Serum-Free Xenogen-Free Culture Conditions Support Human Explant-Derived Cardiac Stem Cell Growth.

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Abstract

Autologous explant-derived cardiac stem cell (EDC) therapies are a promising therapy for ischemic cardiomyopathy, but straightforward clinical translation is limited by traditional culture conditions which are often supplemented with ill-defined and xenobiotic components such as fetal bovine serum. Therefore, we investigated the influence of a commercially sourced serum-free (SF) xenogen-free medium on human EDC yield, phenotype, *in vitro* measures of EDC performance, and post-infarct cardiac repair using an immunodeficient mouse model of acute myocardial infarction. Despite reduced production of several pro-cardiogenic cytokines, SF EDCs promoted similar vessel formation, circulating stem cell recruitment, and cardiogenic differentiation as compared to standard cultures. Transplant of SF EDCs into immunodeficient mice 1 week after myocardial infarction boosted post-ischemic repair beyond that of standard EDCs by enhancing viable myocardium within the infarct. These findings demonstrate that serum-free culture methods provide a superior cardiac-derived cell product with ready clinical translatability.

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List of Abbreviations

αSMA	Alpha smooth muscle actin
ABCG2	ATP-binding cassette sub-family G member 2
ACEI	Angiotensin-converting enzyme inhibitor
ARB	Angiotensin receptor blocker
BMI	Body mass index
CAC	Circulating angiogenic cell
CCS	Canadian Cardiovascular Society
CDC	Cardiosphere-derived cell
CD90	Cluster of differentiation 90
CEM	Cardiac explant medium
c-Kit	Tyrosine receptor-kinase Kit
CSC	Cardiac stem cell
CSp	Cardiosphere
cTnT	Cardiac troponin T
DAPI	4’,6-diamidino-2-phenylindole
ECM	Extracellular matrix
EDC	Explant-derived cardiac stem cell
EF	Ejection fraction
EGF	Epidermal growth factor
EPC	Endothelial progenitor cell
exSF	Expanded serum-free
FAC	Fractional area change
FBS	Fetal bovine serum
GMP	Good manufacturing practices
HF	Heart failure
HGF	Hepatocyte growth factor
HNA	Human nuclear antigen

HUVEC	Human umbilical vein endothelial cell
IL-6	Interleukin 6
LAD	Left anterior descending coronary artery
LV	Left ventricular
LVEF	Left ventricular ejection fraction
MI	Myocardial infarction
MSC	Mesenchymal stem cell
NOD	Non-obese diabetic
PBS	Phosphate buffered saline
qPCR	Quantitative polymerase chain reaction
SCID	Severe combined immunodeficient
SCF	Stem cell factor
SDF-1	Stromal cell-derived factor 1
SF	Serum-free
VEGF	Vascular endothelial growth factor
vWF	von Willebrand's factor
XF	Xenogen-free

1. INTRODUCTION

1.1 Heart failure in Canada

Owing to modern mechanical and pharmaceutical advances in the realm of cardiac care, the acute mortality attributable to myocardial infarction has declined dramatically in recent years.[1, 2] Improved patient survival following ischemic injury has forced health care systems worldwide to accommodate a growing number of patients living with the debilitating symptoms of chronic heart failure (HF). Current estimates suggest that 600,000 Canadians are living with heart failure with 50,000 new diagnoses being made each year, posing an annual cost of almost 3 billion to the Canadian health care system.[3] Despite advances in its medical treatment, the annual mortality associated with HF has only decreased from 27 to 25% over the last decade.[4] This observation reflects the ability of current device and pharmaceutical therapies to slow rather than reverse disease progression, and has rationalized a focus on developing therapies to repair and revascularize damaged hearts.

1.2 Cell-based therapy for heart failure

Over the last two decades, stem cell therapy has emerged as a promising treatment option for heart failure patients. Canonical teaching dictates that stem cells are transplanted into the damaged heart tissue, engraft, and differentiate extensively within the recipient organ to create working myocardium and improve overall cardiac function. However, there is strong evidence that transplanted cells also act by an indirect mechanism, providing repair through the release of immunomodulatory, anti-apoptotic, and pro-angiogenic paracrine factors, and/or the recruitment of host progenitor cells.[5-7] A number of candidate cell types have been explored for their ability to provide cardiac repair, with varying degrees of success. Among these are hematological cell products (bone marrow cells (BMCs), mesenchymal stem cells (MSCs) and endothelial

progenitor cells (EPCs)), pluripotent stem cells (embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs)), and cardiac-derived stem cells (CSCs). Pre-clinical and clinical studies using stem/progenitor cells have yielded promising results and provide valuable insight as the search for an ideal cellular candidate for the treatment of heart failure continues.

