

# **Genetic Modification of Cardiac Stem Cells with Stromal Cell-Derived Factor 1 $\alpha$ to Enhance Myocardial Repair**

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## Abstract

The incidence of heart failure (HF) continues to grow despite advances to current therapies which are effective insofar as slowing disease progression. Cardiac stem cell (CSC) therapy is an emerging treatment for the reversal of HF. We sought to examine the effect of genetically engineering CSCs to over-express stromal cell-derived factor 1 $\alpha$  (SDF1 $\alpha$ ) on myocardial repair. SDF1 $\alpha$  over-expressing CSCs exhibited a broader paracrine signature resulting in increased stimulation of capillary network formation and chemotaxis under in vitro conditions. Using a murine model of myocardial ischemia, we demonstrated over-expression of SDF1 $\alpha$  increased myocardial SDF1 $\alpha$  content, reduced scar burden and increased activation of PI3K/AKT signaling as compared to non-transduced CSC and vehicle controls. These effects improved cardiac function without increasing cell engraftment suggesting that the mechanisms driving these benefits are largely paracrine mediated. Taken together this data suggests that transplantation of CSCs genetically programmed to over-express SDF1 $\alpha$  provides superior cardiac repair by boosting the content of cardio-protective cytokines during the critical healing phase after myocardial infarction.

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## List of Abbreviations

$\alpha$ SMA	Alpha smooth muscle actin
bFGF	Basic fibroblast growth factor
BM	Bone marrow
CAC	Circulating angiogenic cell
CDC	Cardiosphere-derived cell
CD90	Cluster of differentiation 90
CSC	Cardiac stem cell
DPPIV/CD26	Dipeptidyl peptidase IV/cluster of differentiation 26
CSp	Cardiosphere
cTnT	Cardiac troponin T
G-CSF	Granulocyte colony stimulating factor
HF	Heart Failure
HSC	Hematopoietic stem cell
LV	Left ventricle
LVEF	Left ventricular ejection fraction
MCP-1	Monocyte chemoattractant protein-1
MI	Myocardial infarction
MMP	Matrix metalloproteinase
NHDF	Normal human dermal fibroblast
NYHA	New York Heart Association
SDF1 $\alpha$	Stromal cell-derived factor 1 $\alpha$
SP	Side population
TGF- $\beta$	Transforming growth factor $\beta$
TNF- $\beta$	Tumour necrosis factor $\beta$
VEGF	Vascular endothelial growth factor
vWF	Von Willebrand factor

# 1.0 Introduction

## 1.1 Stem cell therapy for heart failure: A new treatment paradigm

In Canada, heart failure (HF) poses a significant burden on society with approximately 500,000 people living with HF and 50,000 new patients diagnosed each year.<sup>1</sup> This has driven remarkable therapeutic advances- including drug, surgical, and lifestyle interventions which improve the immediate outcomes of patients suffering from this debilitating condition. However, medical advances and an aging population have increased the number of patients living with HF. Unfortunately, HF is usually progressive as available therapies are unable to restore normal cardiac function- leaving patients with the prospect of heart transplantation, implantation of mechanical support devices or death from progressive pump failure. Given these bleak prospects, therapies which emphasize disease reversal have become the new focus for the management of HF.

Cell based therapies are an emerging treatment option to reverse the effects of HF. One obstacle facing the field is the choice of an ideal cell candidate. Heavily investigated candidate cell products (i.e., bone marrow progenitor cells,<sup>2-4</sup> mesenchymal stem cells (MSC)<sup>5</sup> and satellite skeletal myoblasts)<sup>6</sup> have been met with variable clinical success and sometimes marked pro-arrhythmia. This has led to the search for ideal cell products which need to be easily obtainable, non-immunogenic, non-carcinogenic, improve cardiac function and be capable of engrafting with proper electromechanical coupling (i.e., non-proarrhythmic). Cardiac stem cell (CSC) based products (i.e., c-Kit+ cells,<sup>7</sup> cardiospheres (CSp),<sup>8-10</sup> and cardiosphere-derived cells (CDCs))<sup>11, 12</sup> have gained traction because of their ability to stimulate cardiac repair through indirect paracrine salvage and straightforward cardiomyogenic differentiation to directly repair the damaged heart. This report will outline the evidence for a self-renewing heart, the characterization of the resident CSC niche, the establishment of CSC therapy, the regenerative mechanisms underlying these effects,

current clinical investigations and the future of CSC therapy with a focus on the role of the cytokine Stromal Cell-Derived Factor 1 $\alpha$  (SDF1 $\alpha$ ).

## **1.2 Evidence for a self-renewing heart**

The human heart has been traditionally viewed as a post-mitotic organ devoid of any self-repair.<sup>13-15</sup> This paradigm stated that the heart was composed of a set population of cardiomyocytes that slowly diminishes over time. It follows that injuries which decrease the content of cardiomyocytes and patients remain in a static state of impaired myocardial function without a reservoir for myocardial turnover and rescue.<sup>16, 17</sup>

Dogma supporting a terminally differentiated heart has been dismissed following the recent discovery of CSCs within the adult heart.<sup>16, 18-20</sup> A number of studies have demonstrated that the adult heart exhibits low level cardiomyocyte renewal throughout the normal life-span.<sup>21, 22</sup> One landmark study took advantage of the ability of atmospheric carbon-14, formed during Cold War era nuclear bomb testing, to integrate into genomic DNA. This “carbon dating” technique permits the accurate identification of post-natal DNA synthesis and cellular turnover.<sup>23</sup> By comparing the levels of carbon-14 content in DNA to established atmospheric carbon-14 levels, the authors were able to demonstrate that almost 50% of adult cardiomyocytes are renewed over an individual’s lifetime. Interestingly, this “repopulation” decreased with advancing years as approximately 1% of cardiomyocytes turnover annually at age 25 as compared to 0.45% at age 75.

In contrast, a recent study by Kajstura et al. (2010) evaluated the effects of age and gender on cardiomyocyte formation within hearts from deceased patients who died of non-cardiac related causes.<sup>16</sup> By measuring the number of senescent and cycling cardiomyocytes using appropriate markers (p16<sup>INK4a</sup> and Ki67) the authors concluded that cardiomyocyte turnover occurs 15 times over the course of a lifespan in women and 11 times in men. Although these investigators precluded confounding factors such as cell fusion and DNA repair, the disparity in the estimated magnitude of the cardiomyocyte

compartment remains. These divergent results may in part be attributed to stimulatory effects of clinical conditions such as diabetes and hypertension but the extent remains controversial.

Despite discrepant findings regarding the extent of myocardial replacement, it remains clear that the normal adult heart undergoes life-long cardiac replacement and has shifted the belief of a non-dividing heart to a self-renewing heart. These findings hint of the possibility to harness endogenous repair in the native heart.

### **1.3 Resident cardiac stem cells– a cell source to support myocardial turnover**

As life-long repopulation of the cardiomyocyte compartment was being established, other groups attempted to establish the source of this phenomenon. Early work supported the concept that myocardial turnover was attributable to the slow, progressive division of existing cardiomyocytes.<sup>24-27</sup> Alternatively, it may due in part to cardiac replacement from a pool of resident CSCs; not unlike those seen in other regenerative organ systems like the liver and brain.<sup>28, 29</sup>

A very small resident population of CSCs was first identified within the post-natal heart following the adaptation of skeletal myoblast isolation and culture techniques to the adult heart.<sup>30</sup> These cells were termed side population (SP) cells and were characterized by their ability to efflux Hoechst dye following enzymatic dissociation from murine hearts. Flow cytometry revealed that SP cells made up ~1% of the total cell number in the heart and were negative for prototypical markers of cardiac identity. The capacity of SP cells for cardiac differentiation was confirmed upon co-culture with mature cardiomyocytes. This study was the first to demonstrate that the adult heart contains a small fraction of primitive CSCs capable of differentiating to form functional myocardium.

A number of other markers of cardiac SP cells have emerged including ATP-binding cassette transporter (ABCG2)<sup>31-34</sup> and stem cell antigen 1 (Sca-1+/CD31-).<sup>35</sup> In keeping with the initial cardiac SP study, antigenically-purified ABCG2+ cells demonstrated a

marked ability to proliferate and differentiate into functional cardiomyocytes.<sup>33</sup> Sca-1+/CD31- SP cells demonstrated a marked ability to migrate within the heart to regions of damage in response to ischemic injury.<sup>36</sup> In this study, transplantation of Sca-1+/CD31- cardiac SP cells attenuated LV dysfunction and adverse structural remodeling through increased neovascularization and salvage of reversibly damaged myocytes. Furthermore, Sca-1 knockout mice displayed reduced healing in response to ischemia, ultimately leading to lowered contractility and hypertrophy.<sup>37</sup> Taken together, these studies implicate the presence of a CSC niche in the form of cardiac SP cells that contributes to repopulating cardiomyocytes during physiological and pathological states.

Numerous groups have characterized resident CSCs beyond the description of cardiac SP cells. Chief among these was the well-known hematopoietic stem cell marker c-Kit (a receptor tyrosine kinase) was evaluated as a potential marker for resident CSCs with recent work suggesting that c-Kit+ CSCs may be the principal source of stem cells within the developing heart.<sup>38</sup> Under normal mechanical stress, local increases in ATP levels stimulate Ca<sup>2+</sup> oscillations in c-Kit+ CSCs which activate proliferation and the gradual loss of pluripotency markers as they assume a cardiac fate. This ATP mediated stimulus of c-Kit+ CSC cardiomyogenesis is further potentiated under increased myocardial stress and concurrent secretion of histamine by mast cells.<sup>39</sup> Here, the authors argue that c-Kit+ cells comprise the CSC compartment and are capable of differentiating into functional myocardium.

The existence of cardiac c-Kit+ cells were first described in 2003 by Beltrami and colleagues who identified multipotent, self-renewing, and clonogenic CSCs that were positive for c-Kit and negative for hematopoietic lineage markers (most notably CD34 and CD45). These cells were largely found within the interstitial space between cardiomyocytes. The distinction between c-Kit+/CD45- and c-Kit+/CD45+ cells is especially important because it discriminates between cardiac c-Kit cells and bone marrow-derived c-Kit cells,

respectively. The cardiac c-Kit CSCs alone favoured the adoption of a cardiomyogenic fate through expression of early cardiac development transcription factors including Nkx2.5, MEF2C, and GATA4. The investigators estimated that the primitive CSC population is extremely small and represented 1 CSC for every 10,000 cardiomyocytes. This is important because it demonstrates that although the heart is not a purely static organ; the level of endogenous repair is incapable of meaningful regeneration in response to cardiac injury.<sup>16,</sup>

18, 40

Further characterization of the resident CSC population by D'Amario et al. (2011) has identified two distinct classes— myogenic (c-Kit+/KDR-) and vasculogenic (c-Kit+/KDR+) CSCs.<sup>41</sup> Myogenic CSCs were found to be located in the myocardial interstitium surrounded by supportive fibroblasts. In contrast, the vasculogenic CSCs are largely endothelial and smooth muscle precursors found throughout the coronary vasculature. Fortunately, both classes of CSCs are readily obtained from endomyocardial biopsies allowing for a clinically relevant cell product.<sup>41</sup>

Collectively this data confirm the presence of a small yet distinct CSC population within the myocardium that boasts a capacity for cardiomyocyte renewal under normal and pathological states and presents a logical cell candidate for cellular cardiomyoplasty.

#### **1.4 CSC-based cell sources for therapeutic application**

Although resident CSCs can replace damaged cardiomyocytes, clinical experience has demonstrated that it is unable to completely repair injured myocardium after significant damage.<sup>18, 40</sup> Obvious limitations include a limited initial number of CSCs (1 in 10,000 cells) and the harsh operating conditions after myocardial damage. These challenges have driven the development of advanced culture techniques to isolate and propagate the CSC pool under *ex vivo* conditions from routine clinical biopsies.<sup>42-45</sup>

In 2004, the Messina group successfully isolated and expanded cells from human and murine hearts.<sup>46</sup> Human biopsies or murine hearts were minced, enzymatically

digested and then plated. After several days in culture, a monolayer of cells (termed cardiac outgrowth or explant derived cells) spontaneously emigrated from plated tissue fragments. The mechanism behind the formation of these cardiac explant-derived cells is largely unknown; however emerging evidence suggests a possible role for Notch-1 mediated induction of epithelial to mesenchymal transition (EMT) in both c-Kit<sup>+</sup> and c-Kit<sup>-</sup> CSCs.<sup>47</sup> The enzymatically collected cardiac outgrowth is composed of a heterogeneous mixture of complementary subpopulations that express stem cell related markers (c-kit, ABCG2, SSEA-1), endothelial progenitor markers (CD31, CD34), and mesenchymal markers (CD90, CD105).<sup>44</sup> These cells can be harvested and grown in suspension to form 3D spherical aggregates (designated as cardiospheres (CSp)) with the putative intent of enriching the c-Kit<sup>+</sup> subpopulation.<sup>42, 43, 48</sup> Cardiosphere formation is increasingly viewed as a process mediated through hydrophobic interactions with knock-on effects to stimulate ERK and VEGF pathways.<sup>48, 49</sup> Cardiosphere therapy has demonstrated a dose-dependent benefit in a number of pre-clinical models.<sup>8, 9</sup>

To circumvent the risk of thrombosis following intra-coronary infusion of large diameter (70-100  $\mu$ m) CSp, Smith et al. dissociated CSp into a single cell suspension and re-plated it in 2D cultures to generate CDCs.<sup>9, 40, 45</sup> Since then, CDCs have been translated from animal trials<sup>50-53</sup> into the clinic<sup>11, 12</sup> to avoid the need for intra-myocardial delivery.

In contrast to the strategy of implanting an aggregate population of complimentary cells, other groups have investigated the effect of implanting antigen selected and expanded cardiac progenitor cells (i.e., c-Kit<sup>+</sup>). These cells are isolated from the cardiac outgrowth of plated cardiac tissue for expansion in defined media to therapeutically relevant “doses”. Preclinical and phase 1 clinical studies have shown that transplantation of c-Kit<sup>+</sup> cells is associated with fewer heart failure symptoms (i.e., lower NYHA class), better myocardial function and more viable myocardium.<sup>7, 54, 55</sup>

In comparing these various CSC products, we have previously shown that direct cardiac outgrowth has a superior intrinsic regenerative capacity while maintaining equivalent paracrine potential; thus effectively eliminating the need for antigenic sub-selection<sup>51</sup> or sphere expansion.<sup>44</sup> Utilizing the heterogeneous cardiac outgrowth is advantageous as it lowers costs and simplifies the culture technique while avoiding the risks of phenotypic drift or malignant transformation associated with prolonged culture.<sup>56</sup> Above all, the c-Kit+ subpopulation of cardiac outgrowth exhibits a capacity for enhanced differentiation to a cardiac phenotype via enhanced expression of the early cardiac markers (Nkx2.5, MEF2C, and GATA4)<sup>16, 18, 45, 55, 57</sup> and pluripotency factors (Nanog, c-myc, Klf-4, OCT4).<sup>58</sup> In keeping with a stem cell character, these cells have demonstrated a capacity for self-renewal, clonogenicity, in addition to their multipotency.<sup>40, 42, 44, 46, 59</sup> Although head to head studies are infrequent, the one study comparing antigen selected c-Kit+ cells with CDCs demonstrated that CDCs were superior in terms of regenerative performance (i.e., better LVEF and lower scar burden).<sup>51</sup> Interestingly, the more heterogeneous outgrowth cells afforded equivalent functional benefits to CDCs with a markedly superior capacity to differentiate into a cardiac lineage.<sup>44</sup>

Thus CSCs from cardiac samples provide promising autologous sources of cells that permit *ex-vivo* amplification and delivery to areas of injury, where they engraft and improve heart function.

### **1.5 Mechanisms driving CSC-mediated cardiac repair**

Although functional improvements have been well documented following the delivery of CSC products into both pre-clinical animal models and phase 1 clinical trials, the underlying mechanisms by which CSC provide myocardial repair remain up for debate.<sup>60, 61</sup>

Early investigations into the efficacy of CSC therapy showed that these cell products are capable of engrafting into the host myocardium without arrhythmogenic consequences.<sup>6, 18,</sup>

<sup>55</sup> CSCs display a marked ability to adopt cardiomyocyte, smooth muscle, and endothelial

fates while imparting increased neovasculogenesis within the damaged myocardium.<sup>45, 52, 62</sup>

As a result, the role of direct differentiation into these lineages is regarded as one of the major hallmarks of CSC action.

