Table 4.1. Baseline clinical characteristics of the patients.

	All patients (N=53)	In vitro studies (n=44)	In vivo studies (n=9)
Age (yrs)	68±2	68±2	67±3
BMI (kg/m^2)	29±1	29±2	30±2
Gender (%male)	69%	67%	78%
Diabetes	37%	40%	25%
Hypertension	74%	76%	63%
Dyslipidemia	65%	67%	56%
Ongoing Smoking	10%	12%	0%
Thyroid disease	9%	8%	14%
Peripheral vascular disease	10%	5%	38%
Coronary Artery Disease	75%	70%	88%
History of MI	22%	21%	25%
Valvular Heart Disease	31%	33%	14%
Congestive Heart Failure	31%	33%	14%
NYHA class	1.7±0.2	1.6±0.2	2.0±0.6
LV ejection fraction	44±13	43±13	53±4
CCS class	1.0±1.3	0.8±1.2	2.8±0.5*
Creatinine (umol/L) / GFR (mL/min)	93±4/0.9±0.1	96±4/0.9±0.1	78±7/1.2±0.1*
Medications:			
Anti-platelet therapy	75%	73%	100%
Beta-blocker	69%	74%	45%
Statins	61%	62%	57%
ACEI or ARB	82%	86%	57%

BMI = Body mass index. MI=Myocardial Infarction. NYHA=New York Heart Association. LV=Left Ventricle. CCS=Canadian Cardiovascular Society. GFR=Glomerular Filtration Rate. ACEI=Angiotensin-Converting Enzyme Inhibitors. ARB=Angiotensin Receptor Blockers. Significant: $p \leq 0.05$ vs. *in vitro* study patient characteristics.

A.



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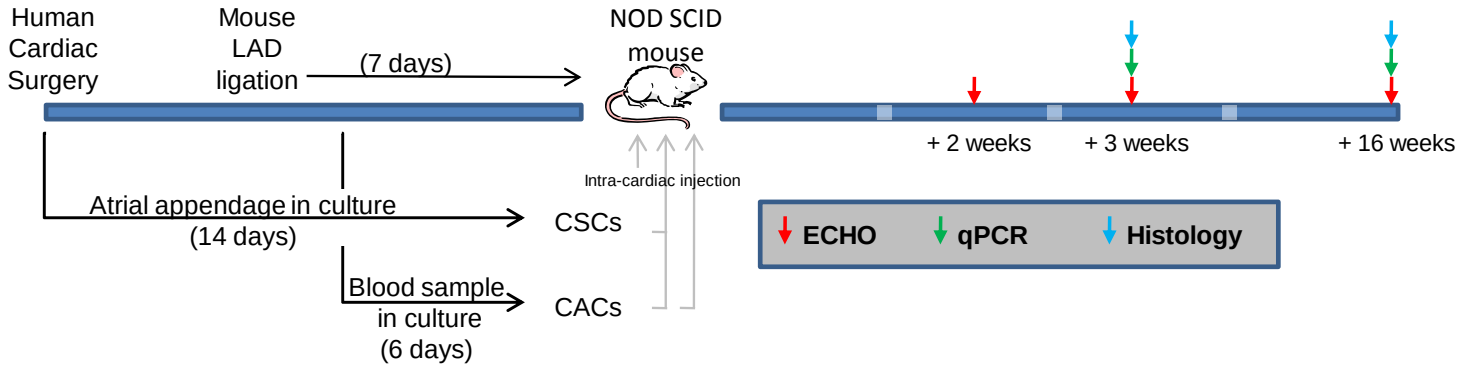
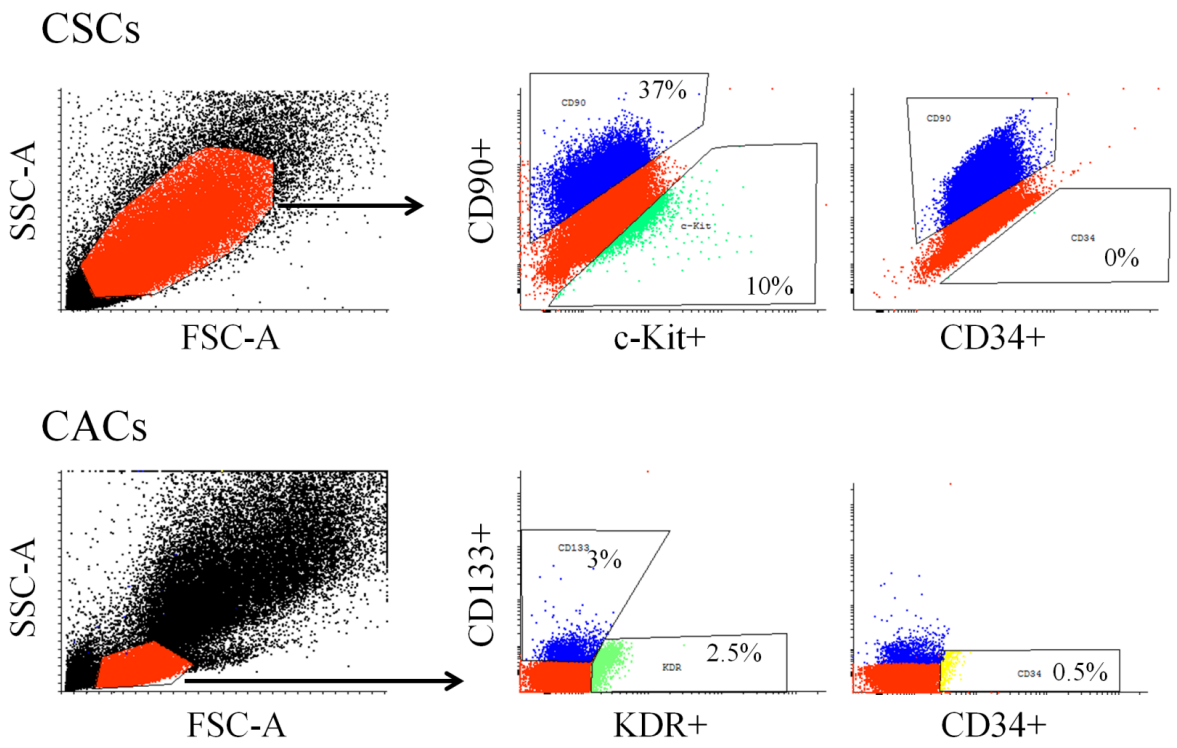


Figure 4.1. Experimental Design. A. Schematic representation of the culture protocol for CACs and CSCs. B. Schematic outlining the timing of the cell culture with animal surgeries, cell transplantation and outcome measures



CSC		CAC	
c-Kit	2.7±1.7%	CD133	1.6±0.4%
CD90	47±9%	KDR	1.0±0.3%
CD34	<0.1%	CD34	0.3±0.2%

Figure 4.2. CAC and CSC surface marker expression. Flow cytometry analysis of the relative proportion of surface marker expression on representative fractions of CSCs and CACs.

4.2 Human CACs express a broader cytokine profile than human CSCs

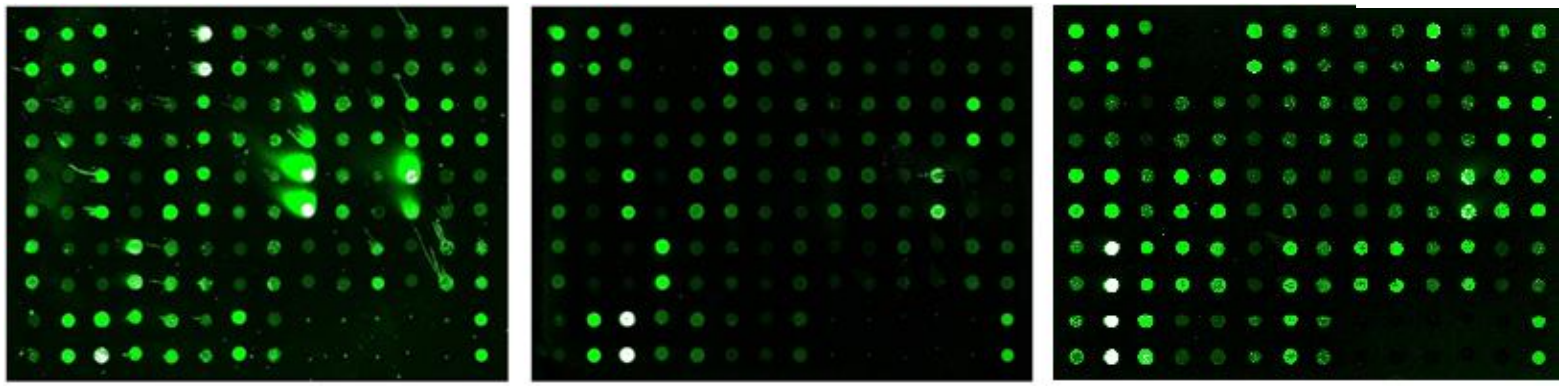
4.2.1 Characterization using cytokine detection arrays

The paracrine profile of human CSCs, CACs and NHDFs was screened using conditioned media with a custom protein array. This array returned a proportional fluorescent signal for the 59 cytokines tested with 2 technical repeats (Figure 4.3). Figure 4.4 demonstrates three representative blots from human CSCs, CACs and NHDFs. As shown in Figure 4.4b, both CACs and CSCs produced a large number of growth factors in excess to NHDF (36 and 5, $p \leq 0.05$ vs. NHDF). Interestingly, the paracrine profile of CACs was significantly broader than CSCs (Chi square value 3.93, $p \leq 0.05$) with rare instances of the same growth factor being over-expressed by both cell types (angiopoetin-1, hepatocyte growth factor (HGF) and vascular endothelial growth factor (VEGF)).