1.2.1 Hematological stem cells

Initial interest in bone marrow cells (BMCs) followed from studies demonstrating self-renewal and a capacity for cardiac transdifferentiation in response to inductive culture conditions.[8] However, while pre-clinical studies using these cells attributed vast improvements in cardiac function to BMC transdifferentiation,[9] subsequent studies using lineage tracing were unable to demonstrate significant BMC engraftment despite showing similar functional improvements.[10] Similar levels of engraftment have been demonstrated in studies using MSCs and other hematological stem cell products, which collectively report long-term retention at less than 3%.[11-13] These studies have given rise to the paracrine hypothesis that the salutary benefits observed with hematological stem cell therapy are owed to the production of cardiogenic cytokines or exosomes that promote endogenous repair and salvage, rather straightforward tissue replacement.[5-7] Despite preclinical data[14-16] demonstrating modest cardiac repair by hematological stem cell products, clinical trials[17-20] using bone barrow derived stem cells have shown little to no effect of these therapies on overall cardiac function.[21] Nonetheless, strategies have been developed to enhance therapeutic products through cell selection and/or modification of culture protocols[22], motivating further clinical investigations. The ongoing phase III BAMI "Bone marrow-derived mononuclear cells on all cause mortality in Acute Myocardial Infarction" trial will enroll 3000 patients in Europe to investigate the hypothesis that BMC therapy improves patient survival 2 years post MI.[23]

1.2.2 Pluripotent stem cells

Given their proven capacity to form all three germ layers and differentiate into all major lineages, it is unsurprising that pluripotent stem cells have garnered significant attention in the field of regenerative medicine. However, this seemingly unlimited potential also poses concerns over the risk of non-cardiac transformation. Given these concerns, much of the work done using ESCs and iPSCs has involved pre-differentiation toward more cardiac-restricted lineages.

Embryonic stem cells (ESCs) are cell lines derived from the inner mass of the blastocyst 4-5 days after fertilization,[24] and can be induced to form beating cardiomyocytes (ESC-CMs) *in vitro*. Pre-clinical studies using ESC-CMs demonstrated improvements in contractile function electrical conduction following transplantation, attributing these improvements to robust engraftment and electrical coupling with host myocardium.[25-29] However, subsequent large animal studies demonstrated ventricular arrhythmias in all animals receiving ESC-CM therapy.[30] While the cause of these findings is unknown, one hypotheses points to larger graft sizes and functional immaturity of engrafted ESC-CMs. As primate hearts received roughly 10-fold larger numbers of ESCs than were used in small animal studies, greater numbers of immature ESC-CM could engraft and differentiate, forming re-entrant circuitry leading to arrhythmia.[31, 32] Others have suggested that injected cells may have been contaminated with ESC-derived pacemaker cells, resulting in early and late afterdepolarizations.[31-33] Nonetheless, these pro-arrhythmic findings have shifted the focus away from ESC-CM therapies.

Akin to ESCs, induced pluripotent stem cells (iPSCs) represent a pluripotent stem cell capable of forming all three germ layers, with similar morphology and surface antigen expression. iPSCs are derived from somatic cells by reprogramming with microRNAs, transcription factors, or small molecules.[34] In addition to representing an autologous candidate cell therapy, the use

of iPSCs obviates some of the ethical concerns surrounding the use of embryonic stem cells. Numerous preclinical animals models have demonstrated safety and therapeutic efficacy of iPSC transplantation,[35-37] with evidence of *in vitro* cardiac differentiation, inhibition of apoptosis, and significant functional benefits.[38] Despite the seemingly unlimited differentiation potential of pluripotent stem cells, iPSCs exhibit modest long-term engraftment,[39] suggesting that the benefits observed are largely paracrine-mediated. This finding, coupled with the pro-arrhythmic risks seen with pluripotent stem cell engraftment,[30-33] has necessitated efforts to identify a safer, more cardiogenic cell therapy.

1.3 Cardiac-derived stem cells (CSCs)

Until recently, prevailing thought saw the adult heart as a terminally-differentiated organ, with a set number of cardiomyocytes slowly declining through an organism's lifespan. It was believed that any adaptation to injury occurred through cellular hypertrophy and/or death, as cardiomyocytes lacked the capacity for self-renewal. Several studies have since demonstrated that adult cardiomyocytes undergo lifelong turnover at a rate of roughly 1% per year.[40, 41] In one such study, Bergmann *et al.* used carbon dating to identify C-14 integration into the genomic DNA of individuals affected by Cold War nuclear testing, estimating a lifetime cardiomyocyte turnover of nearly 50%.[41] Further calculations suggested that this life-long renewal (0.5-1 % per year) declines with age. Others have confirmed these findings using incorporation of the nucleoside analog 5-bromo-2'-deoxyuridine (BrdU) and the cell-cycle marker Ki67 to show cardiomyocyte renewal in healthy and injured hearts, motivating efforts to identify the source of newly formed myocytes in the adult heart.