The effectiveness of direct myocardial replacement is based on the assumption that transplanted cells are significantly retained within the myocardium.<sup>8, 63</sup> However, several reports indicate that transplanted CSC engraftment is modest with only 17% of injected cells reliably detected after 1 hour.<sup>53, 64</sup> But low intra-cardiac retention of injected cells is not restricted to CSCs alone and is attributable to mechanical extrusion, clearance via cardiac lymphatics or cell death associated with the harsh microenvironment after infarction.<sup>53</sup> As such, the potential gains of direct cardiac regeneration have yet to be fully realized with CSC transplant outcomes being heavily leveraged upon paracrine mediated angiogenesis and salvage of reversibly damaged myocardium.<sup>40, 65-68</sup>

### **1.6 From bench to bedside– first generation CSC products in the clinic**

The majority of clinical studies using cell therapy for HF have used bone marrow or blood derived mononuclear cell products because they are easily obtained as a result of the existing infrastructure established to conduct routine bone marrow (BM) transplantation.<sup>69-72</sup> While these studies are admirable for their ease of use within the clinic, the overall efficacy for cardiac and vascular repair is modest.<sup>73, 74</sup> Given that CSC products can adopt all cardiac fates, CSCs attracted clinical attention.

The “Cardiosphere-Derived autologous stem Cells to reverse ventricular dysfunction (CADUCEUS)” trial was a phase 1 study designed to demonstrate the safety of autologous CDC infusion.<sup>11</sup> Patients with an impaired ejection fraction (EF<45%) 2-3 weeks after successful revascularization were enrolled with CDCs cultures established from ventricular biopsies for intra-coronary delivery (4-8 weeks post ventricular biopsy). The primary outcome was to demonstrate 6 months safety data while secondary outcomes probed for an indication of efficacy. CDCs demonstrated safety data within 24 hours and 6

months after transplantation with no significant differences in patient deaths, carcinogenesis or major adverse cardiac events. Magnetic resonance imaging revealed an overall reduction in scar size and increase in viable heart mass 12 months after CDC treatment coupled with a tendency towards an increased LVEF. Thus the authors concluded that autologous intra-coronary infusion of CDCs feasible and safe with warranted extension to a phase 2 study.

CADUCEUS laid the groundwork for a phase 2 trial entitled “Allogeneic Heart Stem Cells to Achieve Myocardial Regeneration” or ALLSTAR. This clinical trial is founded upon work by Malliaras et al. (2012) which demonstrated the safety and efficacy of allogeneic CDC infusion in rats. Extension of the CDC technology to allogeneic delivery minimizes delays associated with harvesting tissue and may provide more timely delivery of CDCs within the immediate post infarct period. Delivery of a cell product on demand may boost the effects observed from CDC transplant as timely delivery has been noted to influence the effects of bone marrow derived cell products (Traverse et al., 2012 LATE-TIME). In ALLSTAR, the major inclusion criteria include diminished myocardial function (LVEF $\leq$ 45%) following revascularization within the preceding 1 to 12 months. Patients will receive an intra-coronary infusion of 25 million CDCs or placebo and will be followed for 12 months.

In contrast, a second phase 1 trial titled “cardiac Stem Cells In Patients with Ischaemic cardiomyopathy (SCIPIO)” examined the safety and efficacy of antigenically purified c-Kit<sup>+</sup> CSCs.<sup>7</sup> In this study, left atrial appendages were harvested from patients with poor myocardial function (EF $<$ 40%) undergoing clinically indicated cardiac surgery. Isolated c-Kit<sup>+</sup> cells were expanded to a therapeutic dose for intra-coronary delivery (1 million c-Kit<sup>+</sup> CSCs).<sup>75</sup> Interim results demonstrated that c-Kit<sup>+</sup> CSC delivery led to marked improvements in LVEF by 3D echocardiography, NYHA class, and quality of life. Since then enrollment of control and treated patients is now complete with more robust data recently published.<sup>54</sup> Global and regional LV function, infarct size, and LV viable tissue were further

characterized using cardiac MRI – the gold standard for such analyses. Cardiac MRI revealed a significant increases in global LVEF in CSC-treated patients ( $27.5 \pm 1.6\%$  to  $41.2 \pm 4.5\%$  at 12 months) and LV viable mass ( $+11.6 \pm 5.1\text{g}$ ) which coincided with decreases in LV nonviable mass ( $-11.9 \pm 2.5\text{g}$ ) and infarct size ( $-9.8 \pm 3.5\text{g}$ ). With these data, the authors maintain that c-Kit+ CSC infusion is easily conducted, safe and consistent with cardiac regeneration.

The final phase 1 clinical trial that has been completed and presented but not published is entitled “AutoLogous Human Cardiac-Derived Stem Cell to Treat Ischemic cArdiomyopathy” or ALCADIA.<sup>10</sup> In this study endomyocardial biopsies were used to generate CDCs using standard techniques. Patients diagnosed with ischemic cardiomyopathy and randomized into the treatment arm (age 20-80 years with  $EF \geq 15\%$  and  $\leq 35\%$ ) will receive intra-myocardial injections of human CSp (500k cells/kg of patient body weight) within a gelatin hydrogel sheet that incorporates basic fibroblast growth factor (bFGF; 200  $\mu\text{g}$ ) during their scheduled CABG. The application of the bFGF secreting sheet is hypothesized to increase the survival and retention of transplants CSCs.<sup>63, 76</sup>

### **1.7 CSC therapy – the next steps...**

Although there is strong evidence to suggest that CSC therapy elicits a positive effect after delivery to damaged myocardium, these benefits observed are inconsistent and modest. The major limitations confronting CSC therapeutics are poor engraftment to permit generation of new working myocytes from transplanted cells and the modest paracrine repertoire of patient sourced CSCs. Efforts to overcome these challenges represent development of the next generation of CSC therapy. These include the application of biomaterials,<sup>77, 78</sup> advanced culture techniques,<sup>8, 63, 79</sup> and genetic engineering.<sup>80, 81</sup> Of these, biomaterials and pre-conditioning with small molecules are the most well studied applications that exhibit functional improvements in the post-ischemic heart.<sup>82, 83</sup>

Recent advances in medical nanotechnology have been shown to boost acute retention of transplanted cells and ultimately improve cardiac function.<sup>63, 84, 85</sup> These bioactive materials largely prevent mechanical extravasation of cells from the site of injection<sup>64</sup> and secrete factors that enhance cell survival and bystander effects involved in tissue repair.<sup>66</sup> These approaches have included labelling with microspheres,<sup>85, 86</sup> extracellular matrix scaffolds,<sup>77, 78, 78, 87</sup> and single cell encapsulation.<sup>66, 88</sup> Mechanical methods to prevent leakage of cells after transplantation by sealing the injection site with fibrin glue have enhanced acute retention and functional benefits.<sup>64</sup> In the end, tactics using biomaterials to improve mechanical delivery, cell survival, and the regenerative capacity of CSCs represent an increasingly promising means of enhancing CSC therapy.

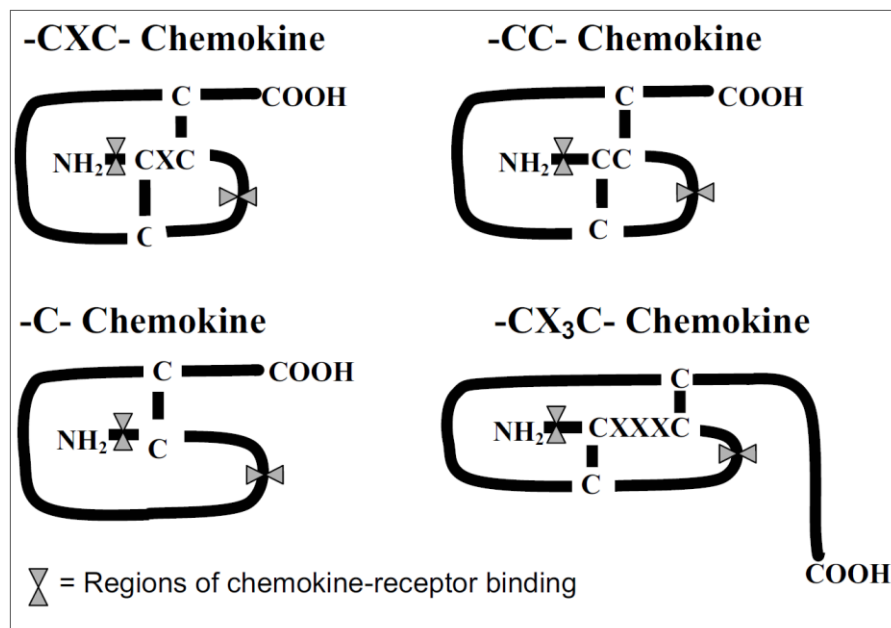
Advanced culture techniques and CSC pre-conditioning with small molecules provide an intrinsic means of improving first generation CSC products.<sup>89</sup> For example, simply culturing CSCs in physiological 5% oxygen instead of atmospheric oxygen levels (20%) reduces chromosomal abnormalities by preventing the formation of toxic reactive oxygen species by “excessive” oxygen stress.<sup>79, 90</sup> Supplementation of blood derived stem cell cultures with small molecules that enhance endothelial nitric oxide synthase (eNOS) expression (AVE-9488<sup>91</sup> and PPAR $\alpha$  agonists<sup>92, 93</sup>) TGF- $\beta$  stimulation,<sup>94</sup> and AKT signaling (statins)<sup>95</sup> have been shown to enhance culture yields. While very little work has been done in the field of CSC culture, pre-conditioning represents an additive and straightforward means of improving CSC therapy outcomes.

Recently, genetic engineering of stem cells has begun to attract attention as genetic alteration of cells to enhance electrical integration,<sup>96, 97</sup> promote cardiomyocyte proliferation,<sup>81</sup> and broaden the cytokine profile of non-CSC stem cell products has been shown to improve transplant outcomes. Given that the majority of benefits seen with non-CSC products reflects the paracrine capacity of these cells, it follows that attempts to increase survival (SDF1 $\alpha$ ,<sup>98</sup> IGF-1,<sup>99</sup> Bcl-2<sup>100</sup>), recruit endogenous progenitor cells (SDF1 $\alpha$ ,

CXCR4),<sup>101</sup> and promote angiogenesis (SDF1 $\alpha$ ,<sup>102</sup> VEGF,<sup>103</sup> eNOS,<sup>104, 105</sup> HIF-1 $\alpha$ <sup>106</sup>) would have benefits. Of all the gene-based approaches, widening the CSC secretome appears to be safest due to the potential risks of oncogenicity associated with cell cycle re-entry of quiescent cardiomyocytes while maintaining the ability for straightforward immune-mediated clearance in case of malignant characteristics.<sup>107</sup>

### 1.8 SDF1 $\alpha$ – the prototypical chemokine to enhance indirect cardiac repair

Chemokines comprise of a large family of basic proteins ranging in 8-14 kDa in size (~70-100 amino acids) that possess a highly conserved tertiary protein structure.<sup>108, 109</sup> They form four sub-families based upon the position of N-terminal cysteine residues with disulfide bridges connecting to downstream cysteine residues. The sub-families include the CXC (i.e. one amino acid residue between the first two cysteines), CC, C, and CX<sub>3</sub>C with corresponding receptors designated CXCR, CCR, CR, and CX<sub>3</sub>CR, respectively (Figure 2).<sup>110, 111</sup> CC chemokines represent the largest subfamily followed by the CXC subfamily which is further divided into two groups. One containing a tripeptide motif glutamate-leucine-arginine (ELR) termed ELR+ and the other ELR-.<sup>112</sup>



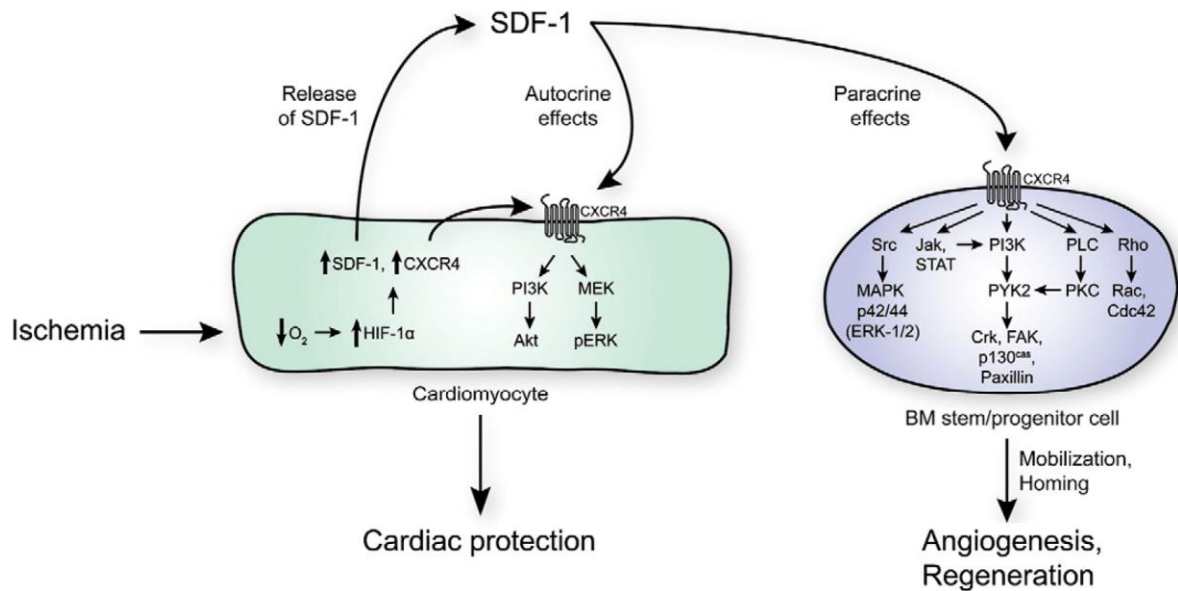
**Figure 1.** Diagram outlining the four chemokine subfamilies.<sup>111</sup>

Generally, chemokines regulate inflammatory and stem cell migration under normal physiological states (homeostatic chemokines) and stress (inducible chemokines).<sup>113</sup> Chemokines were classically regarded as factors that acted predominantly on bone marrow-derived cells; however recent studies report signaling from endothelial cells, cardiomyocytes, circulating angiogenic cells (CACs) and vascular smooth muscle cells that help modify endogenous repair and inflammation.<sup>114</sup>

CSCs produce a broad paracrine profile of cytokines implicated in angiogenesis, survival, and inflammation. These cytokines include angiogenin (Ang), angiopoietin-1 (Ang-1), hepatic growth factor (HGF), interleukin-6 (IL-6) and vascular endothelial growth factor (VEGF). Interestingly, CSCs secrete modest and variable amounts of SDF1 $\alpha$  (0-0.06 pg/ $\mu$ L) - a cytokine strongly implicated in inflammation, bone marrow homeostasis and tissue repair.<sup>44</sup> Thus making SDF1 $\alpha$  a strong candidate for enhanced CSC-mediated cardiac repair.

SDF1 $\alpha$  (also known as CXCL12) is classified as a CXC/ELR- chemokine. It exhibits both steady-state and inducible expression within the BM, heart, liver, kidney, brain, spleen, thymus, and skeletal muscle.<sup>115</sup> SDF1 $\alpha$  plays essential roles in regulating cardiac development, hematopoiesis, lymphocyte homing, B-lineage cell growth, and angiogenesis.<sup>116</sup> The protein sequence of SDF1 $\alpha$  is highly conserved across species and SDF1 $\alpha$  deletion results in vascular defects and embryonic lethality.<sup>117</sup> Initially, two splice variants (SDF1 $\alpha$  and SDF1 $\beta$ ) for the SDF1 gene were identified using signal sequence trap cloning from stromal cells in mouse BM.<sup>116, 118</sup> To date, six isoforms of the human SDF1 gene which spans 88 kb uniquely on chromosome 10 have been identified with SDF1 $\alpha$  and  $\beta$  being the most well characterized.<sup>119, 120</sup> All isoforms bind the N-terminal region to stimulate receptor activation while the C-terminus is required for stabilizing receptor binding.<sup>121</sup> Interestingly, these splice variants display differential pharmacokinetics in the circulation. Although SDF1 $\alpha$  and SDF1 $\beta$  undergo proteolytic degradation at both the N- and

C-terminal regions, the smallest and predominant isoform – SDF1 $\alpha$  – is the most susceptible to degradation.<sup>122</sup> Currently, the literature on all isoforms is severely limited thus future work examining the functional capabilities of each within different physiological and disease models may prove useful.