	A	B	C	D	E	F	G	H	I	J	K	L	M	N
1	POS 1	POS 2	POS 3	NEG	NEG	Angiogenin	Angiopoietin-1	Angiostatin	AR	bFGF	BMP-4	BMP-6	BMP-7	EGF
2	POS 1	POS 2	POS 3	NEG	NEG	Angiogenin	Angiopoietin-1	Angiostatin	AR	bFGF	BMP-4	BMP-6	BMP-7	EGF
3	E-Selectin	FGF-4	FGF-6	FGF-7	FGF-9	GCSF	GM-CSF	HB-EGF	HGF	ICAM-1	IFN-gamma	IGFBP-1	IGFBP-2	IGFBP-3
4	E-Selectin	FGF-4	FGF-6	FGF-7	FGF-9	GCSF	GM-CSF	HB-EGF	HGF	ICAM-1	IFN-gamma	IGFBP-1	IGFBP-2	IGFBP-3
5	IGFBP-4	IGFBP-5	IGFBP-6	IGF-I	IGF-II	IL-1alpha	IL-1beta	IL-10	IL-6	LIF	L-Selectin	MCP-1	M-CSF	MIP-3alpha
6	IGFBP-4	IGFBP-5	IGFBP-6	IGF-I	IGF-II	IL-1alpha	IL-1beta	IL-10	IL-6	LIF	L-Selectin	MCP-1	M-CSF	MIP-3alpha
7	MMP-2	MMP-9	Osteopontin	Osteoprotegerin	PDGF-AA	PDGF-AB	PDGF-BB	PECAM-1	SCF	SDF-1alpha	SDF-1beta	TGF-alpha	TGF-beta 1	TGF-beta 2
8	MMP-2	MMP-9	Osteopontin	Osteoprotegerin	PDGF-AA	PDGF-AB	PDGF-BB	PECAM-1	SCF	SDF-1alpha	SDF-1beta	TGF-alpha	TGF-beta 1	TGF-beta 2
9	TGF-beta 3	TIMP-1	TIMP-2	TNF-alpha	TNF-beta	VCAM-1	VEGF	VEGF R2	Neg	Neg	Neg	Neg	Neg	POS 2
10	TGF-beta 3	TIMP-1	TIMP-2	TNF-alpha	TNF-beta	VCAM-1	VEGF	VEGF R2	Neg	Neg	Neg	Neg	Neg	POS 2

Figure 4.3. Schematic of the custom protein array.

A.



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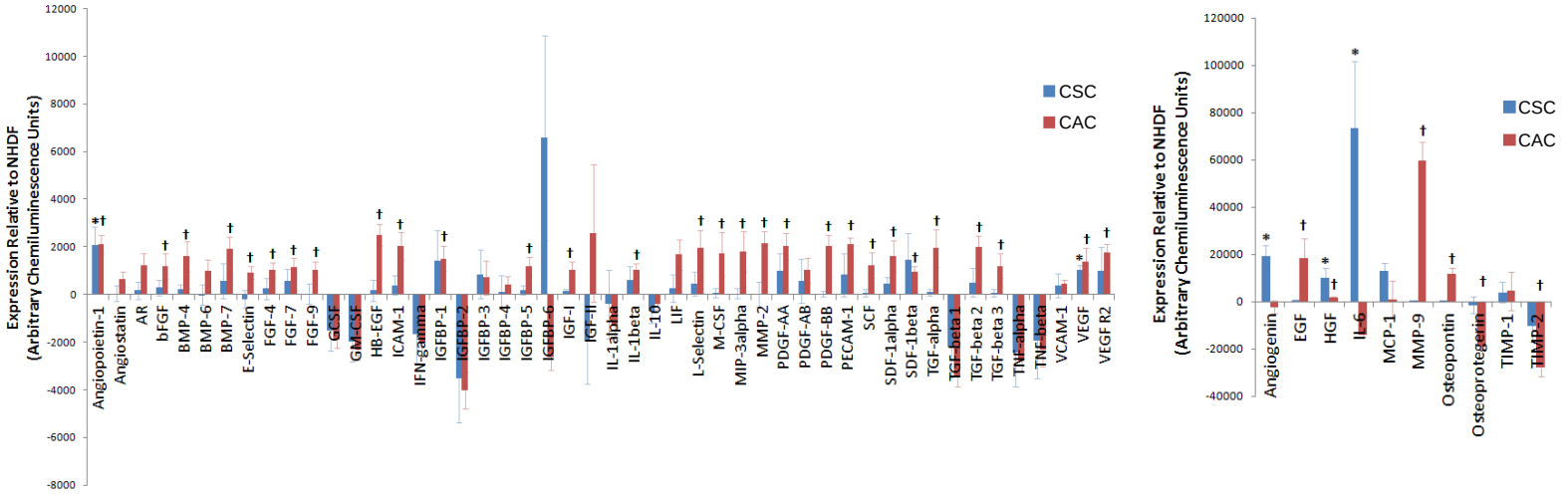


Figure 4.4. Growth factors produced by CACs, CSCs and NHDFs under hypoxic culture conditions. A. Representative images of the custom protein array used to screen conditioned media from CACs, CSCs and NHDFs. Densitometry values were run in duplicates on the same array (Figure 4.3) B. Densitometry analysis of growth factors produced by CACs (n=6) and CSCs (n=8) as compared to NHDF (n=7). *p≤0.05 for CSCs vs. NHDF, †p≤0.05 for CACs vs. NHDF.

4.2.2 Quantitative analysis using ELISA

Confirmatory ELISA analysis was performed on select cytokines based upon high levels of expression or literature supporting a key role in post-infarct repair (Figure 4.5). These assays confirmed that CSCs produced greater amounts of angiogenin, HGF, interleukin-6, stromal cell-derived-factor-1α (SDF-1α) and VEGF whereas CACs produced greater amounts of epidermal growth factor (EGF). The possibility that different combinations of cell types may interact to influence growth factor secretion was analyzed using co-cultures at different confluency ratios. These combination co-cultures corresponded to half the number of cells used in either single stem cell system. (CSC^{low}/CAC^{high} $5.0 \times 10^4/1.5 \times 10^6$; CSC^{high}/CAC^{low} $1.0 \times 10^5/7.5 \times 10^5$; CSC^{high}/CAC^{high} $1.0 \times 10^5/1.5 \times 10^6$). Combination culture did not provide additional production of EGF and HGF in all three co-culture conditions ($p \leq 0.05$ vs. single cultures), whereas angiogenin, SDF-1α and VEGF were all produced in an incremental fashion ($p \leq 0.05$ vs. single cultures). This data suggests that important co-stimulation occurs between the different cell types which may increase the potency of combination therapy when CACs and CSCs are administered together.

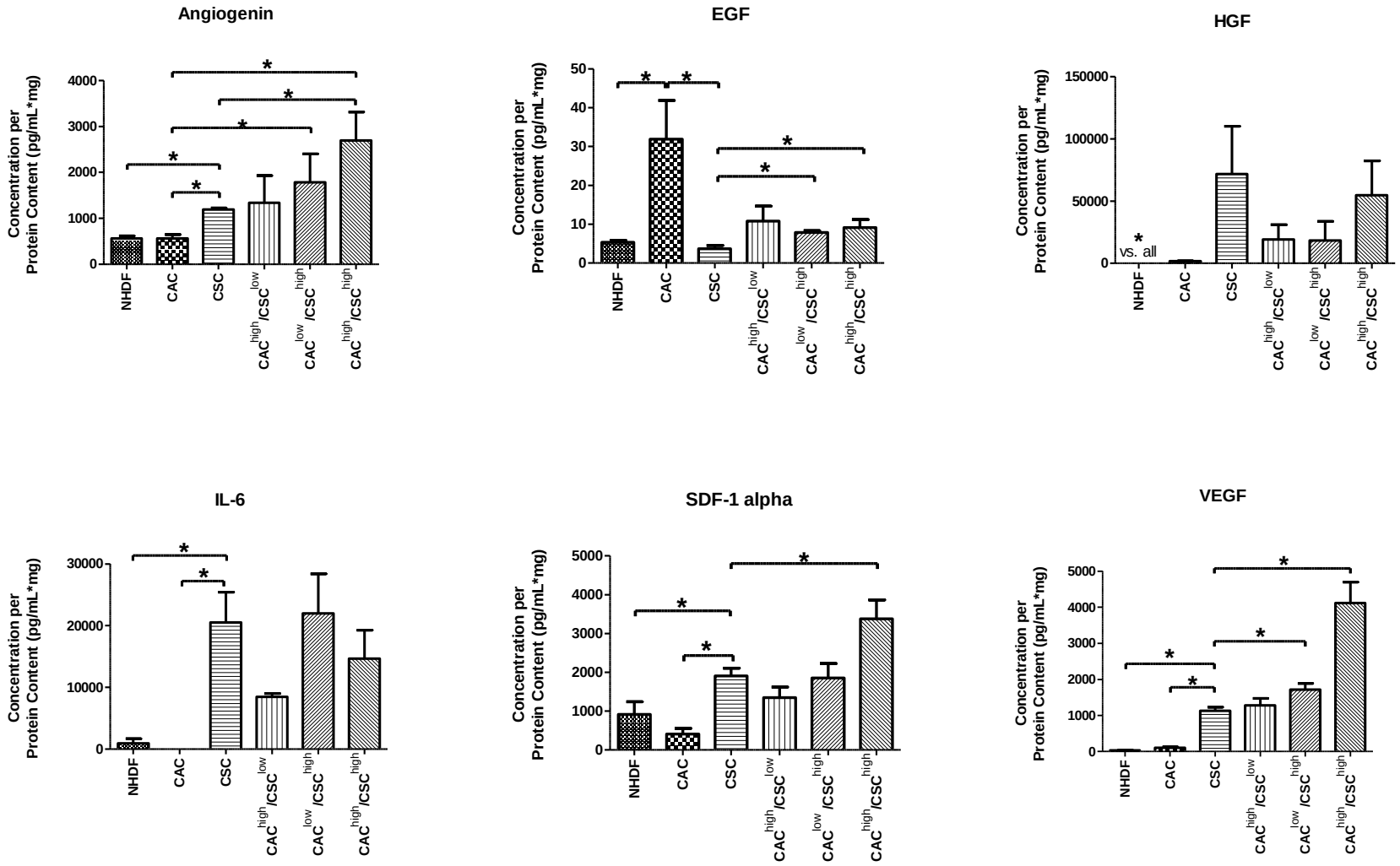


Figure 4.5. Influence of CAC and CSC co-culture on growth factor production under hypoxic conditions. The effect of varying CSC/CAC populations was investigated using different confluency ratios that corresponded to half the number of cells used in either single stem cell system. * $p \leq 0.05$; n = 4 samples per assay.