In 2003, Beltrami *et al.* identified a resident population of cardiac stem cells expressing stem cell surface antigens (e.g. c-Kit, Sca-1) and transcription factors linked to early cardiomyocyte development (e.g. Nkx2.5) and differentiation (MEF2C).[42, 43] Further characterization of resident CSCs demonstrated cells within the atrial appendage positive for the receptor tyrosine kinase Kit (c-Kit) and negative for hematological surface markers such as CD34, CD45, and CD133.[44] In response to acute ischemic injury, resident pools of CSCs are activated and migrate from cardiac niches to the site of injury.[45, 46] However, while resident CSCs possess a capacity for cardiac repair, evidence suggests that this capacity is limited. In 2007, Hsieh *et al.* used a transgenic mouse model with cardiomyocyte-specific expression of GFP to assess the role of resident CSCs in cardiomyocyte renewal, observing little change in GFP expression in healthy hearts over 1 year with greater numbers of GFP$^+$ cells in hearts following ischemic injury. These results suggested that endogenous CSCs undergo minimal cardiomyocyte differentiation in response to cardiac injury, providing modest repair while unable to attenuate ventricular remodeling.[45-47] While suggestive of a minimal role of endogenous CSCs in cardiac repair, these findings motivated efforts to harness and enhance the regenerative potential of CSCs through *ex vivo* culture.

In 2004, Messina *et al.* demonstrated isolation and expansion of cardiac stem cells by culturing digested cardiac tissue sourced from human and murine hearts on fibronectin coated culture dishes.[48] After one week in culture, a population of phase-bright explant-derived cardiac stem cells (EDCs) migrates from plated explant fragments. To elucidate the mechanisms underlying this spontaneous migration, Zakharova and colleagues investigated the expression of molecular markers associated with epithelial-to-mesenchymal transition (EMT) and demonstrated a Notch-1 dependent role of EMT in the formation of c-Kit$^+$ and c-Kit$^-$ cells.[49] Interestingly, EMT has also been shown to be induced in epicardial cells as a physiological response to injury, permitting

activated cells to migrate to a site of myocardial injury.[50, 51] Through re-entry of the cell cycle, emerging explant derived cells proliferate *in vitro.* Following enzymatic lifting, EDCs can be induced to adopt a mature cardiomyocyte phenotype, demonstrating the key properties of stem cells: self-renewal, multipotency, and clonogenicity.[42, 43] The proven capacity to differentiate into all three major cardiac lineages (myocyte, smooth muscle, and endothelial lineages) without evidence for non-cardiac transformation (eg: teratomas) has made CSCs an increasingly promising cellular candidate for cardiac repair.[43]

Several groups have independently developed methods for the isolation and expansion of cardiac-derived stem cell products. Among these are cardiac explant-derived stem cells (EDCs), antigenically-selected c-Kit$^+$ or Sca-1$^+$ cells, and cardiosphere-derived cells (CDCs). In principle, CSC therapy involves obtaining a small myocardial biopsy from the patient using a catheter-guided bioptome. The explant tissue is grown in culture for a number of weeks to allow cellular outgrowth to migrate and proliferate. The enhanced cell product can then be delivered either by intracoronary or intramyocardial route back into the patient's heart at the site of injury to mediate potent repair and functional recovery of the damaged myocardium.

1.3.1 Explant-derived cardiac stem cells (EDCs)

The spontaneous outgrowth obtained from explant tissue is not homogeneous. Rather, the CSC outgrowth contains subpopulations of cells expressing cardiac progenitor (c-Kit+), endothelial progenitor (CD31+, CD34+), and mesenchymal progenitor (CD90+) related surface antigens.[52, 53] While a number of rationales have been put forth for antigenic selection of candidate cells (e.g c-Kit+, Sca-1+), the focus in the Davis lab has been on the aggregate cell product. It has been demonstrated that complementary subpopulations work synergistically to

promote angiogenesis and cardiogenesis both directly through engraftment as well as indirectly through the release of modulatory paracrine factors.[43, 52, 54-57]

Amongst the cell candidates sourced from adult heart tissue, explant-derived cells (EDCs) have become the standard initial cells grown from plated heart tissue for further antigenic selection (e.g., c-Kit^{+} or Sca-1^{+} cells) or sphere culture (i.e., cardiosphere-derived cells (CDCs)).[58-62] Phase I clinical trials using EDC-sourced c-Kit+ cells or CDCs have shown these cells to be safe with efficacy to be confirmed in Phase II trials.[59-61] EDCs provide a complimentary admixture of progenitor cells that promote myocardial repair through indirect paracrine effects and differentiation into myocardium.[63-67] Unfortunately however, direct clinical application of EDCs has historically been limited by a constant return to the proportion of biopsy tissue plated, necessitating a means of cell expansion either by antigenic selection followed by *ex vivo* proliferation (*e.g.* c-Kit^{+} or Sca-1^{+} cells) or culture-guided expansion (*e.g.* CDCs).