**Figure 2.** The role of the SDF1 $\alpha$ :CXCR4 axis in endogenous tissue repair in response to MI. Hypoxia induced upregulation of cardiomyocyte SDF1 $\alpha$  leads to cardiomyocyte salvage and homing of peripheral CXCR4<sup>+</sup> cells to the injured heart.<sup>115</sup>

Unlike the majority of chemokines, only two known receptors for SDF1 $\alpha$  have been identified – CXCR4 and CXCR7 each of which belongs to the seven pass transmembrane G-protein coupled receptor (GPCR) family.<sup>123</sup> Until recently, CXCR4 was regarded as the exclusive receptor for SDF1 $\alpha$ . CXCR4 is expressed on a wide variety of cells ranging from monocytes, CACs, lymphocytes, HSCs, and cardiomyocytes.<sup>74, 124-126</sup> CXCR4 is also associated with pathological disease states, most notably HIV as one of several chemokine receptors required for viral attachment to CD4<sup>+</sup> T cells and in tumour metastasis.<sup>127, 128</sup> Activation of CXCR4 by SDF1 $\alpha$  regulates chemotaxis and cell survival depending on the

target cell and biological state (i.e. steady-state or stress-induced) of the host. Within the context of myocardial ischemia, SDF1 $\alpha$  binding to CXCR4+ on cardiomyocytes increases PI3K/AKT and MAPK/ERK pro-survival signaling (Figure 2). Increased myocardial expression of SDF1 $\alpha$  leads to increased CXCR4 binding on bone marrow derived cells to increase bone marrow mobilization and myocardial homing via JAK/STAT, protein kinase C (PKC), focal adhesion kinase (FAK), p130<sup>Cas</sup>, and PI3K/AKT signal cascades.<sup>115</sup> As with most GPCRs, stimulation with SDF1 $\alpha$  results in CXCR4 and CXCR7 receptor desensitization and internalization that is mediated by  $\beta$ -arrestins. Following receptor internalization,  $\beta$ -arrestin induces p38/MAPK and AKT signaling which further enhances chemotaxis and cell survival.<sup>129-132</sup>

### **1.9 Stem cell mobilization and homing and the role of SDF1 $\alpha$ signaling**

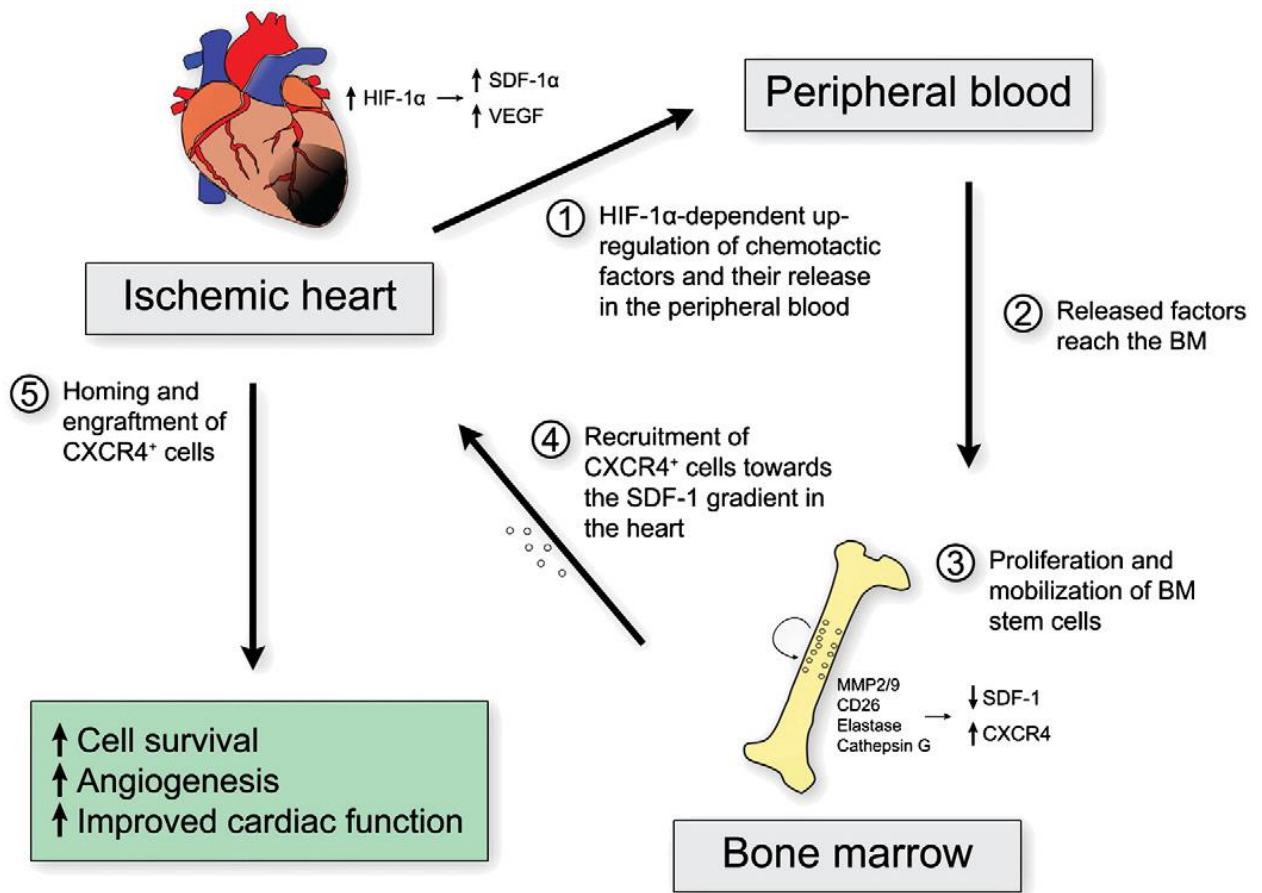
An important portion of the wound healing response to infarction is mediated through the mobilization and homing of circulating bone marrow stem cells to areas of injury.<sup>133</sup> After myocardial infarction, the initial release of chemokines (e.g. SDF1 $\alpha$ ) and microRNA<sup>124</sup> up regulate local production of elastases, CD26, cathepsin G, and matrix metalloproteinases (primarily MMP-2 and MMP-9) to degrade extracellular matrix and cell-cell adhesion proteins that prepare the site of injury for intra-cardiac and transendothelial migration of stem cells.<sup>114, 125, 134, 135</sup>

Under homeostatic conditions within the bone marrow, local binding of SDF1 $\alpha$  to CXCR4 and CXCR7 promotes anchoring of HSCs.<sup>114</sup> Disruption of SDF1 $\alpha$  during the post infarct inflammatory response, results in production of mobilization signaling by factors such as granulocyte colony stimulating factor (G-CSF) to activate neutrophils within the bone marrow to produce elastases that cleave SDF1 $\alpha$  in the bone marrow and results in release of HSCs into the circulation.<sup>115, 136, 137</sup> At the same time, myocardial production of SDF1 $\alpha$  attracts circulating stem cells to areas of injury (Figure 3).<sup>138 101, 139, 140</sup> These extra-cardiac

cells are capable of transdifferentiation into working endothelium while promoting angiogenesis and cardiomyocyte salvage.<sup>141</sup>

Local production of SDF1 $\alpha$  also promotes the intra-cardiac mobilization of endogenous CSCs to areas of cardiac injury.<sup>142</sup> Tang et al. (2011) neatly showed this using a rat model of myocardial ischemia. In this study, MSCs genetically engineered to express VEGF were injected into rats one week after MI induction followed by daily doses of either vehicle (H<sub>2</sub>O) or the CXCR4 antagonist AMD3100. Western blot analysis showed a marked upregulation of myocardial SDF1 $\alpha$  content which was accompanied by increased c-Kit<sup>+</sup> and MDR-1<sup>+</sup> cells within the infarct and peri- infarct regions. This effect was blunted following disruption of SDF1 $\alpha$  signaling by AMD3100, thus implicating the SDF1 $\alpha$ :CXCR4 axis as a principle mediator of endogenous CSC recruitment to the infarct.<sup>139</sup> This finding was corroborated using short hairpin RNAs (shSDF1 $\alpha$ ) that silenced SDF1 $\alpha$  expression. The number of endogenous CSCs that migrated to the infarct after was reduced following shSDF1 $\alpha$  delivery, but not after the administration of control short hairpin RNAs. This demonstrates that SDF1 $\alpha$  has a direct role in stimulating the recruitment of endogenous CSCs to the damaged myocardium. Importantly, increased CSC migration was associated with a reduction in infarct size and improved hemodynamic parameters and as a result underscores the significance in tissue repair. The migratory response of CSCs to an SDF1 $\alpha$  signal appears to be coordinated by downstream AKT signaling.<sup>101</sup> The authors in this study used transwell migration assays and c-Kit<sup>+</sup> CSCs from neonatal mouse hearts to demonstrate that CSC migration was enhanced upon stimulation of the SDF1 $\alpha$ :CXCR4 axis, but was negated by PI3K inhibition using LY294002. Moreover, infarct size was also reduced in an SDF1 $\alpha$ :CXCR4/PI3K dependent manner in agreement with the findings from Tang and colleagues. To sum, these studies illustrate the importance of SDF1 $\alpha$ -mediated recruitment of CSCs and HSCs from the cardiac and BM stem cell niches, respectively for cardiac repair.

Based on these findings, maximal benefits of SDF1 $\alpha$  signaling requires a tightly regulated expression profile with its receptors. Briefly, high myocardial and peripheral blood SDF1 $\alpha$  content must coincide with low BM SDF1 $\alpha$  levels and high receptor expression on cardiomyocytes and homing cells (i.e. HSCs and CSCs).<sup>143, 144</sup> A multivariate analysis of the REPAIR-AMI trial, which assessed the feasibility of BM mononuclear cell infusion, showed that BM cells have time dependent SDF1 $\alpha$  responsiveness that is highest at 4 to 7 days after MI.<sup>145</sup> The Late-TIME randomized clinical trial expanded on this notion by showing that patients that received autologous BM mononuclear cells 2-3 weeks post-MI did not experience a significant change in LVEF, LV volumes, or infarct size.<sup>4</sup> While SDF1 $\alpha$  responsive cells are well primed at 4 to 7 days later, myocardial SDF1 $\alpha$  is already on the decline.<sup>146</sup> This temporal misalignment in the peak expressions of myocardial SDF1 $\alpha$  (24h post-MI) and CXCR4 (96h post-MI) implies sub-optimal SDF1 $\alpha$ :CXCR4 axis activation.<sup>147</sup> As such efforts to align the axis through sustained SDF1 $\alpha$  expression<sup>101, 147-151</sup> and earlier induction of CXCR4 expression have enhanced CSC therapy.<sup>89, 152, 153</sup>



**Figure 3.** SDF1 $\alpha$  signaling promotes CXCR4 $^+$  cell recruitment to the infarct. (1) Myocardial infarction induces stem cell mobilization signals such as SDF1 $\alpha$  and VEGF and their release into the peripheral circulation (2) and eventually acts upon the BM (3). This leads to alterations in the BM microenvironment resulting in proliferation and increased CXCR4 receptor expression of stem and progenitor cells and BM-SDF1 $\alpha$  degradation (4). The change in the BM-SDF1 $\alpha$  gradient causes transmigration of HSCs into the systemic circulation and their homing towards the local myocardial SDF1 $\alpha$  gradient (5). The homing cells ultimately promote myocardial repair through cardioprotective functions and induction of angiogenesis.<sup>115</sup>

## 1.10 Therapeutic applications of SDF1 $\alpha$ for HF

Several studies geared towards taking advantage of SDF1 $\alpha$  and its downstream signaling cascades have shown significant promise in experimental models. Exogenous delivery of SDF1 $\alpha$  (recombinant or cell-based) and inhibition of its proteolytic degradation has been associated with enhanced recruitment of both resident and extra-cardiac stem cells to the infarct, angiogenesis, cardiomyocyte survival, and functional improvements.<sup>98,</sup>

<sup>101, 139-141, 146-149, 154, 154</sup> Conversely, strategies to increase SDF1 $\alpha$  expression on CSC products also leads to greater myocardial homing and concomitant tissue repair.<sup>155</sup>

Delivery	Model	Study endpoint(s)
<b>SDF1<math>\alpha</math> Protein</b>		
Intra-myocardial <sup>151</sup>	Acute MI (mouse)	Reduced apoptosis; decreased infarct size; increased LVEF and FS
Intra-muscular <sup>156</sup>	Acute ligation (mouse)	Increased hindlimb perfusion and capillary density
<b>Gene-based SDF1<math>\alpha</math> over-expression</b>		
Intra-myocardial (plasmid) <sup>157</sup>	Chronic ischemia (Phase I/II clinical trial)	Primary safety endpoint of no MACE at 1 month achieved. Improved 6-min walk distance and quality of life at 4 and 12 months after dosing.
Intra-myocardial (plasmid) <sup>148</sup>	Chronic MI (rat)	Increased vessel density; FS; decreased infarct size
Intra-muscular (plasmid) <sup>102</sup>	Acute ligation (mouse)	Increased hindlimb perfusion
<b>Cell-based over-expression of SDF1<math>\alpha</math></b>		
Intra-myocardial CSCs <sup>101</sup>	Acute MI (mouse)	Increased transplanted cell engraftment; reduced infarct size
Intra-myocardial MSCs <sup>139</sup>	Acute MI (rat)	Increased CSC migration; reduced infarct size;
Intra-venous MSCs <sup>147</sup>	Acute MI (rat)	Reduced apoptosis, Increased FS; Decreased LVEDD and LVESD
Intra-myocardial Skeletal Myoblasts <sup>141</sup>	Acute MI (rat)	Increased LVEF, LVEDD, LVESD; Reduced infarct size

**Table 1 - Trials demonstrating the effects of SDF1 $\alpha$  over-expression.**

Taken together, the promise of SDF1 $\alpha$  to propel cell therapy to the next level has proven fruitful in small animal models and warrants further attention.

## **2.0 Rationale, Research Aim, Hypothesis and Specific Objectives**

Several recent clinical trials have demonstrated the safety of first generation CSC products.<sup>11, 54, 157</sup> However, like other candidate cell types, CSC-mediated repair is limited by modest acute engraftment and extremely poor long-term survival.<sup>59, 158</sup> In the absence of robust engraftment, the benefits seen in trials using current cardiac stem cell products derive largely from the paracrine secretion of growth factors exerting trophic effects on the damaged host tissue. We have shown that the paracrine signature of CSCs has modest and variable amounts of SDF1 $\alpha$ . Given the key role of SDF1 $\alpha$  in myocardial repair and previous work demonstrating the therapeutic potential of exogenous SDF1 $\alpha$  for HF,<sup>101, 139, 147</sup> we explored the effect of over-expressing this key cytokine on CSC-mediated cardiac repair.

### **2.1 Research Aim**

To explore the impact of SDF1 $\alpha$  over-expression on CSC-mediated cardiac repair using an immunodeficient mouse model of myocardial ischemia.

### **2.2 Hypothesis**

Over-expression of SDF1 $\alpha$  in CSCs will enhance post-infarct cardiac repair by improving angiogenesis, resident stem cell recruitment, and transplanted CSC survival following intra-myocardial transplantation into ischemic mouse myocardium.