4.3 Human CACs and CSCs increase angiogenesis and cell migration

The capacity of human CACs and CSCs to form blood vessels was assessed by exposing HUVECs to stem cell conditioned media within a growth factor depleted matrigel assay (Figure 4.6). Media conditioned from CAC and CSC cultures stimulated vessel formation to a similar extent (p=ns). Conditioned media from co-cultures demonstrated an additive effect with more tubule formation ($p \leq 0.05$). Conditioned media from CAC and CSC cultures attracted CACs to a similar extent (p= ns) whereas conditioned media from CSC/CAC co-cultures showed a greater capacity to attract CACs than either single culture alone ($p \leq 0.05$). These results suggest that CSC and CACs have a similar capacity to support angiogenesis while co-culture further enhances this effect.

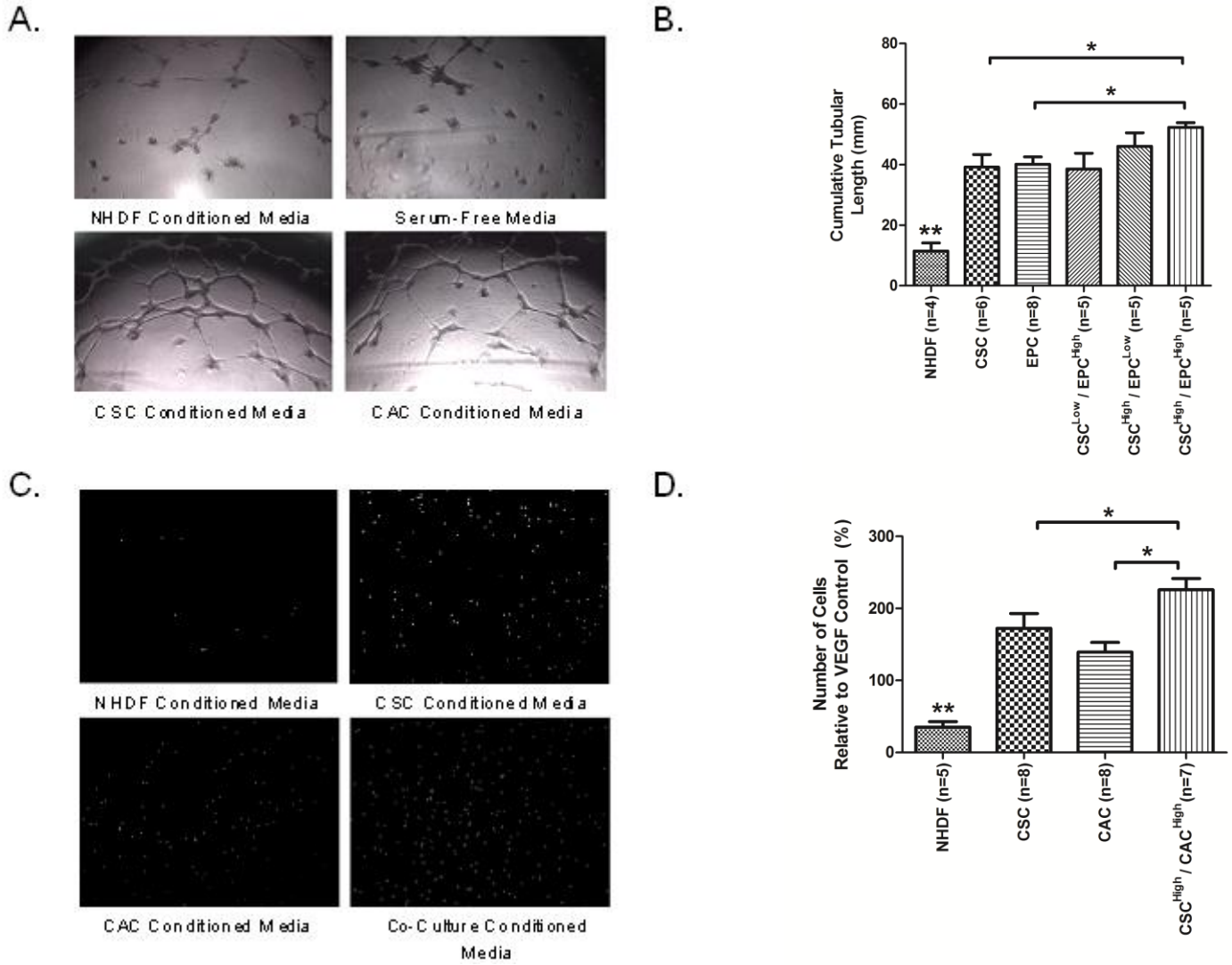


Figure 4.6. Pro-angiogenic effects of CACs and CSCs. A. Representative images of matrigel cultured HUVECs exposed to conditioned media from CACs, CSCs, NHDF and serum free controls. B. Cumulative tubular length analysis demonstrates greater vessel formation in stem cell conditioned media as compared to the negative cellular control. Conditioned media from the co-culture of CACs and CSCs conditioned media further enhanced HUVEC vessel formation. C. Representative images of CACs after migration through the transwell filter when exposed to conditioned media from CACs, CSCs, NHDF and CSC/CAC co-cultures. D. Analysis of the number of cells that migrated through the transwell filter after exposure to conditioned media and normalized to unbiased VEGF-alone stimulation. *$p \leq 0.05$; **$p \leq 0.05$ compared to all other cell cultures.

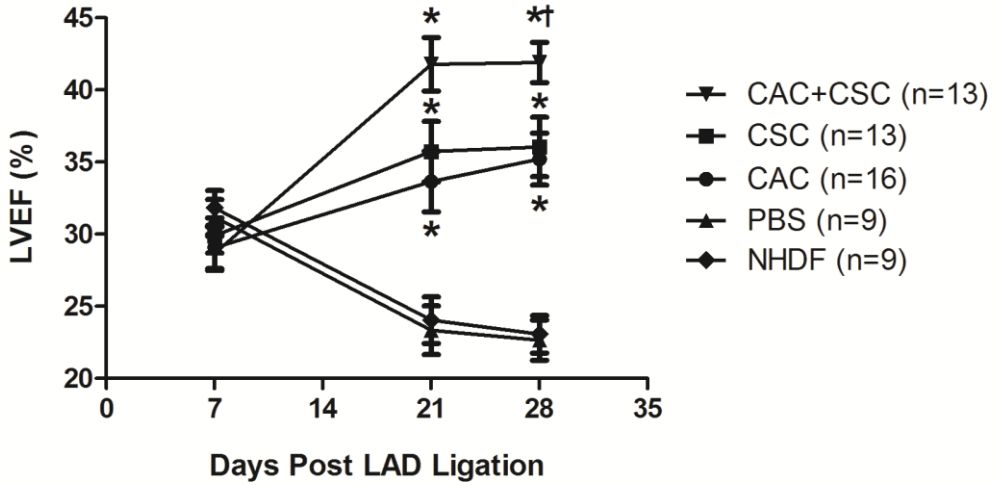
4.4 Human CACs and CSCs provide equivalent myocardial repair with superior benefits using combination therapy

The effect of human CACs and CSCs alone or in combination was assessed after intramyocardial injection into an immunodeficient mouse model of myocardial ischemia. As shown in Figure 4.7a, animals treated with CACs or CSCs alone had a greater ejection fraction (37±2% and 36±2%, respectively) three weeks after LAD ligation than animals treated with NHDF or PBS (22±2% and 23±1%, respectively; $p \leq 0.05$). These benefits were maintained in both individual treatment groups 3 months after LAD ligation (37±2% and 36±2%, respectively; Table 4.2). In a manner consistent with the *in vitro* data, co-transplantation of CACs and CSCs provided greater myocardial repair three weeks after LAD ligation as compared to injection with either cell type alone (Figure 4.7a). Long-term data from a subset of mice (n=4), suggests that these effects are sustained (p=ns, +28 day vs. +16 week post LAD ligation LVEF) as compared to the progressive marked decline in the PBS treatment group (+16 week EF $p \leq 0.05$ compared to CAC or CSC-alone treatment; Figure 4.8a).