1.3.2 Antigenically selected (c-Kit^{+} or Sca-1^{+}) cells

In contrast to the approach of utilizing the aggregate heterogeneous EDC population, one approach has been to antigenically select for a c-Kit^{+} cardiac progenitor subpopulation.[52] Once isolated from primary explant outgrowth, c-Kit^{+} cells are placed back in culture and expanded to obtain a therapeutically-relevant dose. While lineage tracing studies show very limited formation of cardiomyocytes from resident adult c-Kit^{+} cells,[68, 69] *ex vivo* proliferated c-Kit^{+} cells have a demonstrated capacity for cardiomyocyte, vascular smooth muscle, and endothelial differentiation, and have been shown to differentiate and provide functional improvements following delivery to ischemic myocardium.[43, 52] Notably, in the randomized open-label “cardiac Stem Cells In Patients with Ischaemic cardiOmypathy” (SCIPIO) trial, safety and efficacy of intracoronary delivery were established, showing a 12.3% improvement in ejection fraction that

persisted up to two years post-treatment.[58, 59] However, concerns have since been raised surrounding the integrity of the research done on this cell product and its characterization.[70] Ongoing investigations have resulted in the retractions of two papers, leaving several more in question. Despite these troubling findings, the study was carried out in such a way that cells were shipped to a separate institution for delivery and patient follow-up, preserving the integrity of patient outcome data. While still under investigation, there are currently no plans to initiate a phase 2 trial using c-Kit^{+} cells. Still, the cardiogenic potential and operative mechanisms underlying *ex vivo* proliferated c-Kit^{+} cells remain largely undefined, and more preclinical work is necessary before these cells can undergo further clinical testing.

A similar technique has been used by other groups to culture Sca-1^{+} cells isolated from the digestion of whole mouse hearts.[71, 72] Isolated Sca-1^{+} cells are positive for early cardiac transcription factors (GATA4, MEF2C, and Tef-1), and negative for lineage markers.[42] Further, transplantation of Sca-1^{+} cells into a mouse model of ischemia significantly improved left ventricular ejection fraction compared with control, with evidence of cardiomyocyte, smooth muscle, and endothelial differentiation.[72] However, while antigenic selection provides a means of obtaining a homogenous CSC population, clinical translation requires extended time in culture to reach therapeutically relevant doses which creates concerns surrounding phenotypic drift and the potential for malignant transformation.[73] For this reason, several groups have turned towards refining culture techniques and developing culture guided protocols to reach therapeutic doses and facilitate clinical translation.[74]

1.3.3 Cardiosphere-derived cells (CDCs)

A third and distinct cardiac stem cell product is obtained from primary explant cultures by plating EDCs on poly-D-lysine coated dishes to form three-dimensional aggregates termed

cardiospheres. These cardiospheres are then returned to adherent culture and expanded to obtain cardiosphere-derived cells (CDCs). By enabling EDCs to maintain greater numbers of cell-cell interactions, sphering recapitulates a niche-like environment that enhances cell stemness and increases regenerative potency.[48, 55, 75, 76] Through stimulation of the extracellular signal-regulated kinase (ERK) and VEGF pathways, sphering has also been shown to support enhanced cell survival and provide superior functional improvements in small animal studies comparing primary EDCs and CDCs.[75, 77] Importantly, the cardiosphering method also provides a means of expanding cell numbers to clinically relevant doses, without the risks of decreased potency, phenotypic drift, or malignant transformation associated with prolonged time in culture.[55, 73, 76-78]

Like EDCs, CDCs represent a heterogeneous population of cells with subpopulations expressing mesenchymal and stem cell related antigens.[53, 55] In one preclinical murine model, CDC therapy outperformed BMCs and MSCs with respect to paracrine secretion, angiogenesis, cardiomyocyte differentiation and improvements in LVEF when delivered into a setting of acute ischemic injury.[79] More importantly, in the phase 1 randomized “CArdiosphere-Derived aUtologous stem CElls to reverse ventricUlar dySfunction” (CADUCEUS) trial, safety and efficacy of intracoronary infusion of CDCs were established, demonstrating a 12.3% reduction in infarct size 12 months after treatment.[61, 80] To date, CDCs are the only candidate cell-based therapy shown to differentiate and adopt a mature cardiomyocyte phenotype and to electrically couple with surrounding myocardium *in vivo*.[53] Currently ongoing clinical trials employing CDC therapy include the phase I/II HOPE-Duchenne trial (evaluating CDC therapy in males with cardiomyopathy secondary to Duchenne muscular dystrophy), the phase I DYNAMIC trial (evaluating CDC therapy in the setting of advanced HF), and the phase I/II ALLSTAR trial (evaluating the safety and efficacy of CDC therapy after myocardial infarction).[81-83] While patient data from the HOPE-Duchenne trial are pending, six month data from the DYNAMIC trial