### **2.3 Specific Objectives**

1. To characterize the spatiotemporal expression profile of SDF1 $\alpha$  receptors within the heart after MI using qPCR and immunohistochemistry.
2. To determine what sub-population within CSCs is responsible for the production of SDF1 $\alpha$ .
3. To genetically engineer CSCs to over-express SDF1 $\alpha$  using somatic gene transfer to the mesenchymal sub-population (CD90+) within CSCs.

4. To evaluate the effect of SDF1 $\alpha$  over-expression on the overall paracrine signature of CSCs.
5. To quantify the ability of SDF1 $\alpha$  over-expressing CSCs to stimulate capillary network formation, cardiogenic differentiation and stem cell migration.
6. To assess the functional effects of SDF1 $\alpha$  over-expressing CSCs on myocardial repair after intra-myocardial injection into an immunodeficient mouse model of myocardial infarction.
7. To dissect the fundamental mechanisms underlying the effects of SDF1 $\alpha$  over-expression by CSCs on long-term engraftment of transplanted stem cells and stem cell recruitment.

## 3.0 Methods

### 3.1 Expression of CXCR4/CXCR7 within the heart following myocardial infarction

The rationale for the timing of CSC transplantation reflects the logistical realities inherent in cell culture and the need to administer an autologous cell product soon after myocardial infarction (MI). Furthermore, the optimal timing of SDF1 $\alpha$  enhanced cell transplantation is unknown. In theory, ideal timing would be based upon the responsiveness of the heart to SDF1 $\alpha$  signaling which would be reflected by maximal expression of SDF1 $\alpha$  receptors. To develop a protocol that balanced cell culture realities with optimized benefits, we used C57/BL6 mice (Charles River) to characterize the spatiotemporal expression pattern of CXCR4 and CXCR7. Mice were randomized to receive thoracotomy alone (sham control, n = 3) or thoracotomy with left anterior descending (LAD) ligation (n = 24). Mice were sacrificed 1, 7, 14, and 21 days post-LAD ligation (n=6/day). Half of hearts at each time point (n=3) were dissected into three separate regions (infarct, infarct border zone, and off-target (LV posterior wall)). Total RNA was isolated from each region using TRIzol LS reagent (10296-010, Gibco) for qPCR quantification of CXCR4 and CXCR7 expression. .

The remaining 3 hearts at each time point underwent histological sectioning to confirm the observed increases in the CXCR4 receptor using immunohistochemical staining for CXCR4 and CXCR7 in combination with TnT (cTnT; ab10214, Abcam).

Moreover, SDF1 $\alpha$  receptor expression within the representative sub-populations (c-Kit and CD90) of cardiac outgrowth was assessed using flow cytometry. Consequently, the presence of these receptors would render CSCs responsive to SDF1 $\alpha$  and the positive effects associated with its signaling.

### 3.2 Patients and cell culture

Human atrial appendages acquired from patients undergoing clinically-indicated surgery under a protocol approved by the University of Ottawa Heart Institute Research Ethics Board were used to culture CSCs.<sup>44</sup> Patients between the ages of 18 and 80 who were undergoing invasive cardiopulmonary bypass or heart valve repair/replacement were eligible for this study. Patients less than 18 years' old, pregnant women, patients undergoing non-invasive procedures and individuals with infectious disease were excluded.

CSC cultures were established as previously described.<sup>44, 46</sup> Briefly, tissue from the atrial appendage was minced, digested with collagenase IV (Gibco), and explanted on fibronectin coated dishes in cardiac explant media (CEM: Iscove's Modified Dulbecco's Medium, 20% fetal bovine serum, 100U/mL penicillin, 2mmol/L glutamine, 0.1 mmol/L 2-mercaptoethanol; Life Technologies, Burlington Canada). All samples were cultured in physiological oxygen (i.e. 5% O<sub>2</sub>) to increase CSC yield and reduce cellular senescence and chromosomal abnormalities.<sup>79</sup> Cardiac outgrowth was harvested within the first 7-10 days of culture.

The colorimetric WST-8 assay (Cell counting kit 8, Dojindo Molecular Technologies, Inc. Gaithersburg, MD) was used to assess CSC proliferation.<sup>44</sup> Population doubling was calculated with the first colourimetric assay as the starting population [# of population doublings = log<sub>2</sub>(cell number)]. Doubling time was calculated as the difference between the time(t) and cell counts(N) of the starting population(1) and the final population after 48 hours in culture(2) [Doubling time = (t<sub>2</sub>-t<sub>1</sub>)log<sub>2</sub>/(logN<sub>2</sub>-logN<sub>1</sub>)].<sup>159</sup>

Commercially sourced male normal human dermal fibroblasts (NHDF; Promo Cell, Germany) and human umbilical vein endothelial cell (HUVECs; CC-2517 Clonetics) were used in appropriate tests and cultured according to the manufacturer's directions.

Human CACs were obtained from peripheral blood samples of patients undergoing clinically-indicated surgery under the same University of Ottawa Heart Institute Research

Ethics Board approved protocol for transwell migration assays as described previously.<sup>160</sup> In short, density-gradient centrifugation (Histopaque 1077; Sigma-Aldrich, Canada) was used to isolate mononuclear cells which were subsequently cultured on fibronectin coated culture ware for 4-6 days in 20% oxygen 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).

### **3.3 Selective Lentiviral Transduction of CD90+ CSCs to Over-express SDF1 $\alpha$**

Over-expression of SDF1 $\alpha$  in CSCs was achieved using a fourth generation lentiviral vector system. Human SDF1 $\alpha$  complementary DNA was amplified by PCR and cloned into a pLVX-EF1 $\alpha$ -IRES-mCherry backbone expression vector (Clontech). The constitutively active human EF1- $\alpha$  promoter drives the co-expression of SDF1 $\alpha$  and mCherry. Xenogeneic delivery of exogenous SDF1 $\alpha$  did not affect our model as confirmed by protein BLAST which indicated 93% homology between human and mouse SDF1 $\alpha$ . Lentiviral particles were constructed by co-transfection into HEK293T cells and concentration of the viral supernatants over 72 hours. Functional titers were calculated following quantitative PCR using primer probes complementary to the lentiviral packaging sequence from serially transduced HEK293T.<sup>161</sup>

Immunomagnetic sorting separated the CD90+ subpopulation from the remaining cardiac outgrowth and was cultured in parallel with the CD90 depleted population (CD90-). The mesenchymal subpopulation was transduced at 3 MOIs (10, 30, and 50) and recombined with the CD90 depleted population 72 hours later for experimentation. Transduction was confirmed by mCherry detection by fluorescent microscopy.

### **3.4 Conditioned media and paracrine profiling**

Enzyme-linked immunosorbent assay (ELISA; DSA00, R&D Systems) using conditioned media collected 2 days after recombining the CD90+ and CD90- subpopulations

was used to confirm lentiviral-mediated over-expression of SDF1 $\alpha$ . Long-term transgene expression was validated over 2 weeks in culture and compared to conditioned media of non-transduced CSCs. The paracrine profile of non-transduced, mCherry transduced CSCs (empty vector control) and SDF1 $\alpha$  transduced CSCs was characterized using a custom built array (Human Cytokine Antibody Array G Series kit; RayBiotech, USA) on the respective conditioned media. The relative fluorescent signal was calculated using a Genepix 4000B scanner with proprietary software. Fluorescent values were normalized to internal positive and negative controls and expressed as fold change over fluorescent values from NHDF conditioned media.

### **3.5 In vitro angiogenesis assay**

The angiogenic potential of CSC conditioned media was measured by quantifying human umbilical vein endothelial cell (HUVEC) capillary network formation in a matrigel based angiogenesis kit (Millipore ECM625). HUVECs were cultured on matrigel in conditioned media from non-transduced and transduced CSCs for 18 hours. Capillary network formation was manually measured in 6 random fields per well with the NeuronJ, ImageJ plugin. HUVECs cultured in serum-free (i.e. basal) media used as the negative control.<sup>44</sup>

### **3.6 Transwell migration assay**

Transwell migration (07200150, Costar, Pittston, PA, USA) assays were used to measure the chemotactic response of human CACs to CSC-conditioned media and serum-free media (negative control). Transwell membrane inserts (3  $\mu$ m pore size) were loaded with  $5 \times 10^4$  CACs in serum-free media while the lower chamber contained the test media. After 24 hours of incubation, migratory CACs in the bottom well were fixed with 4% paraformaldehyde, washed with PBS, and stained with 4', 6-diamidino-2 phenylindole (DAPI; D9542, Sigma-Aldrich, Canada). Random field analysis of stained nuclei (n = 6 random fields at 10X magnification/well) were counted using the Image-based Tool for

Counting Nuclei (ITCN, ImageJ plugin, NIH). Minimum distance, threshold, and width parameters within the ITCN plugin were consistent for all counts.

### **3.7 In vitro cardiogenic differentiation**

The multipotency of non-transduced and SDF1 $\alpha$  over-expressing CSCs was measured using qPCR for markers of cardiac, smooth muscle cell, and endothelial identity after exposure to conditions known to favour differentiation (Cardiogenic Media [CGM]: DMEM-LG, 40% MCDB-201, 0.75% dimethylsulfoxide, 0.1% 10 mmol/l L-ascorbic acid, 0.01% ITS liquid media supplement, 0.01% linoleic acid-albumin, 0.01% Pen-Strep, 0.0002% 0.25 mmol/l dexamethasone, 0.001% 2-mercaptoethanol, 10 ng/ml recombinant mouse fibroblast growth factor 8b, 100 ng/ml fibroblast growth factor 4, 10 ng/ml recombinant human protein rhDKK-1 and 10 ng/ml recombinant human bone morphogenetic protein).<sup>44</sup> Total RNA was isolated using TRIzol LS reagent for qPCR to measure the expression of troponin T (TnT),  $\alpha$ -smooth muscle actin ( $\alpha$ -SMA), and von Willebrand factor (vWF).

### **3.8 In vivo effects of enhanced SDF1 $\alpha$ production on CSC-mediated cardiac repair**

Eighteen NOD SCID mice (Charles River, Wilmington, MA, USA) were used to determine the effect of CSC therapy on infarcted myocardium. All mice were anaesthetized with 2% isoflurane and subject to standard thoracotomy followed by immediate ligation of the LAD coronary artery. One week after surgery, mice received SDF1 $\alpha$  transduced CSCs (n = 8), non-transduced CSCs (n = 6) or vehicle control (PBS, n = 4). Intra-cardiac injections consisted of two echocardiography guided injections of  $5 \times 10^4$  CSCs into the infarct border zone and apex using a 30G needle.

Two-dimensional echocardiography was performed 7, 21 and 28 days post-MI using the Vevo 770 imaging system (Visual Sonics Inc., Toronto, Canada). Mice were anesthetized with an intraperitoneal injection of ketamine-xylazine (100mg/kg ketamine, 10mg/kg xylazine) before LV images were recorded from the long-axis view. LV dimensions

were manually measured using Visual Sonics version 3.0.0 software from which left ventricular ejection fraction (LVEF) was calculated.

All mice were euthanized after functional assessment at 28 days. Hearts were excised and subjected to either immunohistochemical or qPCR testing. For histology, tissue was washed with 20% sucrose, embedded in optimal cutting temperature (OCT) compound, flash frozen, and sectioned into 5µm slices from the base of the heart to the apex. Masson's

To evaluate stem cell engraftment and differentiation, immunostaining for human nuclear antigen (HNA; SAB4500768, Sigma, Canada) was used to detect engrafted human CSCs. Co-staining with non-specific  $\alpha$ -SMA (ab125266; Abcam), cTnT (ab66133; Abcam) and vWF (11778-1-AP; Proteintech Group, USA) identified engrafted cells that differentiated into smooth muscle, cardiomyocyte and endothelial lineages, respectively.

### **3.9 In vivo effects of enhanced SDF1 $\alpha$ production on rescue of ischemic myocardium**

To explore the mechanism underlying the effect of enhanced SDF1 $\alpha$  production on CSC mediated cardiac repair, we explored the effect of transplanting SDF1 $\alpha$  over-expressing CSCs on myocardial SDF1 $\alpha$  content and stimulation of pro-survival pathways. One week after LAD ligation NOD SCID mice were transplanted with SDF1 $\alpha$  transduced human CSCs (n = 6) or non-transduced human CSCs (n = 6). Hearts were excised at days 7 post-transplantation and dissected into three separate samples (infarct, infarct border and off-target (LV posterior wall) regions) for protein isolation. Western blots were performed to quantify SDF1 $\alpha$  (ab9797, Abcam), Bcl-2 (ab7977, Abcam), Bax (ab32124, Abcam) and GAPDH (sc-25778, Santa Cruz Biotechnology) content to confirm SDF1 $\alpha$  and apoptosis content.

### **3.10 In vivo effects of enhanced SDF1 $\alpha$ production on stem cell recruitment**

Current cell-therapy paradigms contend that functional benefits are largely driven by paracrine mechanisms; however the relative contribution of bone marrow and resident CSC

recruitment is debatable. As such, we used a transgenic bone marrow transplant mouse model to assess the relative contribution of these mechanisms to CSC-mediated cardiac repair and the additive effects of SDF1 $\alpha$  over-expression. Bone marrow from donor male mice was transplanted via tail vein injections ( $5 \times 10^6$  bone marrow cells) into recipient female mice following total body irradiation (cesium source, 900 rads, two courses). After 6 weeks of bone marrow reconstitution, all mice underwent LAD ligation and intra-myocardial injection of vehicle control (PBS, n=5), non-transduced female mouse CSCs (n=6), or SDF1 $\alpha$  transduced female mouse CSCs (n=6) one week post-MI. Mice were euthanized at day 7 and 14 post-MI (n=3 per group and time point). Hearts were subsequently excised for DNA isolation using (GS300, Geneaid) and qPCR to detect the presence of murine Y chromosome (i.e. RNA-binding motif protein, Y-linked, family 1, member A1: RBMY) in the damaged female heart which represents the degree of bone marrow cell recruitment.<sup>162</sup>

### **3.11 Statistical Analysis**

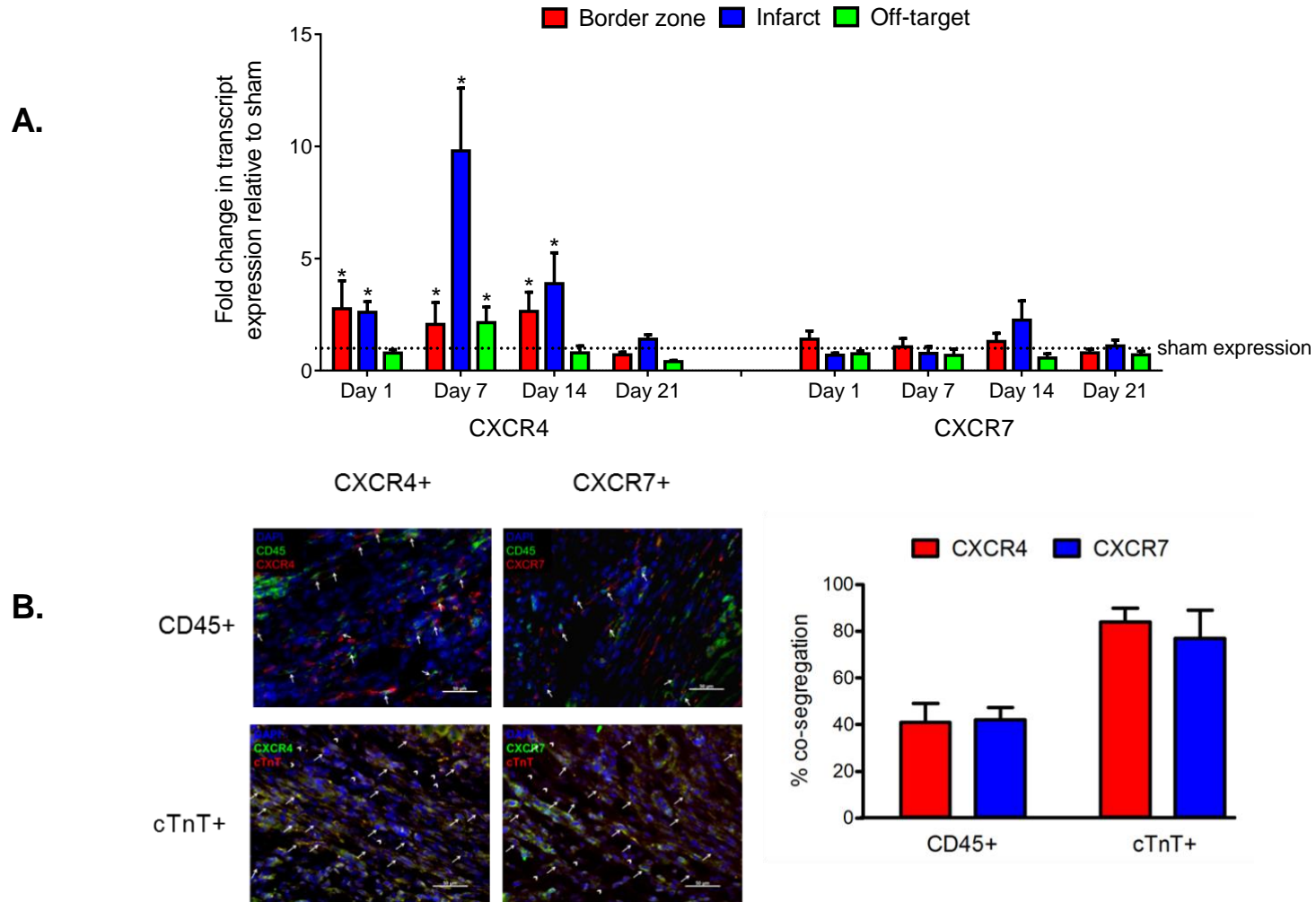
All data is presented as the mean  $\pm$  standard error of the mean (SEM). Differences within groups were first analyzed using one-way analysis of variance (ANOVA). Post hoc testing was conducted using t-test following confirmation of differences outlined by ANOVA. Differences in categorical data were assessed using Chi Square testing. A final value of  $P \leq 0.05$  (i.e.  $\alpha = 0.05$ /confidence interval 95%) was considered significant for all analyses.