These functional benefits occurred in spite of very modest retention of injected cells (Figure 4.7b). Furthermore despite equivalent degrees of myocardial repair, fewer CSCs were found 21 days after intra-myocardial injection as compared to CACs (0.5±0.1% vs. 3.6±1.1%, $p<0.05$). Combination therapy with both CACs and CSCs did not enhance engraftment or cell retention 21 days after injection (p=ns) even though superior effects on myocardial repair were observed. Long term engraftment data demonstrated that while human CSCs continued to persist in the mouse myocardium whereas CAC retention dwindled to comparable numbers by 16 weeks after transplantation (Figure 4.8b). Taken

together, this data hints that the benefits observed with first generation CAC and CSC products are independent of long term myocardial retention and reflect the contribution of growth factors supplied in the first weeks after cell injection.

A.



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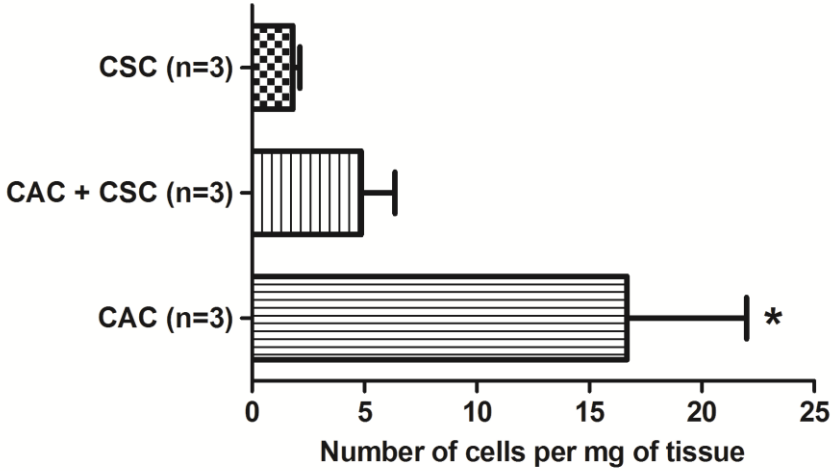


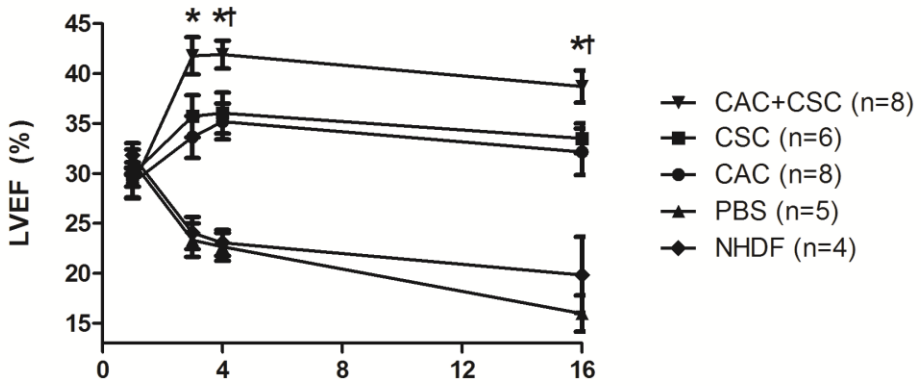
Figure 4.7. Effects of CAC and CSC treatment on myocardial repair and survival. A. Comparison of the effect of cell treatment on LVEF. *****p≤0.05 vs. NHDF or PBS controls using repeated ANOVA; **†**p≤0.05 vs. CAC or CSC alone using repeated ANOVA. B. Quantitive PCR for human alu sequences demonstrating modest long term engraftment of transplanted cells. *p≤0.05 vs. CSC alone.

Table 4.2. Echocardiographic measurements of left ventricle over 16 week follow-up period.

		End Diastolic Volume (µL)	End Systolic Volume (µL)	Stroke Volume (µL)	Ejection Fraction (%)
1 week post MI	NHDF	69.8±5.6	48.5±3.9	22.3±2.2	31.8±1.2
	PBS	60.4±4.3	41.7±3.3	18.7±1.3	31.2±1.2
	CAC	64.7±2.8	46.2±2.3	18.6±1.2	29.1±1.5
	CSC	63.6±3.8	45.1±2.6	18.6±1.7	29.9±1.2
	CAC+CSC	58.5±3.2	40.1±2.1	18.34±1.8	28.7±1.2
3 weeks post MI	NHDF	79.8±11.2	41.5±9.7	18.3±1.8	24.0±1.6
	PBS	74.3±7.4	57.7±6.6	16.6±1.2	23.3±1.7
	CAC	90.3±5.6	61.1±5.1	29.2±2.1*	33.7±2.1*
	CSC	83.3±6.1	52.9±5.6	30.4±2.2*	35.7±2.1*
	CAC+CSC	77.1±2.8	42.9±1.8	34.1±2.2*	41.8±1.5*†
4 weeks post MI	NHDF	85.6±10.0	66.4±8.7	19.1±1.8	23.1±1.3
	PBS	81.4±7.7	63.5±6.5	18.0±1.5	22.7±1.4
	CAC	88.4±5.4	57.7±4.8	30.8±1.4*	35.2±1.8*
	CSC	87.7±7.8	57.1±5.4	30.6±3.5*	36.1±2.1*
	CAC+CSC	75.4±5.1*T	43.5±2.8	31.9±2.7*	41.9±1.4*†
16 weeks post MI	NHDF	124.5±37.9	104.1±37.7	20.4±1.2	19.8±3.8
	PBS	85.0±13.7	70.8±11.7	14.2±2.3	16.0±1.8
	CAC	92.4±5.5	62.4±4.9	30.0±3.1*	32.2±2.3*
	CSC	84.8±7.5	56.8±6.1	27.9±1.2*	33.5±1.5*
	CAC+CSC	89.7±5.2	55.5±4.2	34.2±1.3*	38.7±1.6*†

*****$p \leq 0.05$ vs. PBS or NHDF treatment; †$p \leq 0.05$ vs. CAC and CSC treatment.

A.



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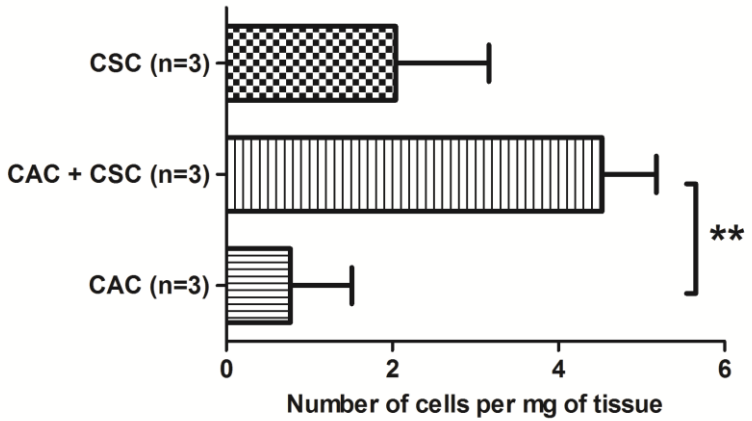

Figure 4.8. Long term (16 week) effects of CAC and CSC transplantation upon myocardial function. A. Week 16 echocardiograms demonstrate the long term effects of CAC and CSC transplantation upon LVEF. B. Quantitive PCR for human alu sequences demonstrating the modest long term (16 week) engraftment of first generation CAC and CSC therapies. *****p≤0.05 vs. pre-transplant (day 7) LVEF; †p≤0.05 vs. PBS treatment at week 16; **p≤0.05 vs.CAC transplantation.

4.5 Transplantation of human CAC and CSCs reduce ventricular scar burden with superior effects using combination therapy

Scar formation and tissue viability within the infarct zone was analyzed using Masson's trichrome stained sections 21 days after stem cell injection (Figure 4.9). Both CAC and CSC transplantation reduced scar formation when compared to PBS treated animals (16.7±1.0% and 13.9±0.8% vs. 23.0±1.6% respectively; $p \leq 0.05$). CSC therapy alone prevented scar formation to a greater degree as compared to CAC treated animals ($p \leq 0.05$) despite equivalent effects on myocardial function. Transplantation of both cell types in combination reduced ventricular scar burden to a greater degree as compared to either cell type alone (8.9±1.0%; $p \leq 0.05$). Cell-mediated effects on ventricular scarring were sustained over long-term follow up (Figure 4.10). CAC and CSC treaded animals demonstrated a significantly higher capillary density within the peri-infarct region compared to PBS treated controls (27.8±3.2% and 22.2.9±2.1% vs. 15.1±2.2% respectively; $p \leq 0.05$). While single cell therapies yielded comparable capillary densities, combination cell therapy improved capillary density over all treatment groups (42.6±2.2%; $p \leq 0.05$; Supplementary Figure 4.11).