showed no incidence of adverse events following triple-vessel infusion of 37.5 to 75 million CDCs.[84] Phase I of the ALLSTAR trial has yielded similarly promising results with no treatment-related adverse events reported in the 14 patients treated.[85] Phase II, estimated to enroll 134 patients, will report relative improvement in cardiac MRI-assessed infarct size at 6 and 12 months post-treatment, and is set for completion in 2021.[83]

1.4 Future directions for cardiac stem cell therapy

Despite the promising results of preclinical and clinical studies, CSC therapy has yet to realize its full therapeutic potential due to limitations in cell yield and modest efficacy of CSCs cultured from patients with co-morbidities.[86, 87] Additional challenges surround boosting the modest long-term engraftment of CSCs, and achieving clinical compliance, as traditional culture media are typically supplemented with ill-defined or xenobiotic components such as fetal bovine serum.[88] Current and future research initiatives focusing on overcoming these limitations and enhancing CSC therapy will be necessary to develop next generation therapies with direct clinical applicability.

1.4.1 Effect of patient co-morbidities on CSC efficacy

One of the most significant hurdles confronting autologous CSC therapy surrounds the effects of patient co-morbidities on cell yield and potency. To date, studies have implicated advanced patient age, male gender, diabetes, and pulmonary arterial hypertension as factors contributing to reduced CSC yields and efficacy.[55, 65, 67, 76, 89-91] These findings are unsurprising, given that similar correlations have been established for other tissue-specific stem cells,[92] but these correlations have only been established for CSCs in recent years. In 2014, Molgat *et al.* demonstrated a reduced cardiac repair, angiogenesis, and chemotactic capacity in CSCs

cultured from diabetic patients (hemoglobin A1c 10±2%). Interestingly, attenuation of this hyperglycemia-induced CSC dysfunction could be achieved using by forced overexpression of glyoxalase 1, an enzyme responsible for the detoxification of reactive dicarbonyls.[65] This link is perhaps unsurprising, given that diabetes is a vascular disease and an established predictor of future cardiac events.[93] However, hyperglycemia alone is not a sufficient predictor of reduced CSC function. A second study by Mayfield and colleagues used a Long Term Stratification (LTS) for survivors of acute coronary syndromes[94] score system to collectively assess the impact of multiple cardiovascular risk factors on CSC function.[91] Interestingly, CSCs cultured from patients with greater numbers of cardiovascular risk factors exhibited decreased production of exosomes and the pro-healing cytokine SDF-1α. These differences translated to decreased regenerative performance, as demonstrated using *in vitro* measures of angiogenesis and chemotaxis, as well as decreased cardiac repair in a mouse model of acute ischemic injury.[91] As autologous CSCs are cultured directly from patient tissue biopsies from the same individuals who will require the therapy in the future, more mechanistic insight will be needed in order understand and mitigate these effects and improve CSC therapies.

1.4.2 Allogeneic CSC therapy

One potential solution to the negative effects of donor co-morbidities has been to explore the avenue of allogeneic therapy. In addition to reducing inter-patient variability, an allogeneic therapy would alleviate time constraints (CSCs require 3-5 weeks of culture),[55] reduce manufacturing expense and risk and facilitate quality control. The development of an allogeneic paradigm would allow for a uniform, “off-the-shelf” cell product that could be sourced from healthy donors and administered to patients sooner following ischemic injury. In addition, whole hearts could be obtained from an organ procurement organization, allowing cell manufacturing to be scaled to provide several therapeutic doses.

The first experimental evidence to support allogeneic CSC therapy came from *in vitro* studies demonstrating the immune-privileged nature of CDCs. When cocultured with lymphocytes, allogeneic CDCs did not elicit lymphocyte proliferation or produce inflammatory cytokines.[95] In addition, rats receiving allogeneic CDCs without immunosuppression showed no evidence of systemic immunogenicity and saw equivalent functional improvements as those receiving syngeneic CDCs, 6 months following treatment.[95]

Safety and efficacy of allogeneic CDC therapy were subsequently demonstrated in a porcine model of MI, where mild mononuclear infiltration but no systemic immunity were observed, and MRI revealed improved global cardiac function and decreased myocardial scar size 2 months following treatment.[60] This study served as a pre-clinical validation for the "ALLogeneic heart STem cells to Achieve myocardial Regeneration" (ALLSTAR) and "Dilated cardiomYopathy iNtervention with Allogeneic MyocardIally-regenerative Cells" (DYNAMIC) trials, discussed above, which are currently underway and have shown promising early results.[82, 83]

1.4.3 Modification of CSCs to enhance therapeutic repair

The limited long-term engraftment and functional improvements seen with first generation CSC therapies highlight an opportunity for enhancing the regenerative potential of existing cell products. To this end, several studies have explored direct genetic modification as a means of enhancing stem cells.[96, 97] While paracrine engineering approaches have been met with a considerable success in non-cardiac derived cell therapies,[98-101] limited work has been done using these approaches to enhance CSC products.