## **4.0 Results**

### **4.1 CXCR4 expression is globally upregulated following myocardial infarction**

While the cardioprotective role of SDF1 $\alpha$  after ischemic injury has been well established,<sup>101, 147, 151</sup> we profiled the expression of SDF1 $\alpha$  receptors (CXCR4 and CXCR7) after MI in C57/BL6 mice to better understand when the myocardium is maximally SDF1 $\alpha$  responsive (Figure 4). Quantitative PCR revealed that the CXCR4 transcript was increased as early as one day after LAD ligation and this increase was sustained for at least 2 weeks

after infarction. CXCR4 transcript levels were greatest 7 days after MI and returned to baseline by day 21 after MI (Figure 4A). Interestingly, a disparity in SDF1 $\alpha$  receptor expression was observed as CXCR7 transcript levels remained constant following ischemia (Figure 4A). Immunohistochemistry confirmed that both SDF1 $\alpha$  receptors were widely expressed by cardiomyocytes and BM cells (Figure 4B). These results demonstrate that, while both CXCR4 and CXCR7 are expressed within the native myocardium, infarction stimulates the up-regulation of CXCR4 to enhance pro-survival signalling by SDF1 $\alpha$ . Taken together, this data rationalizes the enhancement of an SDF1 $\alpha$  signal soon after MI to stimulate the rescue of reversibly damaged myocardium. To this end, SDF1 $\alpha$  was genetically over-expressed within transplant ready human CSCs to investigate the impact of an increased myocardial salvage and recruitment of SDF1 $\alpha$  responsive stem cells on CSC-mediated cardiac repair.



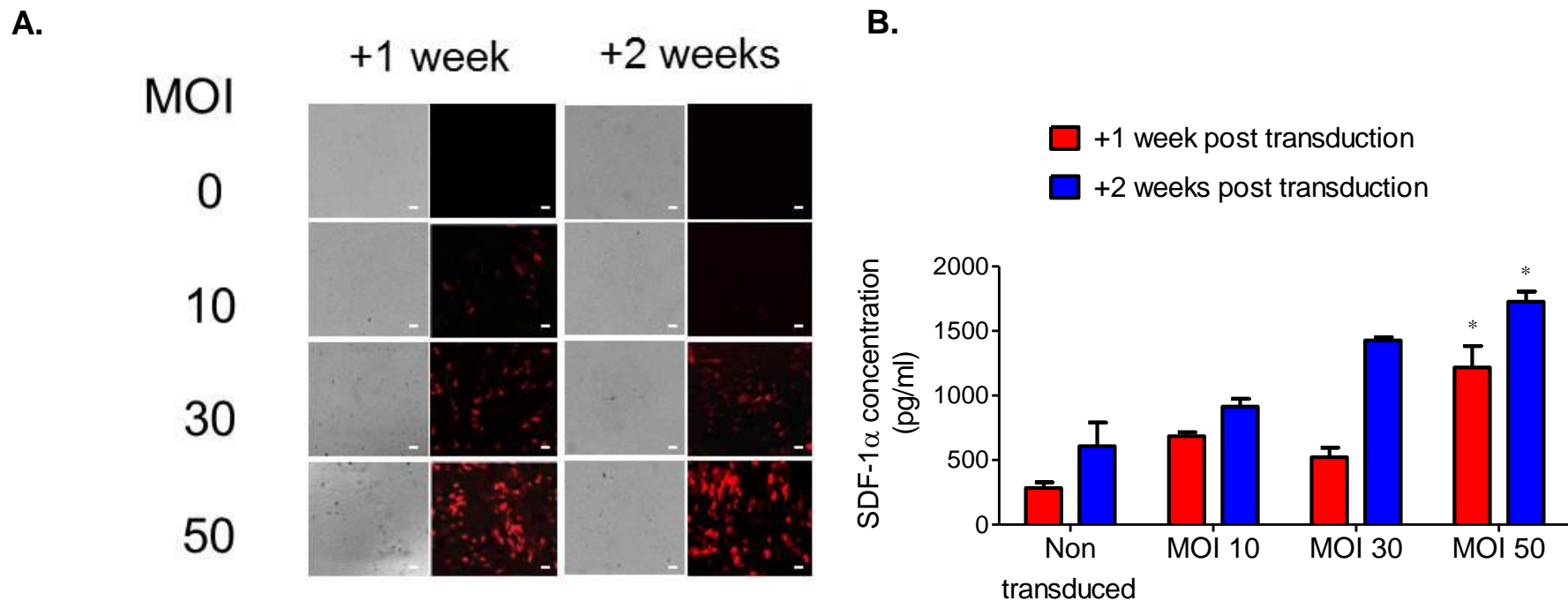
**Figure 4.** Spatiotemporal expression of SDF1 $\alpha$  receptors post-MI. CXCR4 expression is increased at day 7 following ischemia (A). CXCR7 expression does not change post-MI. Significance was calculated using pair-wise reallocation randomisation.<sup>163</sup> Stars indicate  $p < 0.05$  vs. sham-operated mice. Representative images of myocardial sections stained for CXCR4 (green), CXCR7 (green), cTnT (red) and DAPI (blue) at day 7 post-MI (B). Images were taken using a confocal microscope at 100x magnification with oil immersion. Scale bar indicates 50 $\mu$ m (n=3 mice/time point).

## 4.2 Lentiviral gene transfer yields long term SDF1 $\alpha$ expression without impairing the CSC secretome

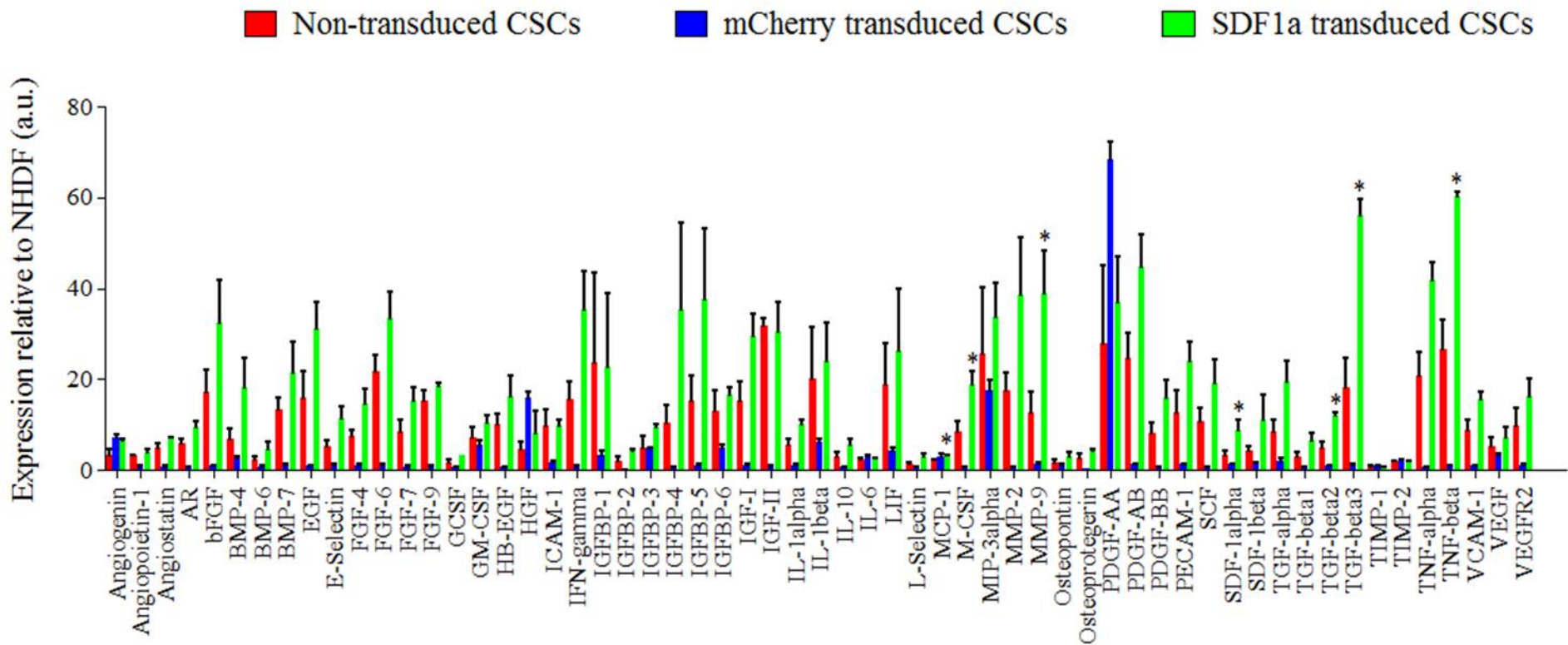
Lentiviral-mediated transduction of the mesenchymal subpopulation (i.e., CD90+ cells) within CSCs resulted in a dose dependent increase in SDF1 $\alpha$  transgene expression (Figure 5). This strategy was motivated by the suggestion that the CD90+ cells may not markedly contribute to CSC regenerative performance.<sup>51</sup> Maximal transduction occurred at an MOI of 50 with negligible effects on *in vitro* proliferation (Appendix 1). Peak SDF1 $\alpha$  transgene expression following recombination of the transduced CD90+ fraction with the CD90 depleted fraction was achieved with an MOI of 50 (4.7 $\pm$ 1.2 fold increase in SDF1 $\alpha$  expression vs. non-treated CSCs,  $p\leq 0.05$ ). Long term overexpression of SDF1 $\alpha$  was sustained over 2 weeks of *in vitro* culture (Figure 5). Given that treatment of CSCs with an MOI of 50 produced the most robust long-term transgene expression with negligible effects on proliferation, this MOI was chosen for all subsequent experiments.

Given that a recent report suggested that disruption of the CSC paracrine profile by cytokine over-expression may blunt the regenerative potential of CSCs,<sup>80</sup> conditioned media from LV-mCherry, LV-SDF1 $\alpha$  and non-transduced CSCs was characterized as a means of “quality control” to ensure that over-expression of SDF1 $\alpha$  did not have a negative impact upon the overall paracrine signature of CSCs (Figure 6). Transduction with the LV-mCherry backbone vector narrowed the CSC secretome when compared to non-transduced CSCs (15 vs 39 cytokines produced in excess to NHDF,  $P\leq 0.05$  versus cytokine levels detected within NHDF-conditioned media;  $\chi^2$  value, 15.72;  $P\leq 0.001$  versus the expected frequency of cytokines elevated in NHDF conditioned media). These data suggest that the CD90 subpopulation within CSCs contributes a significant proportion of the regenerative cytokines secreted by CSCs and that treatment with lentivirus treatment significantly impairs this ability. Treatment of CSCs with LV-SDF1 $\alpha$  enhanced SDF1 $\alpha$  expression (2.8 $\pm$ 0.7 fold increase,  $p\leq 0.05$ ) and rescued the paracrine signature of CSCs (49 vs. 39 cytokines

produced in excess to NHDF conditioned media,  $p \leq 0.05$  versus cytokine levels detected within NHDF-conditioned media;  $\chi^2$  value, 2.386;  $p=0.12$  versus the expected frequency of cytokines elevated in non-transduced CSC conditioned media). Interestingly, flow cytometry revealed that CXCR4 was highly expressed by the vast majority of CSCs. The c-Kit-/CD90- subpopulation represented the most SDF1 $\alpha$  responsive element within CSCs as CXCR4 was not expressed by the majority of c-Kit+/CD90- and c-Kit-/CD90+ cells (93 $\pm$ 2% vs. 43 $\pm$ 0.3% and 47 $\pm$ 11%, respectively; Appendix 2). Overall, this data suggests that salvage of the CSC paracrine profile after lentiviral transduction occurs through autocrine signaling by SDF1 $\alpha$  between complimentary CSC sub-populations.



**Figure 5.** Lentiviral transduction provides long-term expression of SDF1 $\alpha$  in vitro and does not impair the paracrine signature of CSCs. Reporter mCherry expression is sustained over 2 weeks in culture when MOI's of 30 and 50 are applied. Fluorescent images taken at 10x magnification at 590 nm are shown (A). SDF1 $\alpha$  transgene expression persists long-term in vitro. Optimal transgene expression is obtained using a MOI of 50. Quantification of SDF1 $\alpha$  concentrations within conditioned media using ELISA following LV transduction (B). Bars indicate standard error of the mean (n=3/time pt).



**Figure 6.** The paracrine signature of CSCs is not impaired following lentiviral transduction. Seven cytokines were increased after LV-SDF1 $\alpha$  transduction (MCP-1, M-CSF, MMP-9, SDF1 $\alpha$ , TNF- $\beta$ , TGF- $\beta$ 2, TGF- $\beta$ 3). Stars indicate  $p < 0.05$  vs. non-transduced CSC conditioned media (n=4).