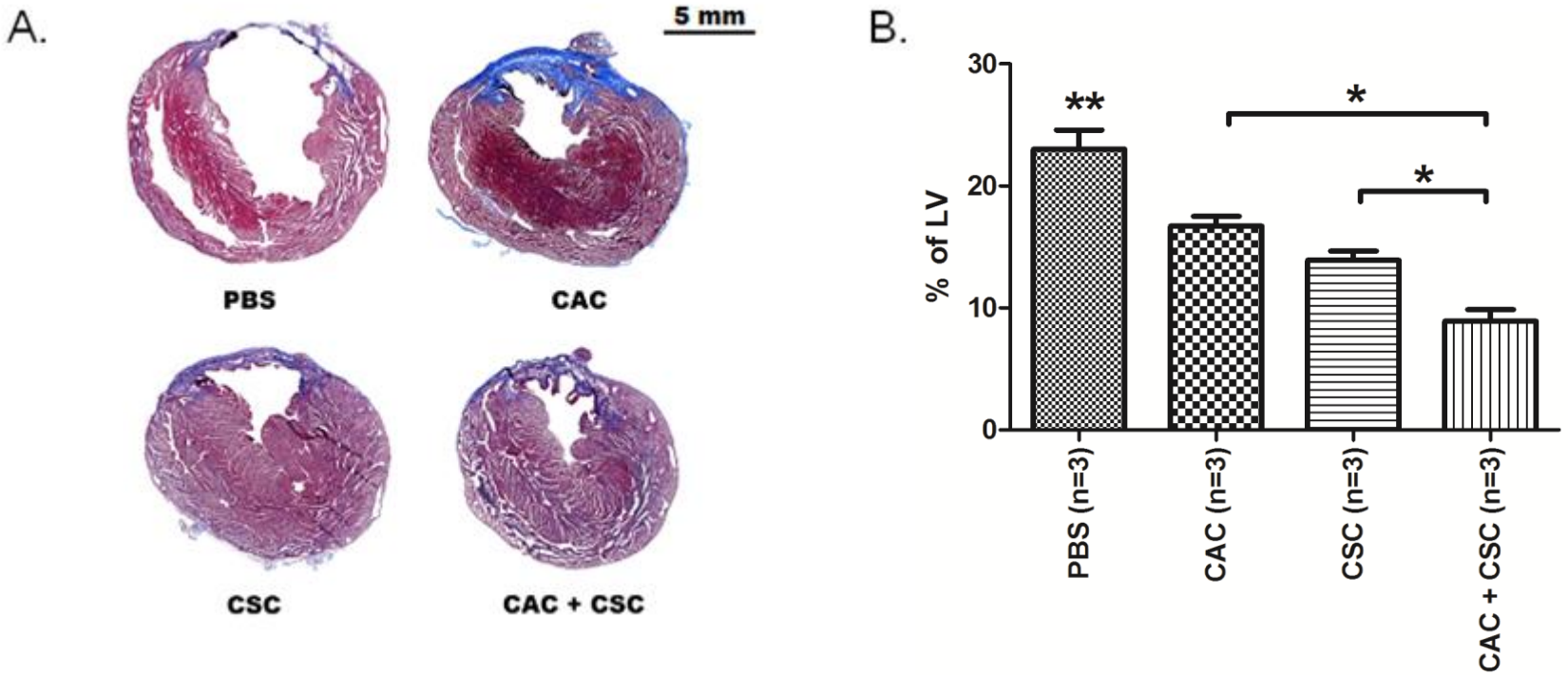

Figure 4.9. Effects of CAC and CSC transplantation on ventricular scar burden after LAD ligation. A. Representative histology images from each stem cell treatment 28 days after myocardial infarction demonstrating scar burden using Masson's trichrome satin. B. Quantification of the scar tissue present in the left ventricle (LV) 28 days after myocardial infarction. . * $p \leq 0.05$ vs. single-cell therapies ** $p \leq 0.05$ vs. CACs or CSCs or CAC+CSCs.

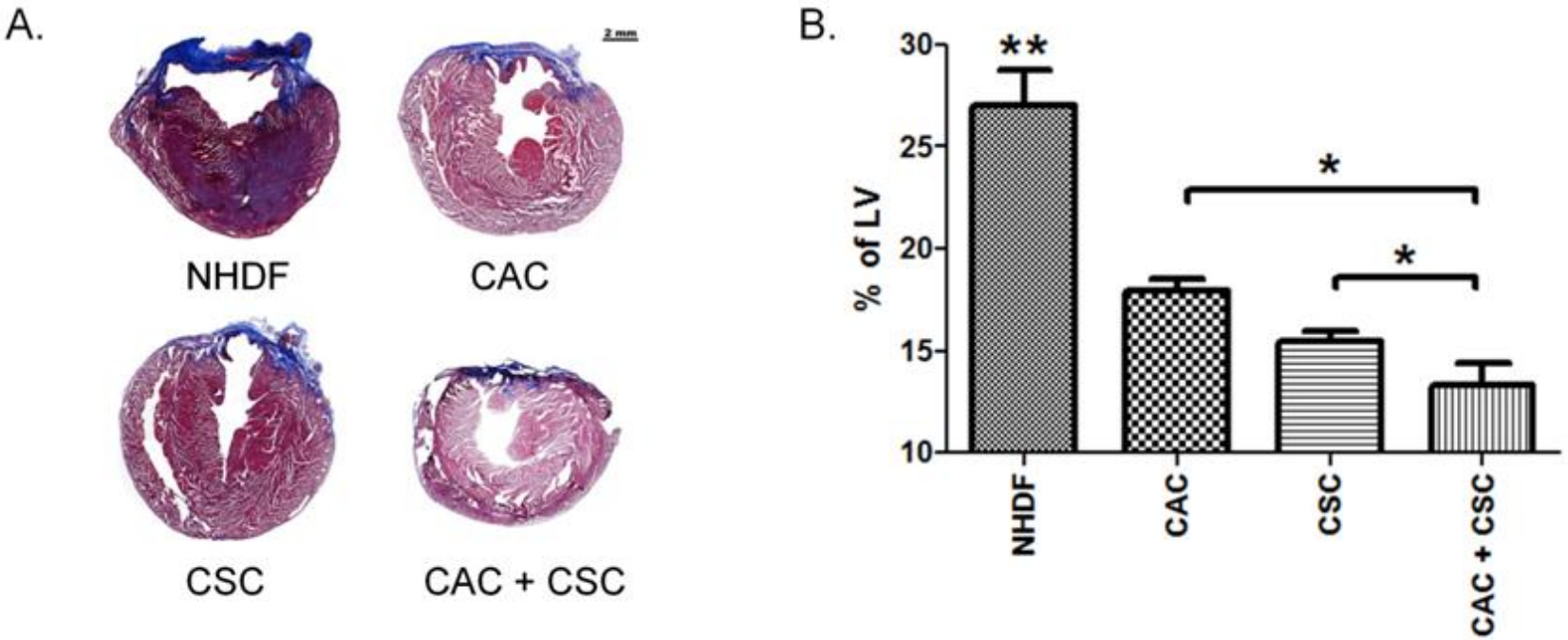

Figure 4.10. Long term (16 week) effects of CAC and CSC transplantation on left ventricular scar burden. A. Representative Masson's trichrome images of each cell therapy 16 weeks after myocardial infarction. B. Percentage scar formation within the left ventricle was assessed using ImageJ software. CAC/CSC co-transplantation resulted in decreased scar burden when compared to single cell therapy, while all three therapies demonstrated enhanced effects over NHDF treated animals (n=3 per group). * $p \leq 0.05$ vs. single-cell therapies ** $p \leq 0.05$ vs. all other cell therapies.