In 2011, Bonios *et al.* used a lentivirus to constitutively express hypoxia-inducible factor-1α (HIF-1α), a factor previously shown to promote stem cell angiogenesis and engraftment, in CDCs.[102] Despite displaying similar engraftment (~2% at 4 weeks post-infarction), HIF-1α CDCs did not improve LV ejection fraction as normal CDCs, and produced higher levels of endothelin-1. Further, constitutive HIF-1α expression in CDCs was found to negate the beneficial effects of VEGF and IGF-1.[102] These findings highlight the possibility of undesirable pleiotropic effects in paracrine engineering, and suggest that paracrine balance is critical to maintaining cellular regenerative potency.

More successful paracrine engineering outcomes have been achieved through lentiviral overexpression of insulin-like growth factor 1 (IGF-1) and stromal cell derived factor-1α (SDF-1α) to improve transplanted EDC survival.[63, 64] Using lentivirus mediated gene transfer of SDF-1α to $CD90^+$ EDCs, Tilokee *et al.* demonstrated an increase in blood vessel formation (2.3±0.1 fold increase), chemotaxis (2.9±0.1 fold increase), and cardiogenic differentiation compared with nontransduced EDCs.[63] Transplant of SDF-1α overexpressing EDCs into an immunodeficient mouse model of ischemic injury also resulted in superior functional improvements (LVEF of 39±2 vs 33±1 %) and recruitment of endogenous progenitor cells compared with nontransduced EDCs.

In a similar study, Jackson and colleagues demonstrated that overexpression of IGF-1 in $CD90^+$ EDCs results in enhanced proliferation and increased expression of pro-survival transcripts under hypoxic, low serum conditions.[64] Transplantation of IGF-1 overexpressing EDCs into a murine model of myocardial infarction enhanced engraftment (9.1±3.6 fold increase in cell retention at 21 days) and functional improvements (LVEF of 41±2 vs. 37±1 %, $p \leq 0.01$)

compared with control mice. These improvements were attributed to the anti-apoptotic action of IGF-1, evidenced by reduced expression of the pro-apoptotic factors Bax and p53, and increased expression of the anti-apoptotic transcript Bcl-2 within the infarct border zone of mice injected with IGF-1 overexpressing EDCs. These findings highlight the importance of cell persistence in achieving myocardial repair and validate a targeted means of enhancing cardiac stem cell therapies.

Some of the most significant improvements in CSC engraftment have been through the use of biomaterials. Biomaterials offer biocompatible support for transplanted cells by providing signals for cell attachment, proliferation, and migration, allowing cells to survive and persist longer following transplantation.[103] Successful biomaterials approaches to date have included the use of a fibrin glue to seal the site of cell injection, magnetic targeting to maintain transplanted cells at the site of injection for longer, and three-dimensional scaffolds to anchor and provide attachment signals to transplanted cells.[87, 104-108] Of note, one such biomaterials study investigated EDC encapsulation in matrix-enriched hydrogel capsules and demonstrated enhanced angiogenesis and chemotaxis, attributing these effects to the enhanced cell survival through attachment signals provided by the capsules.[109] Injection of encapsulated EDCs into an immunodeficient mouse model 1 week following infarction improved acute retention (3±1 fold vs. non-encapsulated EDCs) and long-term retention (10±1 vs. 4±1 % for non-encapsulated EDCs at 3 weeks), and provided a 10±2% greater increase in LVEF three weeks after transplant.[109] These significant improvements in cell engraftment and repair have further bolstered the potential regenerative capacity of CSC therapy.

1.5 Overcoming barriers to clinical translation

Despite promising strategies to enhance CSC therapies, barriers surrounding clinical compliance remain, as traditional culture media are supplemented with ill-defined or xenobiotic components such as fetal bovine serum.[88] Current research initiatives are focused upon overcoming these limitations and enhancing CSC culture to develop next generation therapies which are ready for direct clinical translation.

1.5.1 Compliance with current Good Manufacturing Practices (cGMP)

Clinical translation of a cell-based therapy, autologous or allogeneic, requires protocol optimization and compliance with established regulatory standards. Cells must be manufactured, transported, and clinically delivered in a way that maintains product safety, identity, and efficacy. Current Good Manufacturing Practices (cGMP) guidelines provide a set of quality and safety standards which cover all aspects of the manufacturing process, and cGMP compliance requires extensive testing and validation of all proposed protocols and reagents.