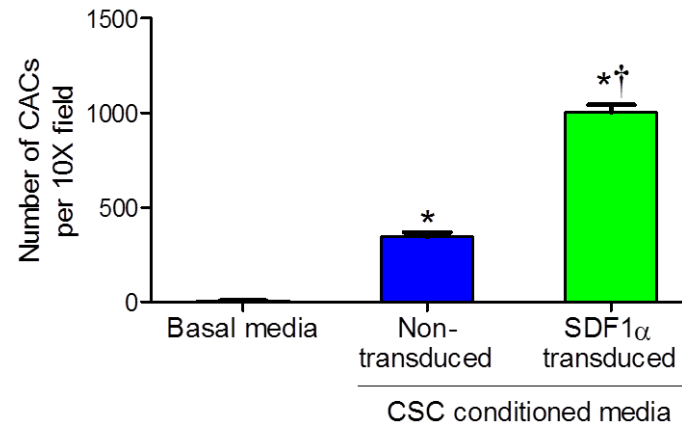
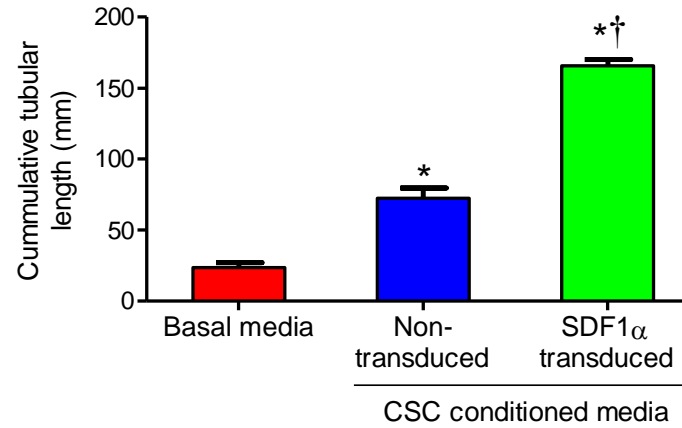
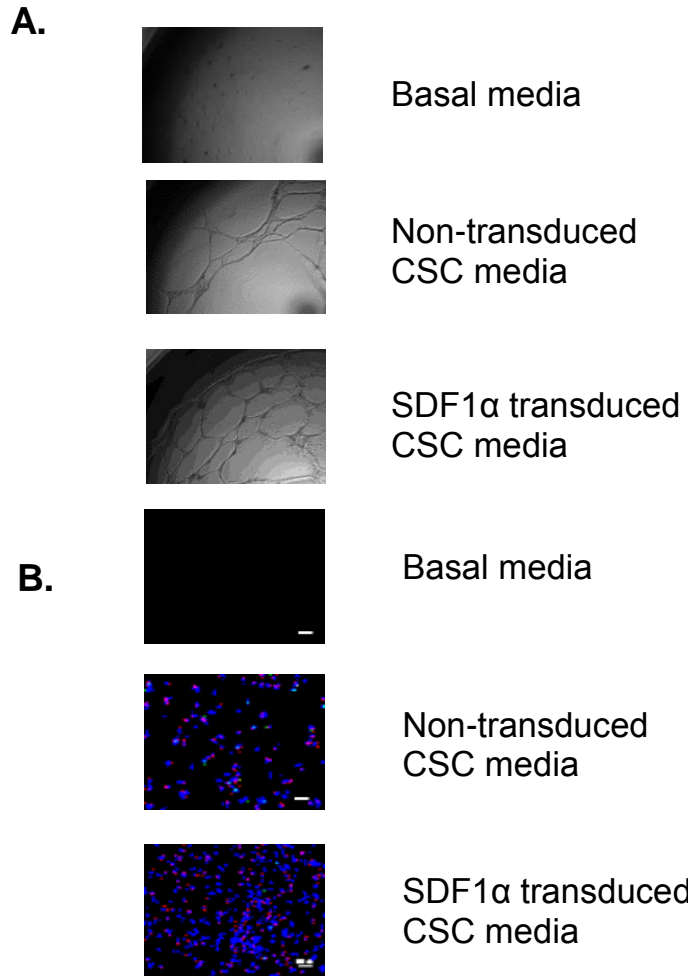
### **4.3 SDF1 $\alpha$ over-expression enhances angiogenesis, cardiac and endothelial lineage marker expression, and stem cell recruitment**

*In vitro* angiogenesis assays were used to assess the angiogenic capacity of CSC conditioned media on HUVECs. As shown in figure 7A, media conditioned CSCs that over-express SDF1 $\alpha$  demonstrated a superior ability to promote capillary network formation *in vitro* (165.7 $\pm$ 4.3 vs 72.5 $\pm$ 7.1 mm cumulative tubule length,  $p\leq 0.05$ ).

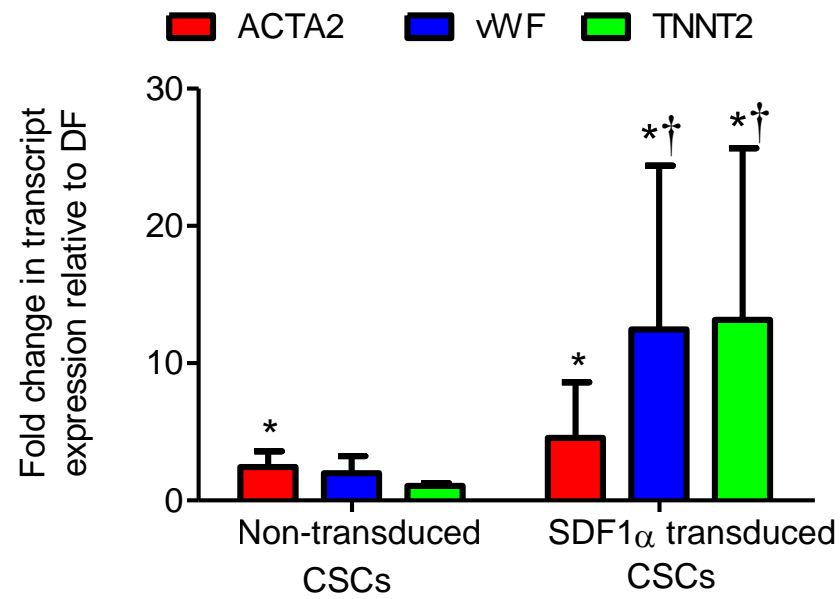
The ability of CSC conditioned media to promote the recruitment of blood derived CACs was evaluated using a standard transwell migration assay. Chemotactic CACs were visualized under a fluorescent microscope after 24 hours of incubation with serum-free media or conditioned media from CSC or CSCs that over-express SDF1 $\alpha$ . As shown in Figure 7B, chemotaxis was enhanced after exposure to media conditioned by CSCs that over-express SDF1 $\alpha$  (1001 $\pm$ 42 vs. 343 $\pm$ 25 CACs per field as compared to untreated CSCs,  $p\leq 0.05$ ).

Although the majority of CSC-mediated benefits are conferred through paracrine mechanisms, these cells are capable of enhancing cardiac repair through direct replacement of injured myocardium. After one week of culture within conditions known to favour a cardiac phenotype, qPCR analysis demonstrated that over-expression of SDF1 $\alpha$  drove increased transcript levels of TNNT2 and vWF when compared to non-transduced CSCs ( $p\leq 0.05$ ; Figure 8).

Taken together, this data suggests that over-expression of SDF1 $\alpha$  by CSCs promotes angiogenesis and stem cell recruitment while enhanced expression of cardiac and endothelial lineage markers hints toward an increased propensity to adopt these fates.



**Figure 7.** Over-expression of SDF1 $\alpha$  augments tube formation (A). Over-expression of SDF1 $\alpha$  improves the CAC chemotactic response in vitro. Migrated CACs stained with DAPI (B). Migration was expressed as the number of CAC nuclei per high powered field. Bars indicate standard error of the mean. Stars and crosses indicate  $p < 0.05$  vs NHDF and non-transduced CSCs, respectively ( $n = 3/\text{group}$ ).

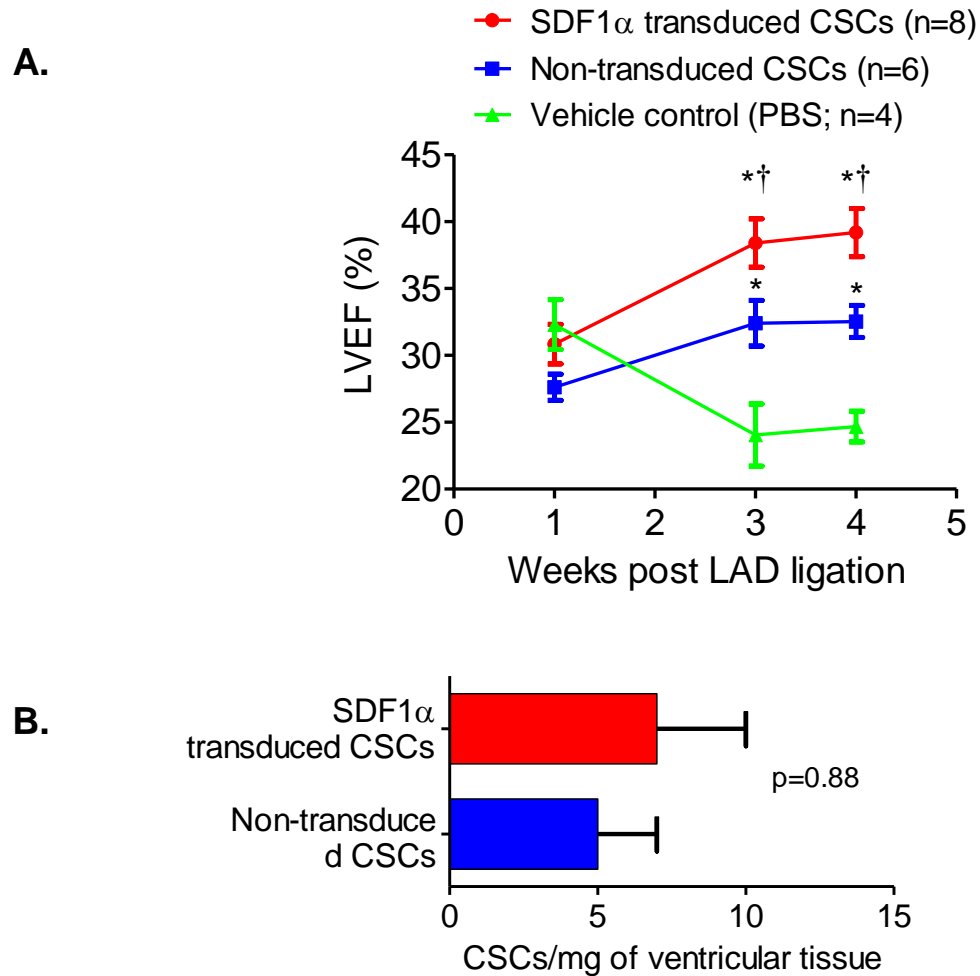


**Figure 8.** SDF1 $\alpha$  overexpressing CSCs have an enhanced capacity for in vitro differentiation. Stars and crosses indicated  $p < 0.05$  vs NHDF and non-transduced CSCs, respectively. Bars indicate standard error of the mean (n=2).

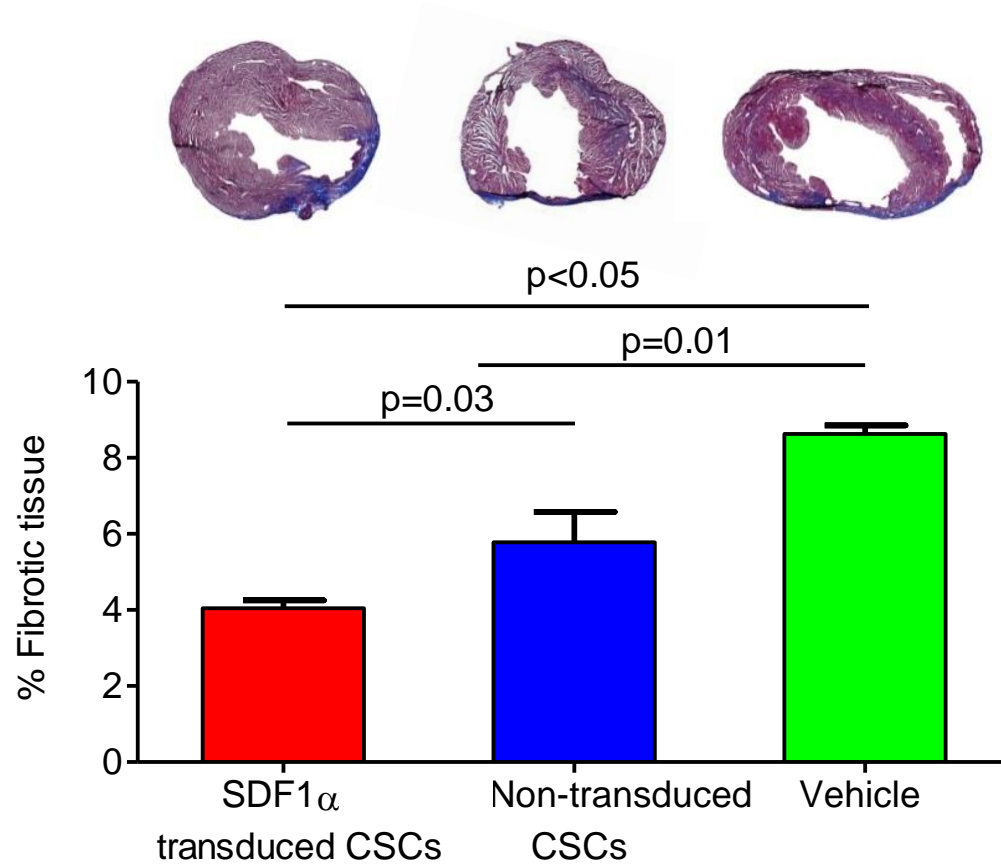
#### 4.4 Transplantation of CSCs that over-express SDF1 $\alpha$ enhances cardiac repair

Intra-myocardial delivery of SDF1 $\alpha$  transduced CSCs into the peri-infarct region of infarcted SCID mice enhanced cardiac repair as compared to non-transduced CSCs and vehicle control (Figure 9A). As anticipated, mice receiving PBS experienced a progressive decline in LVEF from the time of injection to the end of follow up ( $31.1\pm 1.9\%$  vs.  $25.1\pm 1.4\%$ , respectively;  $p\leq 0.05$ ). Administration of non-transduced CSCs gradually improved LV function over 3 weeks ( $27.6\pm 0.9\%$  to  $32.5\pm 1.0\%$ , respectively;  $p\leq 0.05$ ). Transplantation of CSCs over-expressing SDF1 $\alpha$  provided the most marked progressive benefit in LV function ( $30.8\pm 1.3\%$  to  $39.2\pm 1.6\%$ , respectively;  $p\leq 0.05$  as compared to LVEF 1 week post MI and LVEF of non-transduced CSCs 4 weeks post MI). These beneficial effects were confirmed with Masson's trichrome stained sections demonstrating reduced scar formation and increased tissue viability within at risk regions after treatment with CSCs over-expressing SDF1 $\alpha$  as compared to both non-treated CSCs and vehicle injections ( $4.0\pm 0.2$  vs  $5.8\pm 0.8\%$  fibrotic tissue,  $p\leq 0.05$ ; Figure 10).

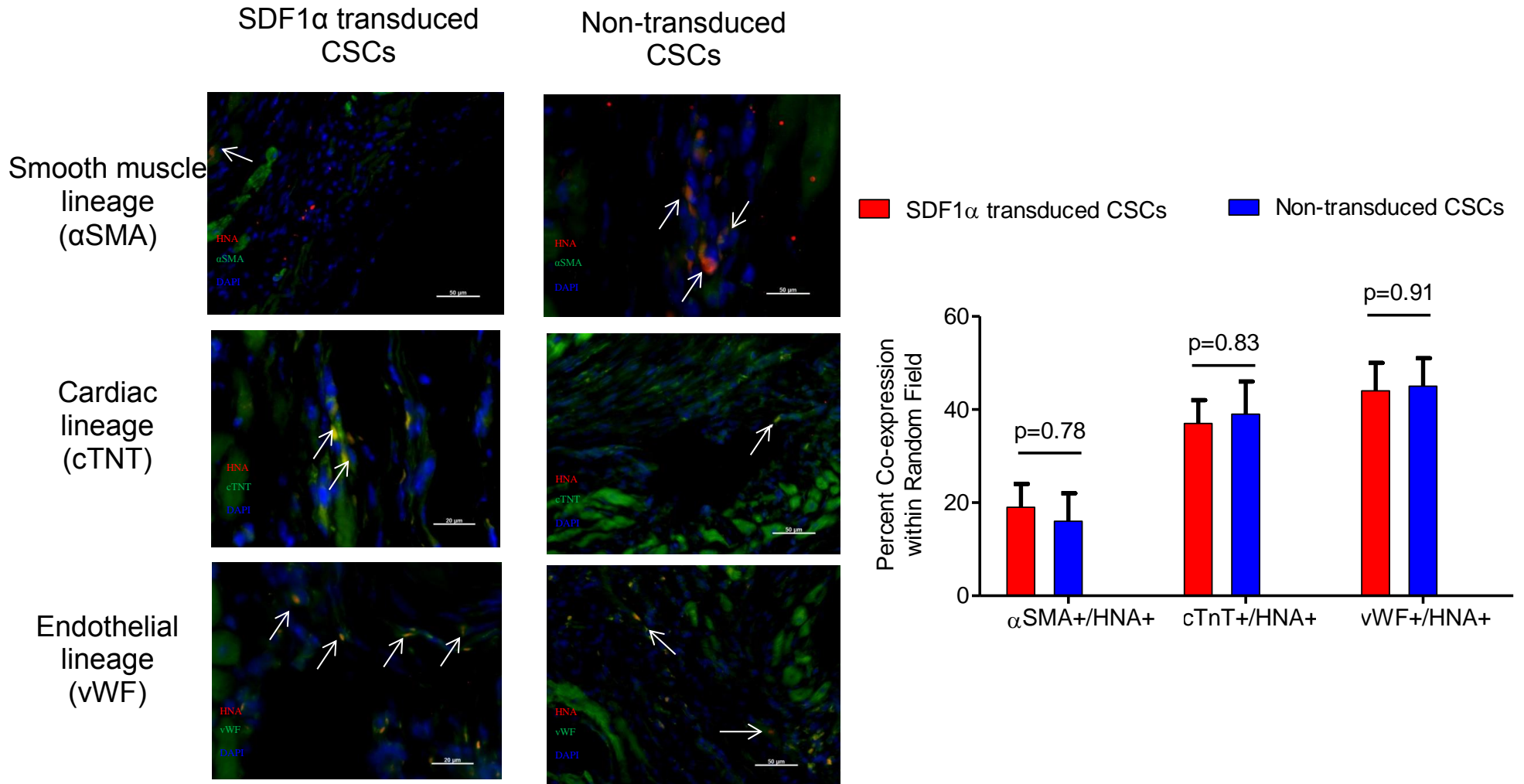
Despite markedly better post-infarct repair, transplant of CSCs that over-expressed SDF1 $\alpha$  did not increase engraftment or cell retention 21 days after injection as compared to intra-myocardial injection of unmodified CSCs ( $5\pm 2$  vs.  $7\pm 3$  human CSCs/mg of protein lysate, respectively;  $p=0.73$ ; Figure 9B). Additionally while *in vitro* studies suggested that over-expression of SDF1 $\alpha$  primes CSCs to adopt a cardiac fate, random field analysis of stained ventricular sections demonstrated that over-expression of SDF1 $\alpha$  did not alter the identity of human cells found on follow-up (Figure 11). These results highlight the observation that persistence of transplanted cells is not necessary for marked post infarct repair after transplantation. Furthermore, broadening the paracrine profile of CSCs prior to transplantation significantly improves indirect myocardial healing to boost functional outcomes.