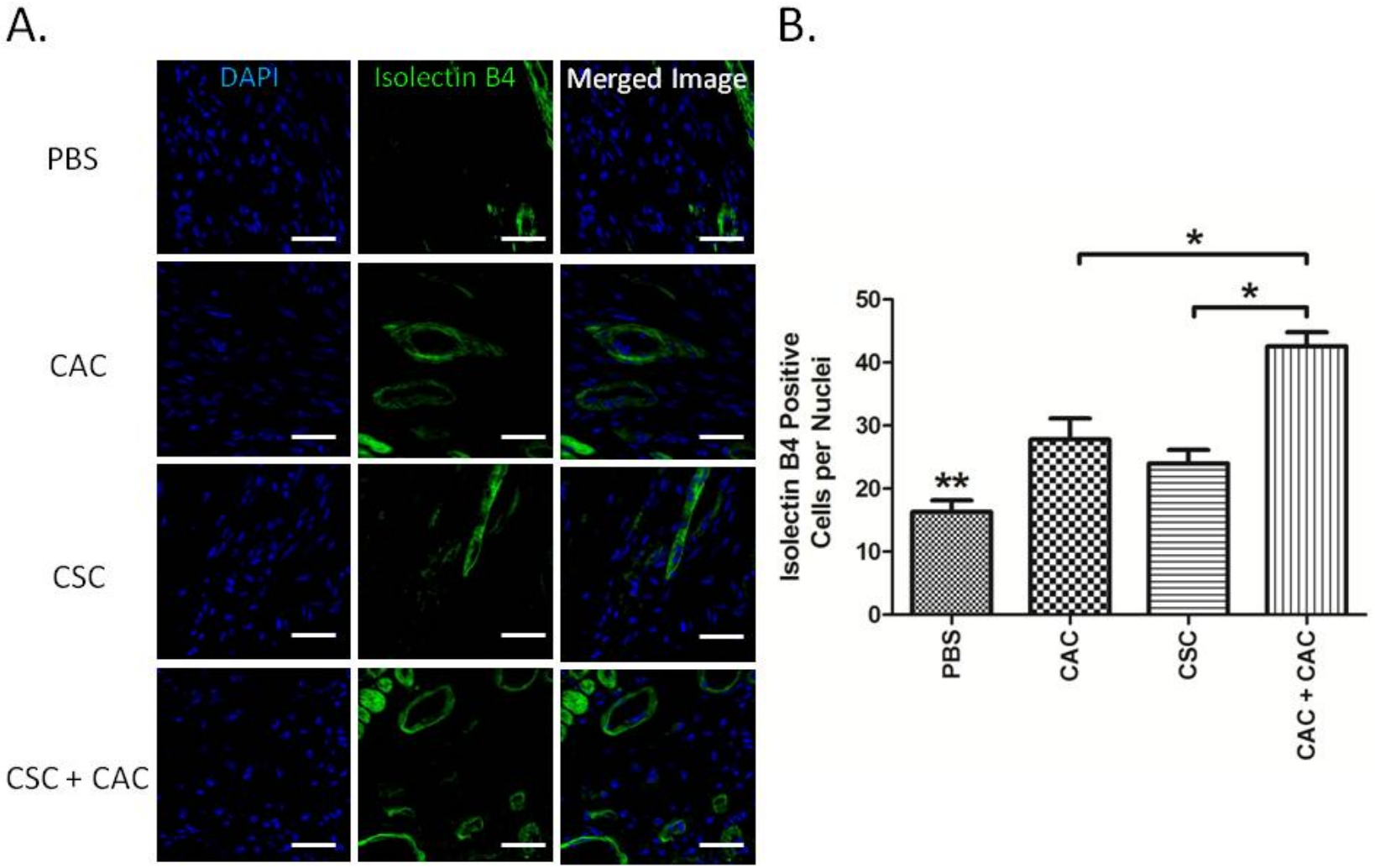

Figure 4.11. Capillary density within the border zone of the ventricular infarcts 28 days after cell transplantation. A. Representative images of the capillary density between treatment groups one microscope field from the infarct border zone. Scale bar = 30 µm. B. Comparison of the percentage of isolectin B4 positive capillaries within the border zone between treatment groups (n=3 per group). CAC/CSC co-transplantation resulted in increased capillary density when compared to single cell therapy, while all three therapies demonstrated enhanced effects over control animals. * $p \leq 0.05$ vs. single-cell therapies ** $p \leq 0.05$ vs. all other cell therapies.

4.6 Small “clusters” of differentiated human cells persist within the infarct and peri-infarct regions

To evaluate stem cell engraftment and differentiation, histological sections were labeled with human nuclear antigen in conjunction with markers for cardiac lineage (smooth muscle (α-smooth muscle actin; α-SMA), vascular cells (Von Willebrand Factor; vWF) and myocytes (cardiac troponin T; cTnT); Figure 4.12). Small clusters of human cells were identified 21 days after stem cell transplantation in each treatment group within the peri-infarct region as well as the infarct itself. This indicates that each stem cell treatment provided cells capable of engrafting and differentiating into functional cells within the damaged myocardium, albeit at a modest degree. Animals transplanted with human CACs alone had only human cells of vascular identity found on follow-up histology (Figure 4.13). In contrast, animals treated with either CSCs alone or combination CACs+CSCs had human cells of all three lineages found-demonstrating the inherent multi-lineage potential of CSCs.

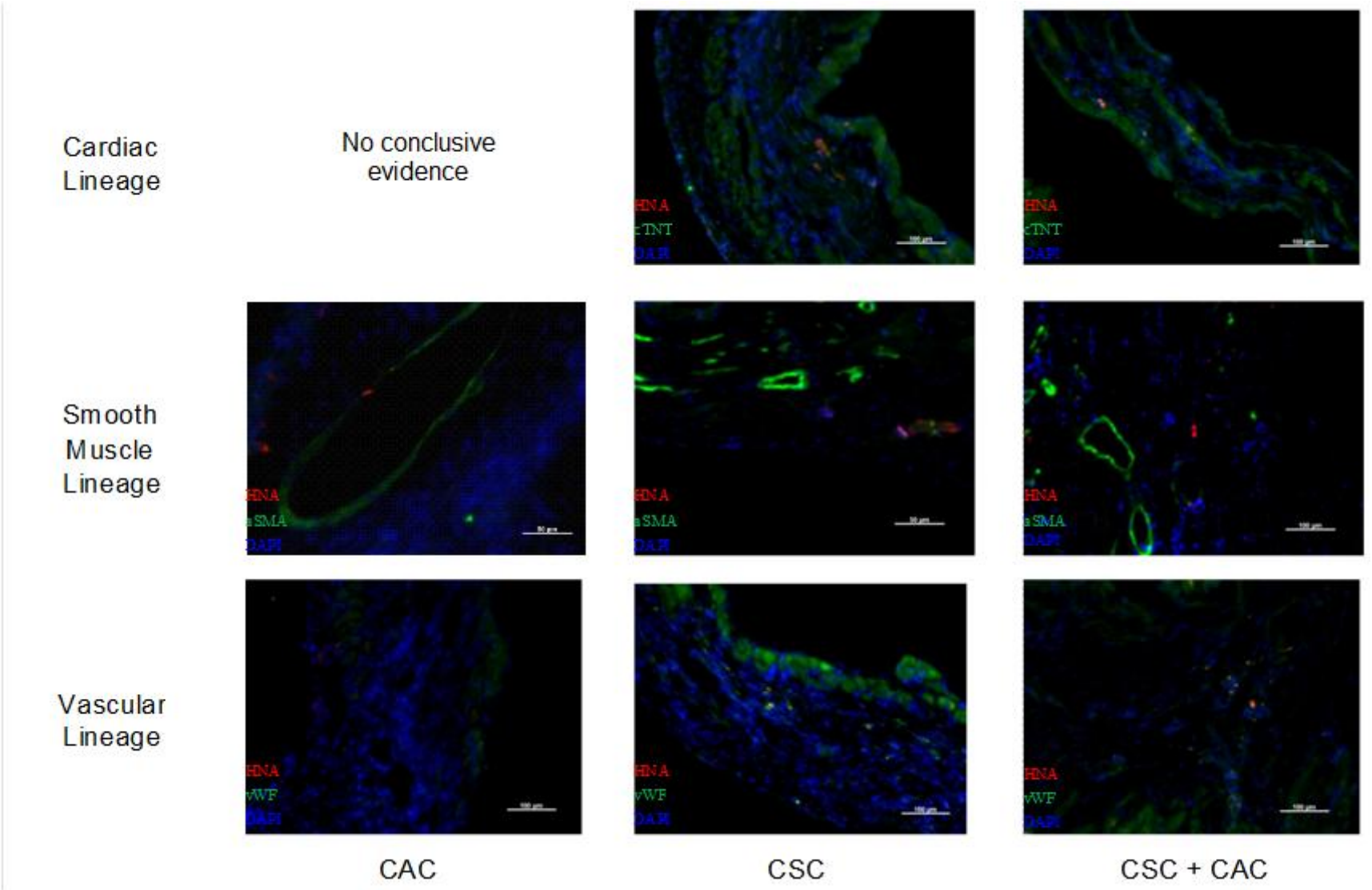

Figure 4.12. Clusters of differentiated human cells persist within the peri-infarct and infarct regions. Representative images of each stem cell treated group demonstrating human cells that co-segregate with markers of cardiac lineage. HNA = Human Nuclear Antigen. αSMA = Alpha Smooth Muscle Actin. cTnT = Cardiac Troponin. vWF = Von Willebrand Factor.

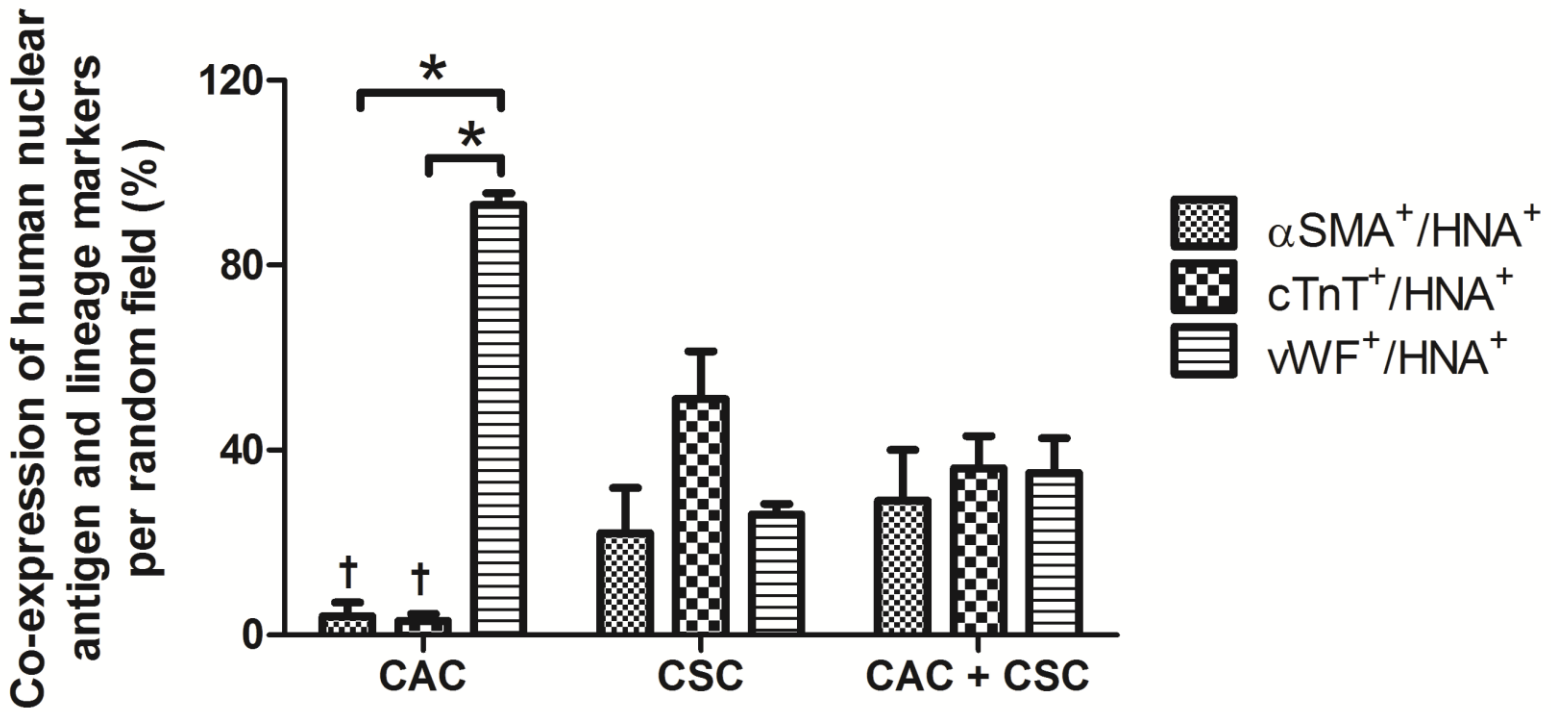

Figure 4.13. Lineage fate of retained human stem cells 28 days after transplantation. Random field analysis from histological sections demonstrating the co-segregation of human nuclear antigen positive cells with markers of cardiac (cTnT), smooth muscle (αSMA) or endothelial (vWF) identity (n=3 per group). * $p \leq 0.05$ vs. vWF^+/HNA^+ expression in CAC treated hearts. † $p \leq 0.05$ vs. αSMA+/HNA+ or cTnT+/HNA+ expression in CSC and CAC+CSC treated hearts.