1.5.2 Fetal bovine serum in culture media

Fetal bovine serum (FBS) is the most widely used supplement in cell culture media, providing high levels of hormones and growth factors to support cell proliferation and expansion *in vitro*.[110] However, the highly-variable and ill-defined nature of FBS make it difficult to characterize and, given its xenogeneic origin, pose difficulties for clinical translation.

The issue of lot-to-lot variability in levels of chemical, protein, and endocrine components in fetal bovine serum formulations within and between suppliers has been known since 1975.[111]

Varying levels of immunoglobulins, transcription factors, and growth factors (platelet-derived growth factor, epidermal growth factor, insulin-like growth factor, fibroblast growth factor, and nerve growth factor) have been shown to alter cellular function, cause significant changes in phenotype, and lead to unexpected growth characteristics or cytotoxicity.[112-114] Further variability arises from the presence of unidentified components within FBS.[115, 116] As tests to characterize individual formulations are underdeveloped and costly given the high volumes of serum used, many laboratories perform their own screening to select and secure entire lots of FBS to reduce variability in their experiments.[110] Still, these lots eventually run out, and the reproducibility of results from FBS-dependent cultures by other laboratories remains in question.

Other concerns surrounding the use of FBS in cell therapeutics surround the risks of immunogenicity and transmission of viral and prion agents.[117-119] One study reported anaphylactic immune reactions in patients following the infusion of lymphocytes cultured in an FBS-containing medium.[120] While no such effects have been reported in patients receiving hMSCs derived from FBS containing culture, the potential of humoral immunogenicity remains in question. Given the highly variable and poorly-defined composition of fetal bovine serum, efforts to limit or replace the use of FBS is a critical step in the translation of candidate cell therapies to the clinic.

1.5.4 Efforts to eliminate serum from cell therapeutics

To date, a number of efforts to replace fetal bovine serum have used alternatives such human sourced serum or platelet lysate. Autologous human serum has proven an effective and even superior alternative supplement in some MSC and ESC cultures, preserving differentiation capacity, increasing proliferation, and improving cellular migration.[121-123] However, results using commercial or allogeneic sera have been more contradictory. Several studies examining MSCs

cultured in allogeneic human serum have found that MSCs exhibit proliferative senescence and growth arrest.[121, 124-126] In one such study, human MSCs cultured in allogeneic serum-supplemented media exhibited low attachment and colony formation, failing to proliferate beyond passage 1, while cultures supplemented with autologous or xenogeneic serum displayed normal growth characteristics.[126] These differences were attributed to unidentified allogeneic proteins exhibiting growth inhibition. A second group demonstrated poor cell yields, morphological deterioration, and extensive cell death in human MSCs cultured with commercial human serum,[127] and found that rat MSC culture in heterologous serum required growth factor supplementation to achieve normal growth.[128] These findings highlight the variability inherent in using serum-sourced supplements, and demonstrate the challenges of finding suitable animal serum replacement strategies for stem cell culture.

With respect to CSCs, one study examined the possibility of replacing FBS in cardiosphere and CDC cultures.[129] This study, published by Chimenti *et al.* in 2014, investigated alternative serum and supplement formulations, culturing CDCs using autologous human serum, commercially available AB human serum, and gamma-irradiated FBS (giFBS). Interestingly, cardiac explants cultured in 20% autologous serum grew faster during the initial 3 weeks in culture, but these EDCs were unable to form cardiospheres in secondary culture and stopped growing. Similarly, explants cultured in commercial human sera displayed faster outgrowth initially, but after one to two weeks took on a flat, vacuole-rich morphology indicative of senescence, exhibited a shift toward endothelial commitment (homogeneous CD31 expression), and stopped proliferating. Interestingly, the inhibitory effect of commercial human serum on EDC growth was dose-dependent, with EDCs exhibiting increased proliferation in 3% versus 20% serum – the opposite trend seen with FBS. Gamma-irradiation, a virus-inactivation treatment for FBS, resulted in similar undesirable outcomes in two of the formulations examined

(senescent-like morphology and growth arrest), with one formulation providing comparable culture outcomes and GMP-compliance.[129] However, the issues of variability and reproducibility remain with such an approach.

The conflicting findings of these studies reflect the inherent variability and lack of product definition that accompany the use of blood-derived media supplements, rationalizing a focus on developing serum-free alternatives.