**Figure 9.** Transplant of CSCs that over-express SDF1 $\alpha$  one week after LAD ligation improves LVEF (A). CSCs confer modest benefits to the ischemic heart, whereas cardiac function declines notably following vehicle injection. Mean LVEF's are shown for each group at each time point. Stars and crosses indicate p<0.05 vs. vehicle control and non-transduced CSCs, respectively. SDF-1 $\alpha$  over-expression did not augment cell retention at day 28 (n=3 mice/group)(B). All bars indicate standard error of the mean.



**Figure 10.** Over expression of SDF1 $\alpha$  reduces scar burden at day 28 post-MI. Masson's trichrome staining. All bars indicate standard error of the mean (n=3 mice/group).



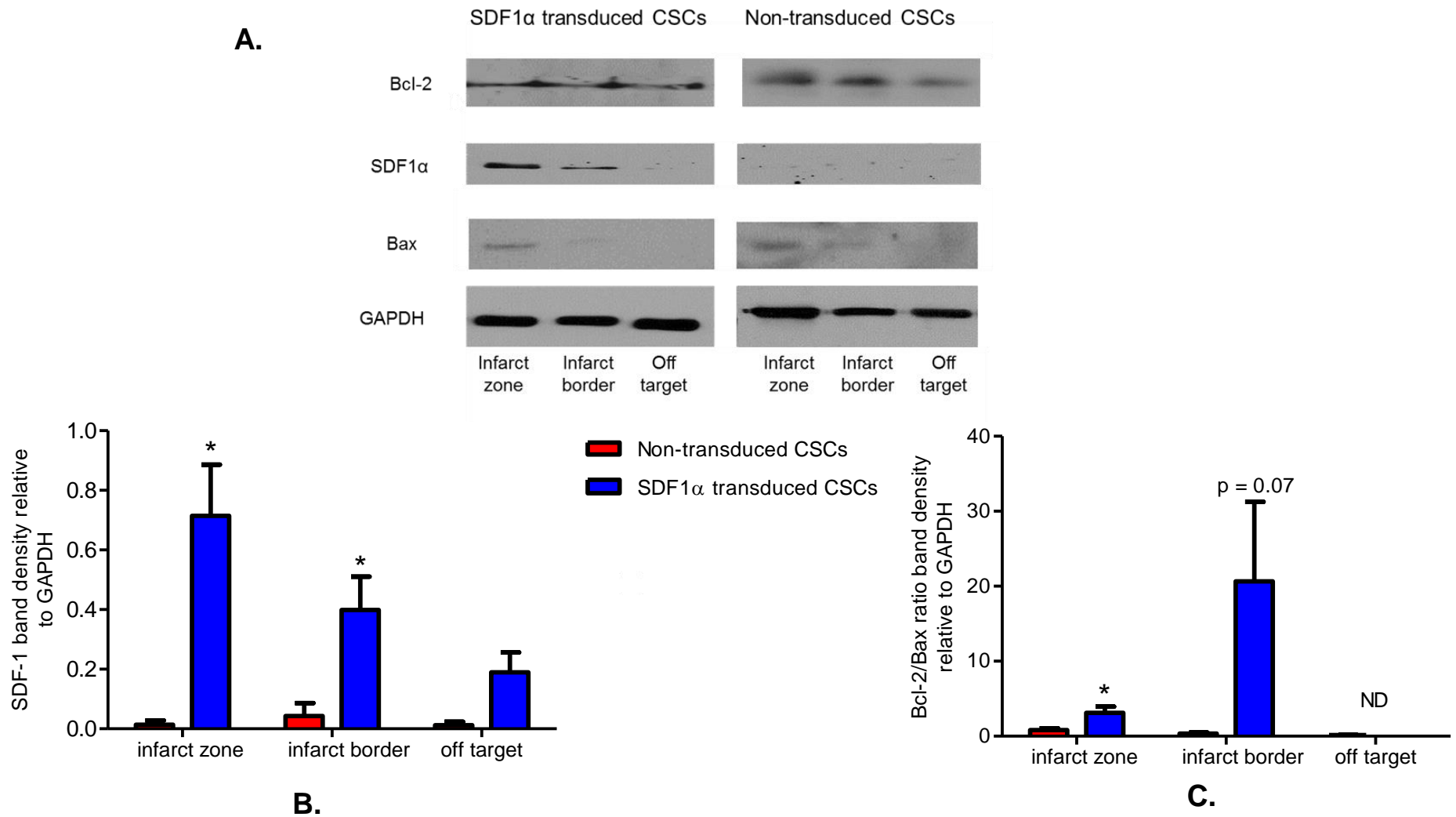
**Figure 11.** SDF-1 $\alpha$  over-expression did not alter the fate of successfully engrafted cells as scored from histological sections. Bars indicate standard error of the mean (n=3 mice/group).

#### **4.5 Transplantation of SDF1 $\alpha$ over-expressing CSCs increases local SDF1 $\alpha$ content while reducing apoptosis and enhancing resident stem cell recruitment**

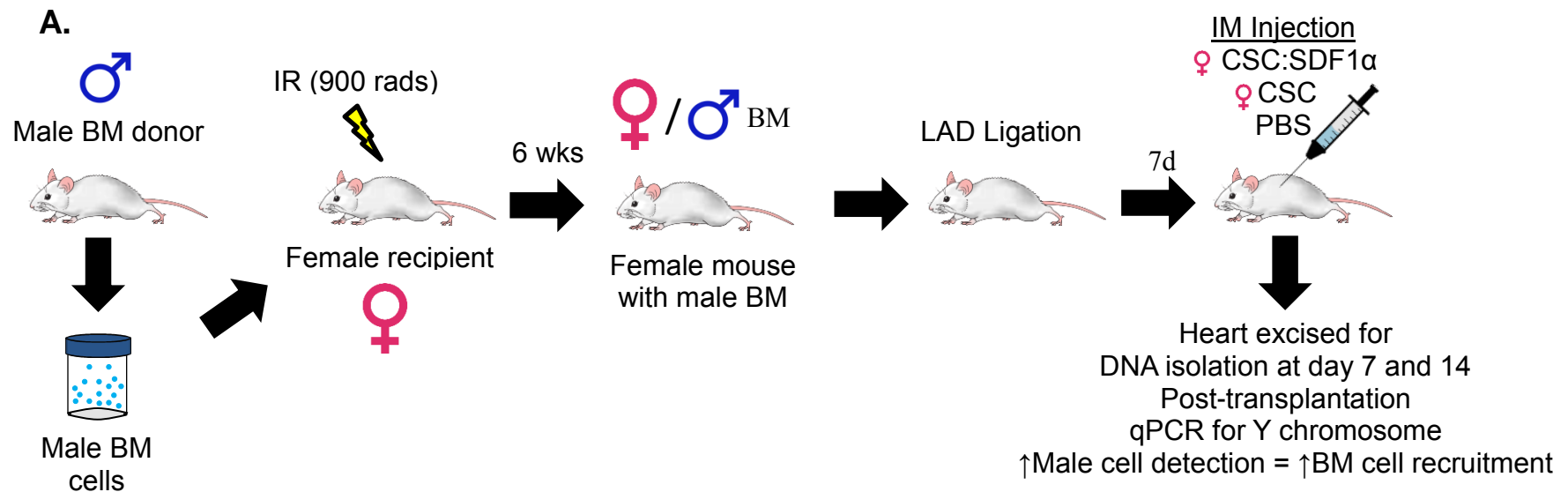
The mechanism underlying benefits seen after transplantation of SDF1 $\alpha$  over-expressing CSCs was probed using a series of infarcted NOD SCID mice sacrificed 7 after CSC injection (Figure 12). Analysis of the treated ventricles demonstrated that transplantation of SDF1 $\alpha$  over-expressing CSCs markedly increased local SDF1 $\alpha$  content in the infarct, infarct border and off-target regions 7 days after transplantation ( $51\pm 12$ ,  $7\pm 1$  and  $16\pm 5$  fold over myocardial section transplanted with non-transduced CSCs,  $p\leq 0.05$ ). These local increases in SDF1 $\alpha$  content were faithfully paralleled by a reduction in apoptosis as indicated by a marked disparity in the ratio of the apoptosis inhibitor Bcl-2 to the apoptosis promoter Bax (Figure 12C).

#### **4.6 Transplantation of SDF1 $\alpha$ over-expressing CSCs increases recruitment of blood derived cells**

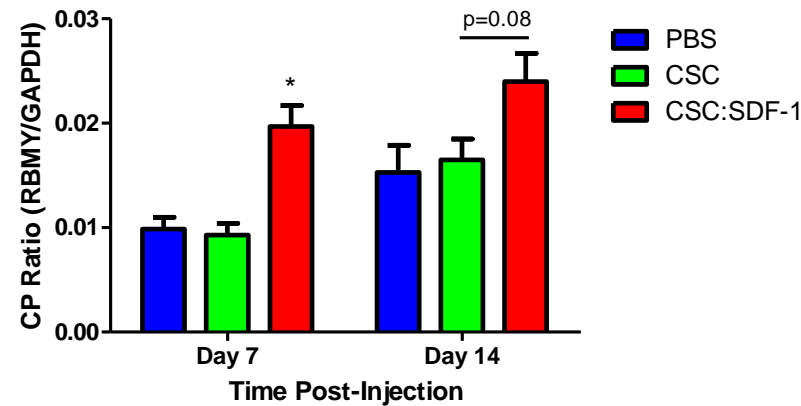
A sex mismatch bone marrow transplant model was used to quantify the recruitment of bone marrow cells to the injured myocardium after administration of PBS, non-transduced CSCs or SDF1 $\alpha$  transduced CSCs. Quantitative PCR for RBMY revealed that transplantation of SDF1 $\alpha$  transduced CSCs was associated with a  $2.3\pm 0.5$  fold increase in bone marrow cell recruitment over transplantation of non-transduced CSCs (Figure 13,  $p\leq 0.05$ ). Interestingly, the effect of transplanting SDF1 $\alpha$  transduced CSCs on bone marrow recruitment was negligible by 14 days after transplantation ( $1.5\pm 0.3$  fold change over non-transduced CSCs;  $p = 0.08$ ). Taken together, this data suggests that transplant of SDF1 $\alpha$  transduced CSCs recruits circulating blood cells largely during the first week after injection and this effect diminished as the number of engrafted CSCs diminishes.



**Figure 12.** Representative images of Western blots (A). SDF1α over-expressing CSC transplantation increased myocardial SDF1α (B) while increasing the Bcl-2/Bax ratio (C). Bars indicate standard error of the mean. Stars indicate  $p < 0.05$  vs. transplantation of non-transduced CSCs ( $n = 3$  mice/group). ND is not detected.



**B.**



**Figure 13.** Schematic of BM transplant and CSC therapy (A). SDF1 $\alpha$  over-expressing CSCs increase BM cell recruitment to the heart 7 days after transplantation (B). Bars indicate standard error of the mean. Star indicates  $p < 0.05$  vs. non-transduced CSC transplantation ( $n=3$  mice/group).

## 5.0 Discussion

The discovery of the self-renewing heart sparked the emergence of CSC therapy as a promising new means of managing HF. A wealth of evidence continues to develop in pre-clinical models that demonstrate consistent improvements in cardiac function with exciting data detailing the fundamental mechanisms.<sup>40, 101, 139</sup> Furthermore, clinical trials are underway and confirm the positive effects observed in animal trials.<sup>11, 54, 76, 157</sup> Because of the adult heart provides a poor host and low cell retention is observed, benefits seen after CSC therapy are leveraged through quick local delivery of paracrine factors that stimulate cardioprotective effects on reversibly damaged cardiomyocytes while enhancing the homing of resident CSCs and BM progenitors to the injured region to ultimately promote angiogenesis and cardiomyogenesis.<sup>142, 164, 165</sup> The paracrine profile of CSCs has been characterized and CSCs do not produce significant amounts of the cardioprotective cytokine SDF1 $\alpha$ ; thus rationalizing the current study to better understand the effects of SDF1 $\alpha$  over-expression on CSC-mediated cardiac repair.

Several groups have demonstrated exogenous SDF1 $\alpha$  can be delivered through recombinant protein administration, direct gene therapy (i.e. plasmid transfection) or cell-based gene therapies (Table 1). The novelty of the current study is that: 1) this is the first published effort to genetically engineer ex vivo proliferated CSCs 2) to target SDF1 $\alpha$  to explicitly engineer the paracrine profile of these cells and 3) to quantitatively characterize the magnitude of BM cell recruited to the heart by CSCs. The CSC product used is advantageous as it is a cell source that does not carry the risks accompanied by long-term culture which are unavoidable after antigenic sub-selection and sphere formation.

A gene-based cell therapy approach as opposed to a strictly gene-based or biomaterial approach was chosen as this approach allows for optimal tissue repair via sustained paracrine-mediated mechanisms, direct CSC differentiation into functional

myocardium, and cell-cell adhesion stimulated pathways while minimizing off-target delivery and SDF1 $\alpha$  loss due to cytokine degradation. Prolonged exposure to SDF1 $\alpha$  may in the end be oncogenic. By providing a cell-based source we allow for the eventual natural clearance of potentially harmful cells as opposed to a biomaterial which may remain intact. Moreover, a lentiviral vector was well suited for this study when an appropriate MOI is used because they have transduction efficiencies approaching 90%, integrate into the host genome and thus the inserted genes are propagated through subsequent progeny, do not affect cell replication and can deliver fairly large gene products.

In this thesis, we demonstrated that over-expression of SDF1 $\alpha$  by CSCs enhances endogenous stem cell recruitment, angiogenesis and expression of cardiac and endothelial lineage markers. Transplantation of SDF1 $\alpha$  over-expressing CSCs one week after MI provided the greatest improvement in LV function over a 3 week follow-up when compared to standard CSC therapy and vehicle control. Accordingly, the paracrine profile of first generation CSCs can be broadened with enriched SDF1 $\alpha$  expression to augment the efficacy of CSC therapy for HF.