5. Discussion

5.1 A new treatment paradigm for heart failure

CSC therapy holds the hope of mending the broken heart. With recent studies demonstrating lifelong cardiac repair and identification of cell candidates capable of proving myocardial repair, the field of adult CSCs is rapidly progressing towards clinical application. Similar to other stem cell sources,[222] current first generation CSC products appear to provide cardiac repair largely through local delivery of cardioprotective cytokines that either recruit endogenous progenitors or salvage reversibly damaged myocytes. Given that these non-CSC cells act shortly after a cardiac event with limited returns following delayed administration, the window for non-CSC transplantation and the need for long term persistence of engrafted cells is limited.[223, 224] CSC therapy provides an attractive alternative source of true cardiac progenitor cells capable of differentiating into new working myocardium while providing a supportive paracrine profile.[74, 77, 79] These unique features open prospects for durable cardiac repair and possibly late delivery for patients with established heart failure. This also rules out allogeneic treatment with “healthy” cell sources because CSCs must be capable of efficient acute engraftment and robust long term persistence. The rapid progress in this field has been encouraging and new phase two trials of current first generation therapies will emerge in the next few years while work continues to enable the rational design of future cell-based therapeutics.

This study demonstrates that CACs and CSCs provide unique paracrine repertoires with equivalent effects on angiogenesis, stem cell migration and myocardial repair. In a manner consistent with previous work, CSCs possess a superior capacity to differentiate into cardiac tissue.[57, 74, 82] Combination therapy with both first generation cell products synergistically

improved post infarct myocardial function greater than either therapy alone. This synergy is likely mediated by the complimentary paracrine signatures that promote revascularization and the growth of new myocardium.

5.2 Cardiac cell therapies with contrasting ontogenies

Early promising clinical and pre-clinical data has propelled culture selected CACs to the forefront of cardiac cell therapy with data demonstrating significant neovascularization in humans.[207, 225, 226] Autologous transplanted CACs secrete stimulatory cytokines that modify growth factors within the local milieu and increase the recruitment of other progenitor cells.[227, 228] While experimental evidence supports the direct differentiation into new blood vessels, this has been rarely observed in vivo and vascularization from paracrine/humoral factors or secondary recruitment of host stem cells are likely the main mechanisms leading to functional improvement.[101, 217, 229-233]

Recently, CSCs have garnered increased attention as candidates for myocardial repair.[92, 93] In contrast to CAC therapy, it is estimated that direct cardiomyocyte and vascular transdifferentiation provide up to 20% of the overall increase in cardiac tissue while indirect effects on tissue preservation and/or recruitment of endogenous progenitors provide the remainder.[77] The first generation CSC product used in this study[74] represents the initial cellular outgrowth from cardiac samples without recourse to cardiosphere expansion[92] or antigenic sub-selection and prolonged culture.[93] The general appeal of this study is further enhanced by using the common starting product being deployed in clinical trials. Although not in clinical trials, this CSC product provides a timely source of complementary CSC sub-populations with an equivalent[74] or superior[57] capacity to improve post MI cardiac function.

Given that these two cell types represent the two leading autologous cell sources in phase 1 and 2 cardiac repair trials, we chose to contrast cell products from patients undergoing clinically indicated cardiac surgery – precisely the same patients enrolled in current trials and in need of cellular cardiomyoplasty in the future. To provide an unbiased comparison of stem cell potency, *in vitro* experiments contrasted CACs and CSCs acquired at the time of cardiac surgery. The *in vivo* study was designed such that atrial appendages were acquired at the time of cardiac surgery with the CAC blood draws being performed 9 days after surgery. This strategy strives to reflect the logistical realities inherent in CAC/CSC cell culture and the administration of an autologous cell product soon after myocardial infarction. Although this approach may have favored CAC function and viability,[217, 234] this bias is tempered by the observation that 1) the effects of vascular damage on CAC function are very transient, 2) the results observed in both the *in vivo* and *in vitro* experiments are consistent, and 3) the phenotypic profile of both injected cells sources is consistent with those used in clinical trials (Figure 4.2).

5.3 Effects of individual cell therapy

Several studies have contrasted the effect of complimentary cell types on myocardial repair.[212-214, 235] Early attempts focused upon a comparison between blood derived angiogenic progenitor cells and skeletal muscle progenitors.[212, 213] While these studies demonstrated both cell types reduced scar size and improved ventricular function to an equivalent degree, this data is not readily translatable into clinical practice given that skeletal myoblast transplantation has been plagued with ventricular tachy-arrhythmias and sudden cardiac death.[6, 236] More contemporary data has shown that two cell sources derived from the adult heart (i.e., epicardium-derived cells and antibody selected cardiac progenitor cells) are capable of providing equivalent post-MI benefits.[214] While these two

cell products are not in clinical trials (and may represent two of the sub-populations found within the direct outgrowth of myocardial samples),[74] they provided equivalent myocardial repair in a manner similar to direct injection of sub-populations from expanded CSC sources.[57, 237] A recent paper compared expanded CSCs (i.e., cardiosphere derived cells, CDCs) from patients to several stem cell products (i.e., bone marrow-derived medenchymal stem cells, adipose tissue derived stem cells and bone marrow mononuclear cells) from healthy volunteers. Importantly, this study demonstrated that CDCs had the greatest functional benefit after experimental myocardial infarction an effect attributed to a more balanced paracrine profile.[57]

The current work extends these previous studies by providing the first unambiguous head to head comparison of autologous CACs and CSCs from clinical patients. While earlier work from our lab demonstrated that expanded populations of CSCs from human cardiac samples secrete significant quantities of VGEF, IGF-1 and HGF, we used a wide-ranging cytokine protein array to show that CSCs also secrete significant amounts of angiopoeitin-1, angiogenin and IL-6.[74, 77] Interestingly, the CSC paracrine signature had very few overlap cytokines (angiopoeitin-1, VEGF and HGF) with the more elaborate CAC paracrine profile. Despite these differences, the angiogenic response to conditioned media was similar between the two cells types suggesting this plays an important role in mediating cardiac repair. Although CSCs differentiated more readily into a cardiac phenotype, effects on cardiac repair were equivalent. This result may be explained by the PCR engraftment data demonstrating very modest long term retention (<1% of transplanted cells detected 3 weeks after injection). Despite this finding, long term (+16 weeks post LAD ligation) benefits are sustained notwithstanding very low numbers of injected cells. These findings suggest that the benefit of CAC and CSC transplantation occurs immediately after injection and the

persistence of large numbers of injected cells is not necessary to sustain effects on myocardial repair.

5.4 Effects of combination cell therapy

Given the number of cardiac stem cell candidates of dissimilar ontogeny, it follows that treatment with complementary cell types may provide synergistic benefits. This hypothesis is based on the premise that cells supporting the surrounding host tissue may increase the retention and proliferative capacity of cells capable of forming true contractile elements. The very limited published data to date supports this notion as treatment with non-clinical cell types (i.e., angiogenic cells + skeletal myoblasts or epicardium-derived cells + cardiac progenitor cells) demonstrates superior effects when combination cell products are injected.[212-214]

This work agrees with these reports and provides direct evidence that application of the two leading pre-clinical agents for myocardial repair provides synergistic benefits when applied after myocardial infarction. *In vitro* experiments highlight the additive "dose" response benefits obtained when increasing amounts of CACs and CSCs are applied towards angiogenesis, cardiogenesis and cytokine production. Combination therapy with CACs and CSCs did not increase long term cell retention- a finding that underscores the importance of cardioprotective/vasculogenic cytokines in mediating the benefits observed in cardiac repair using these first generation stem cell products.

5.5 Proposed mechanisms governing cell synergy

While identifying the mechanism underlying the enhanced therapeutic benefits from a dual cell therapy cannot be directly ascertained from this work, the conditioned media experiments demonstrating a boost in cytokine production when combining CACs and CSCs in culture hints that these improvements are likely mediated through autocrine and paracrine communication between these stem cell populations. As summarized in Figure 5.1a, CACs and CSCs used in this study release a comprehensive, yet divergent cytokine profile, highlighting the disparities in function between these stem cell sources. In addition to acting upon the transplanted stem cell population in a synergistic fashion, these complementary paracrine profiles likely act in synchrony by modulating the host environment to induce myocardial salvage and by recruiting endogenous stem cell populations to stimulate regeneration. Therefore, we propose a biological model hypothesizing several means of communication that contribute to the overall improvements seen with this combinational cell product (Figure 5.1b).

This model proposes several direct and indirect means of communication that stimulate cell performance once transplanted into areas of ischemic damage. Previous work using mesenchymal stem cells (MSCs) has demonstrated that when these cells are directly cultured with cardiomyocytes, the cell-cell contact triggers a cardiomyogenic response by MSCs that is lost when direct contact is substituted with conditioned media.[238, 239] This indicates that direct cell-cell interactions between CACs and CSCs may “prime” these cells, enhancing overall cardiomyogenic performance. From an indirect perspective, it is likely that when combined with CSCs, CACs stimulate an augmented cytokine production from one or both cell sources through paracrine and autocrine messaging. This is supported by preliminary work in our lab demonstrating that virally inducing CSC to over-express specific cytokines such as SDF-1α[240] and IGF-1[241], triggers an autocrine response by these cells, amplifying the release of other cytokines involved in myocardial salvage and regeneration.

Thus the extensive paracrine repertoire provided by CACs is likely acting in a synergistic fashion, boosting the cytokine production by CSCs and vice versa.

It is likely that CACs further contribute to CSC performance by creating a niche environment that stimulates anti-apoptotic, cell growth and proliferation pathways through the release of cytokines such as BMP(4,6), EGF, FGF(2,4,7,9), HGF and IGF-1. This notion is supported by preliminary data in our lab suggesting that virally induced overexpression of IGF-1 in CSCs stimulates an autocrine response that activates ERK, MAPK and PI3/AKT pathways, which are potent anti-apoptotic and pro-survival cascades.[241, 242] These pro-survival cytokines provided by CACs in conjunction with the inflammatory modulating IL-6 secreted by CSCs may also play a significant role influencing the host environment, once transplanted *in vivo*. Since the paracrine signature of both cell types combined covers a more complete range of therapeutically relevant messengers, it stands to reason that the combinational cell product would have a more significant role in cardio-protection, endogenous stem cell recruitment and direct regeneration. Early data examining the *in vivo* effects of IGF-1 and SDF-1α have demonstrated that receptor expression is upregulated 7 days post-MI in mouse LAD ligation models and remains elevated for 14 days post-MI, supporting the notion that these cytokines play an integral part in myocardial salvage and regeneration.[240, 241]

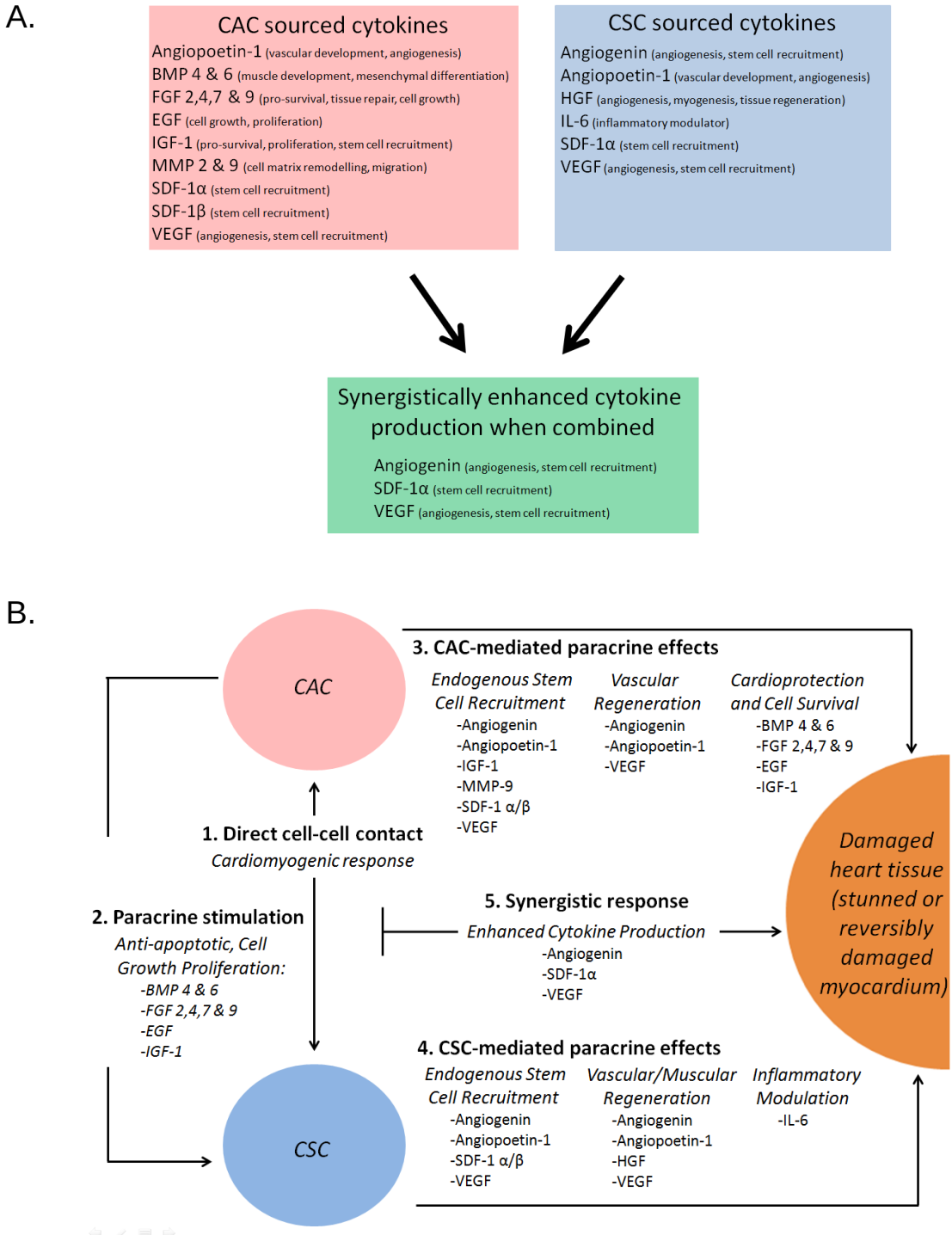

Figure 5.1. Overview of paracrine mediated contributions from each stem cell source. A. Disparate cytokine profiles from CACs and CSCs combine to provide enhanced therapeutic benefits after cell transplantation. B. CACs stimulate CSC growth, proliferation and anti-apoptotic pathways through direct and indirect signaling, while stimulating *de novo* vascular growth within the infarct regions. Furthermore, combining CACs with CSC therapy augments cytokine production through a synergistic response that promotes myocardial and vascular regeneration.

5.6 Limitations of this current work and hurdles before clinical translation

Despite comparing and contrasting the therapeutic capacity of CACs and CSCs both alone and as a dual cell product, this small animal study has several issues that must be addressed prior to clinical translation. One of the most important issues that must be addressed is the generation of a clinically applicable number of cells to therapeutically "doses" from limited tissue constraints. This study injected $1.0x10^5$ cells per animal in a single dose. The clinical dosing for a similar cell product in humans is 15 million cells per intra-coronary injection.[92] Although we combined an equal ratio of CACs:CSCs as an *in vivo* therapy, it would be pertinent to determine if enhanced cell-mediated cardiac repair could be obtained by altering the stem cell ratio.

While this study demonstrated enhanced cytokine production by combining CACs with CSCs, the origin of each elevated cytokine still remains unclear. We hypothesize that the cytokines supplied by the CACs stimulated CSCs (and themselves) within the combined stem cell milieu, however identifying the specific roles that each stem cell plays in this cellular interaction may help to indicate a more appropriate ratio of CAC:CSC to maximize cytokine production and therapeutic benefits. It is important to note however, that a long term study in order to test the oncogenicity/safety of the dual cell product is required as many of the cytokines are directly involved in tumorigenesis.[243]

5.7 Future directions

In light of encouraging phase-1 clinical trial results demonstrating a modest therapeutic benefit after CSC injection, the next step in this research is amplification to a therapeutic dosing and investigation of alternative culture methods. The most pertinent of these

methods is to investigate a sphered cell product (akin to cardiospheres) that comprises CSCs and CACs. Recent work by Lee et al., has demonstrated that direct transplantation of cardiospheres into large animal models of acute myocardial ischemia provides superior benefits to those observed from single cell expanded products.[244] We hypothesize that this stem cell enrichment technology may result in a functionally superior culture technique to combine CACs and CSCs, as cells the cells would be grown in a 3-dimensional niche environment supporting stem cell enrichment leading to a more robust paracrine profile. Furthermore, this culture technique is able to forego harsh trypsinization to lift the cells from the culture dish moments before transplantation and this may lead to improved cell survival and enhanced engraftment upon delivery.

This work lends to future work developing next generation CSC therapy, as a number of cytokines have been identified as being key modulators of myocardial regeneration and salvage. These cytokines may be incorporated into future cell matrix materials and combined with CSC/CAC therapy in order to enhance the efficacy of single/dual cell therapy. Alternatively, this work has created a foundation for work examining the effects virally mediated overexpression/silencing of specific cytokines, to help build a better understanding of the specific interactions between the CSC and the infarct area. Ideally, this would help lead to enhanced engraftment and overall therapeutic efficacy.

6. Conclusions

Human blood and cardiac stem cells provide equivalent degrees of myocardial repair when administered one week after experimental myocardial infarction. This improved myocardial function persisted on long term follow-up despite only modest engraftment of the transplanted cells. Paracrine profiling demonstrated that both cell types secrete a complementary array of growth factors with equivalent angiogenic effects. Co-transplantation of both cell types further enhanced post infarct cardiac repair with negligible effects on long-term cell retention. These synergistic effects may be explained by overlapping or improved paracrine signatures.

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