### **5.1 Effects of SDF1 $\alpha$ over-expression on the CSC secretome and implications for BM cell recruitment**

Recent reports hint that exposure to lentiviral vectors may narrow the range of cytokines secreted by CSCs.<sup>80</sup> As a safeguard against this, only the CD90 sub-population was treated with a lentiviral vector. Interestingly, empty vector transduction blunted the CSC secretome in comparison to non-transduced CSCs; however this effect was not observed upon transduction of SDF1 $\alpha$ . This data demonstrates that the CD90 subpopulation is a critical sub-population within the CSC admixture as the negative effects of its selective transduction impaired the overall CSC secretome. These findings are in keeping with a recent report from Li et al. (2012) in which infarcted mice injected with

“complete” CDCs had enhanced cardiac function in comparison to mice injected with c-Kit purified CDCs.<sup>51</sup> As a result, only non-transduced CSCs and SDF1 $\alpha$  over-expressing CSCs were evaluated in the remaining experiments on the premise that the impaired CSCs treated with empty vector are inferior. Flow cytometry demonstrated that expression of CXCR4 within the c-Kit+/CD90-, c-Kit-/CD90+ and c-Kit+/CD90+ renders them responsive to SDF1 $\alpha$  signaling. As such, it is likely that the observed effects are due in part to paracrine “priming” of CSCs by SDF1 $\alpha$  from neighboring cells within the CSC admixture.

In addition to rescuing the paracrine signature of CSCs, SDF1 $\alpha$  over-expression improved the expression of six cytokines above levels observed in non-transduced CSCs. These included monocyte chemoattractant protein-1 (MCP-1), macrophage colony stimulating factor (M-CSF), matrix metalloproteinase 9 (MMP-9), tumor necrosis factor  $\beta$  (TNF- $\beta$ ), and transforming growth factor  $\beta$ 1 and  $\beta$ 2 (TGF- $\beta$ 2 and TGF- $\beta$ 3). These cytokines are implicated in BM cell mobilization (including HSC and macrophages) which play vital roles in the wound healing process<sup>166-168</sup> These data provide possible insight into potential downstream, SDF1 $\alpha$  initiated signaling pathways that promote BM cell chemotaxis. They also hint towards a molecular mechanism driving the increased chemotactic response of CACs in the transwell migration assays and in the male BM cells in the *in vivo* BM sex mismatch model. Although transplantation of CSCs that over-expressed SDF1 $\alpha$  led to enhanced BM cell recruitment one week later, this effect was not present two weeks later. The observed basal levels of BM cell recruitment in the absence of exogenous SDF1 $\alpha$  suggest that background levels of SDF1 $\alpha$  associated indirect cytokines may have a role in trafficking BM cells to the heart. For instance, a study by Morimoto et al (2006) used a transgenic mouse that over-expressed MCP-1 under the myosin heavy chain locus, thus all cardiomyocytes expressed increased levels of the protein.<sup>169</sup> Using an ischemia/reperfusion approach, the investigators showed that the transgenic mice were better protected than wildtype mice suggesting a possible role for the recruitment of monocytes in cardiac repair.

Thus overall, it is most likely that a basal repertoire of chemokines appear to mediate background levels of BM cell homing with increased homing as a consequence of exogenous stimulation shortly after transplantation. The outcome we observed suggests that accelerating the inflammatory response during the early stages after LAD ligation may help to reduce the scar burden observed at day 28.<sup>109</sup>

In the end, SDF1 $\alpha$  over-expression enhances the CSC paracrine profile in a way that primes CSC therapy to increase the mobilization and homing of endogenous stem and progenitor cells which is likely a driving force in the noted improvement to cardiac function.

## **5.2 Effects of SDF1 $\alpha$ over-expression on transplanted CSC fate and pro-survival signaling**

CSCs also possess a capacity for adopting a cardiomyocyte fate. SDF1 $\alpha$  transduction may have increased the tendency of CSCs to adopt either a cardiac or endothelial identity by enhancing the transcript levels of TNNT2 and vWF. This finding has traction in the literature as the SDF1 $\alpha$ :CXCR4 axis enhances adoption of a cardiomyogenic or vasculogenic fate. Unfortunately, this effect did not boost the number of engrafted and functional transplanted cardiomyocytes seen 3 weeks after post-transplantation. This finding undoubtedly reflects a combination of limited 1 hour engraftment (10-15% of the injected cells acutely retained) and underscores the limited capacity for SDF1 $\alpha$  to prevent ongoing cell loss after transplantation.

Within the same animal model, local SDF1 $\alpha$  over-expression was confirmed at one week after injection and demonstrated that downstream signaling pathways associated with SDF1 $\alpha$  were activated. The pro-survival signaling effect we observed was consistent with a previous report from Wang et al (2012) which showed that the administration of c-Kit+ purified CSCs over-expressing SDF1 $\alpha$  enhanced cardiac function specifically through CXCR4-PI3K signaling. The specificity of this claim was founded upon the loss of cardiac benefits upon co-administration of CSCs with AMD3100 or LY294002 (CXCR4 antagonist

and PI3K inhibitor, respectively). In that study, however, the level of pro-survival signaling was not quantified.<sup>101</sup> We achieved this by Western blotting for Bcl-2 (anti-apoptotic) and Bax (pro-apoptotic) which are downstream factors in the PI3K/AKT pathway.

Western blot showed an increase in the Bcl-2/Bax ratio indicating a reduction in apoptosis within the sites of injury within the heart. These data suggest that over-expression of SDF1 $\alpha$  by CSCs confers a pro-survival effect upon reversibly damaged myocardium. These analyses did not distinguish between exogenous SDF1 $\alpha$  provided by transplanted CSCs and endogenous SDF1 $\alpha$  content. Previous work has demonstrated that CACs produce significant quantities of SDF1 $\alpha$ .<sup>65</sup> As a result, the spike in SDF1 $\alpha$  content observed may be attributable to transplantation of CSCs that over-express SDF1 $\alpha$ , increased secretion by endogenous cardiomyocytes, and extra-cardiac SDF1 $\alpha$ -secreting cells that have successfully homed to the heart.

Taken together, transplantation CSCs that over-express SDF1 $\alpha$  enhances myocardial SDF1 $\alpha$  content which imparts a pro-survival effect on the damaged myocardium through CXCR4-PI3K signaling.

### **5.3 Study limitations and future directions**

Although this study demonstrated enhanced myocardial SDF1 $\alpha$  content intra-myocardial injection of CSCs that over-express SDF1 $\alpha$ , it is important to note that the relative source(s) of SDF1 $\alpha$  are not wholly defined. This is due in part to the highly conserved nature of SDF1 $\alpha$  which makes discrimination between human and murine origin not possible. One potential approach to this sourcing problem would be to treat CSCs with shSDF1 $\alpha$  shortly prior to injection to eliminate suppress (i.e. human) SDF1 $\alpha$ . Any myocardial SDF1 $\alpha$  detected would be from endogenous reserves either cardiomyocyte or recruited extra-cardiac cells. With this, the contribution of endogenous SDF1 $\alpha$  could be assessed directly, with the transplanted CSC-derived SDF1 $\alpha$  inferred from CSCs treated

with control short hairpin treatment in which SDF1 $\alpha$  would not be silenced. As such, the relative effects of host and transplanted SDF1 $\alpha$  could be compared and contrasted.

The relative activity of SDF1 $\alpha$  with each of its receptors has not been examined and insight into the potentially differential roles of activation of each may prove important. While the role of CXCR7 within the context of cardiac physiology is relatively unknown, recent evidence suggests that CXCR7 heterodimerizes with CXCR4 to elicit  $\beta$ -arrestin to enhance cell migration and cell survival upon exposure to SDF1 $\alpha$ .<sup>129, 131</sup> As such, a better understanding of the interplay between SDF1 $\alpha$ , CXCR4, CXCR7, and CXCR4/7 dimers might open up new avenues that could be exploited for enhanced CSC therapy. Studies that disrupt CXCR4 and/or CXCR7 by using small molecule inhibitors for instance, would represent one way of discerning between the effects of CXCR4, CXCR7, and CXCR4/7 stimulation.

The temporal misalignment in peak expression of SDF1 $\alpha$  and CXCR4 is well documented and efforts to extend SDF1 $\alpha$  peak expression to coincide with that of its receptor have proven useful (Table 1). Promoting earlier expression of CXCR4 to harmonize with SDF1 $\alpha$  peak expression represents an alternate way of optimizing the axis. Cencioni and colleagues (2013) used acidic preconditioning of mouse BM cells to increase their responsiveness to SDF1 $\alpha$ . In this study, preconditioned cells which exhibited increased CXCR4 expression were transplanted into ischemic hindlimb muscle and accelerated vasculogenesis and muscle fibre formation in comparison to non-conditioned cells thus implicating CXCR4 as a therapeutic target.<sup>155</sup> In addition, trials examining the effects of CXCR4 over-expression in cardiomyocytes and/or in endogenous CSCs would provide more insight into the therapeutic potential of CXCR4 within the context of heart failure. For instance, increasing CXCR4 expression on endogenous cardiomyocytes and CSCs may offer enhanced cardioprotection and recruitment of resident CSCs to the damaged area. Moreover, studies that emphasize earlier stimulation of CXCR4 expression

in combination with prolonged SDF1 $\alpha$  expression by complementary cell types may prove to impart the most pronounced benefits to cardiac function.

Before large animal and clinical translation of this CSC product begin, the significant limitation of generating a feasible number of autologous cells for human injection must be attended to. Mice in this study were treated with 10<sup>5</sup> CSCs, however a suitable intra-coronary injection into human patients would require a dose of 25 million cells as used in CADUCEUS and ALLSTAR. Should ALLSTAR prove allogeneic CSCs to be safe and effective, however, autologous CSC products may become irrelevant. A second hurdle to overcome includes the long term safety of this CSC therapy. Activation of pro-survival pathways such as AKT/PI3K and p38/MAPK are ideal targets for cardiomyocyte salvage, however, are also oncogenic. For this reason, investigations into the long-term oncogenicity of these cells are important. The need for such study is furthered by the use of genetic manipulation. Although gene-based therapies are widely in progress around the world,<sup>105, 157, 170</sup> the selection of a suitable vector is paramount. This study used a lentiviral approach which proved effective; however, retroviral vectors that integrate randomly into the genome have an increased propensity for oncogenicity. As such, the specific cell therapy in this study is not clinically relevant and other vectors such as adeno-associated virus and non-viral vectors such as mini-circle DNA (i.e. only the plasmid and its promoter) will most likely be applied in later studies. We profiled the expression profile of SDF1 $\alpha$  receptors in a mouse model and based the timing of cell delivery upon that, however, the SDF1 $\alpha$  responsive of the heart may vary in humans and as such, more studies that build upon the limited knowledge of the human profile within the context of an MI is needed before establishing clinical protocols. Lastly, this study focused upon treating HF in an acute MI model. The majority of HF patients are unable to receive therapy during this phase and as such, inquiry into the efficacy of this cell product in a model of chronic ischemia is warranted.

In addition to addressing the limitations outlined above, a number of techniques can be used to acquire an enhanced SDF1 $\alpha$ -based cell therapy. For instance, efforts to prevent the proteolytic degradation of SDF1 $\alpha$  within the heart have proven beneficial. In one study, the administration of parathyroid hormone, a potent DPP-IV/CD26 inhibitor, led to enhanced myocardial SDF1 $\alpha$  content and increased recruitment of CXCR4+ BM cells to the ischemic heart.<sup>154</sup> The specificity of this effect through SDF1 $\alpha$  was confirmed with co-administration of AMD3100. Alternatively, the delivery of recombinant SDF1 $\alpha$  constructs to safeguard against DPP-IV/CD26 degradation and the co-administration of G-CSF to enhance BM cell mobilization have also been effective in experimental HF models.<sup>137, 146, 150, 171</sup> Those approaches all emphasize manipulation of SDF1 $\alpha$  levels, however, enhancing SDF1 $\alpha$  receptor expression/activation is equally justified.<sup>89, 155</sup> Thus possibilities for manipulation of SDF1 $\alpha$  and its receptor activation are abundant with many showing significant promise.

## **6.0 Conclusion**

Human CSCs genetically engineered to over-express SDF1 $\alpha$  impart an enhanced benefit to cardiac function when delivered one week after permanent coronary ligation in an immunodeficient mouse model in spite of low cell retention three weeks later. Lentiviral transduction to over-express SDF1 $\alpha$  broadened the CSC paracrine repertoire with corresponding effects on cell recruitment to the heart. Increased myocardial SDF1 $\alpha$  content was associated with a marked reduction in apoptosis. Together, the improvement in BM cell recruitment and pro-survival effects observed on the myocardium combined to enhance LV function.

## **7.0 Reference List**

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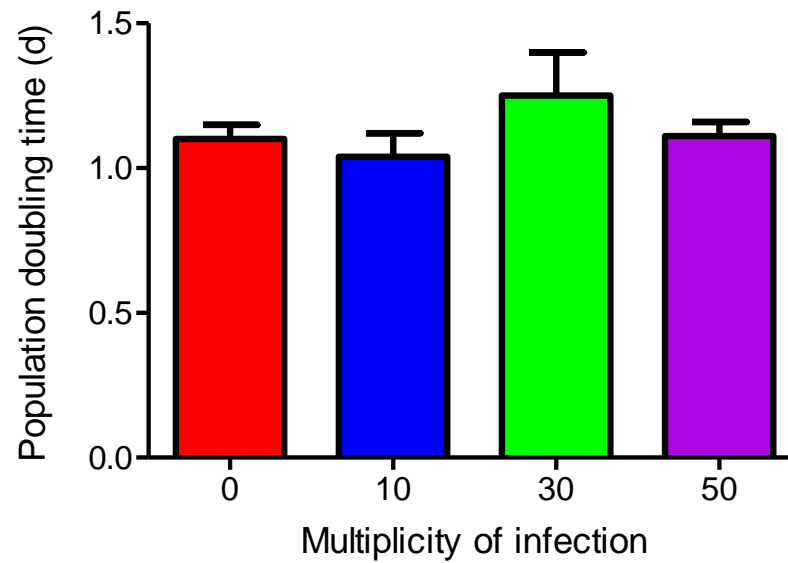
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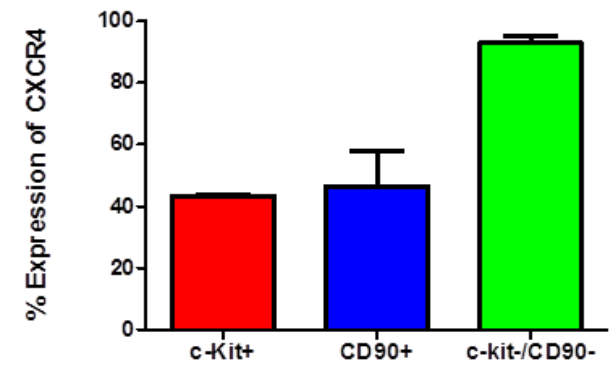
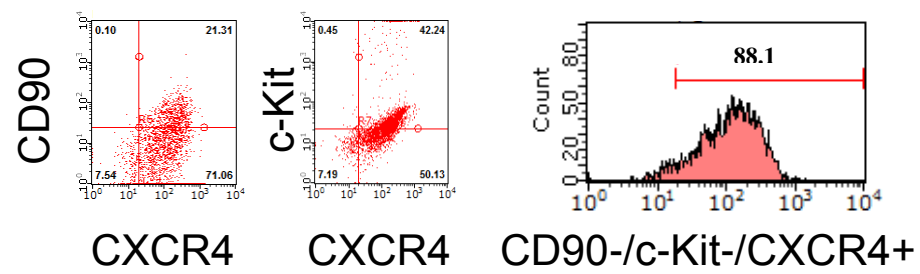
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## 8.0 Appendices



**Appendix 1.** Lentiviral transduction of CSCs does not impair CSC proliferation in vitro. Doubling time was calculated following CCK-8 assay of CSCs cultured over 48 hours. No significant difference between groups was achieved. Bars indicate standard error of the mean (n=3).



**Appendix 2.** Flow cytometry indicates that CXCR4 is expressed in ex vivo proliferated CSCs (n=3).