1.6 Study overview

While recent studies have investigated the effects of replacing fetal bovine serum with commercially-available human-sourced alternatives, the inherent variability and potential for infectious or toxic contaminants make these approaches sub-optimal. Furthermore, altering EDC culture conditions is not straightforward as several divergent culture methods have been shown to profoundly impair the regenerative performance of cardiac-derived cell products.[55, 130, 131] Therefore, we investigate here the influence of a commercially sourced serum-free xenogen-free medium on human explant-derived cardiac stem cell (EDC) yield, phenotype, *in vitro* measures of EDC performance and post infarct cardiac repair. Given that EDCs are the initial cell product used for sphering or antigenic selection, EDCs provide a useful platform for testing the immediate effects of altered culture conditions as it permits early detection of adverse changes that would directly influence the regenerative performance of downstream progeny. To eliminate the possibility of contamination by materials used in tissue or cell processing, the need for each constituent of the EDC culture protocol was first established then, if needed, replaced with a good manufacturing practices (GMP) compatible standard prior to testing in vitro and in vivo measures of EDC regenerative performance. We then investigated the effect of

straightforward expansion of this serum-free xenogen-free EDC product on the cell-mediated repair of injured myocardium as a means of simplifying expansion to clinically relevant “doses” while avoiding culture-acquired drift and the risk of malignant transformation.[73] Finally, the impact of altered culture practices on measures of EDC viability and stability were established to eliminate logistical concerns surrounding transport and clinical delivery to patients.

1.7 Preclinical study design

To ensure clinical translatability, a number of necessary steps were taken to maintain accuracy and ensure reproducibility of the data obtained. Investigator bias in reporting key findings such as ejection fraction was avoided as technicians performing cell injections and ECHO measurements were blinded to the treatment received.

2. STUDY RATIONALE, AIMS & HYPOTHESES

2.1 Rationale

This project is designed to transition EDC culture practices from bench grade to good manufacturing practices. This is the next critical step in developing next generation EDC therapies for clinical use.

2.2 Research Aims

1. To explore the capacity of serum-free, xeno-free culture medium to proliferate and expand human EDC cultures.
2. To explore the ability of serum-free, xeno-free EDCs to mediate post-infarct cardiac repair in a murine model of acute ischemic injury.
3. To investigate the ability of human EDCs to be delivered via clinically approved coronary artery perfusion and intramyocardial injection catheters.

2.3 Hypotheses

1. Transition of EDC cultures to a serum-free, xeno-free culture medium will support cell growth to a degree equivalent to traditional serum culture.
2. Adherent expansion of EDCs within serum-free, xeno-free culture medium will provide a means of reaching therapeutically relevant doses in a timely fashion.
3. Serum-free, xeno-free EDCs will demonstrate equivalent overall therapeutic efficacy as measured by echocardiography and histology.
4. Expanded EDCs will demonstrate equivalent overall therapeutic efficacy as measured by echocardiography and histology.
5. Delivery of EDCs through clinically approved catheters will preserve cell viability while ensuring product delivery to the peri-infarct zone.

3. MATERIALS AND METHODS

3.1 Cell Isolation and culture

Explant-derived cardiac stem cell cultures were derived from atrial appendages or ventricular biopsy tissue obtained from patients undergoing clinically-indicated surgical procedures at the University of Ottawa Heart Institute. All protocols performed were first approved by the Research Ethics Board at the University of Ottawa Heart Institute. Criteria for the inclusion of tissue donors were patients aged 18 to 80 requiring cardiac surgery for coronary artery bypass graft(s) and/or valve surgery. Patients were excluded on the basis of chronic and/or infectious diseases (HIV, hepatitis), pregnancy, or active sepsis. Tissue samples were minced, briefly washed, and digested using either collagenase IV (Life Technologies) or a GMP-grade blend of collagenase I/II (Liberase; Roche)[132] before plating explant tissue on GMP-grade fibronectin-coated culture plates in standard cardiac explant media (Iscove's Modified Dulbecco's Medium with 20% fetal bovine serum, 100 ug/ml streptomycin, 100 U/ml penicillin G, 0.1 mmol/l 2-mercaptoethanol, and 2 mmol/l L-glutamine; Life Technologies) or GMP-grade serum-free xeno-free medium (SF; Nutristem XF, Biological Industries) at physiological (5%) oxygen tension level.[63-65, 67, 109, 133, 134] To overcome tissue losses associated with impaired tissue adhesion observed in plating with SF media alone, SF cultures were supplemented with 2% serum for an initial period of 48 hours, at which time a full replacement of media was made. Supplementation improved tissue biopsy adhesion, as subsequent tissue losses from the cultureware were drastically reduced. EDCs that spontaneously emigrated from plated tissue fragments were enzymatically harvested up to 4 times every seven days with TrypLE Select (Life Technologies), with manual counts of cells taken using a Neubauer hemocytometer. The effects of static expansion on cell numbers and phenotype were investigated using aliquots of harvested cells seeded at 10% confluency on fibronectin coated cultureware for 7 days.

Circulating angiogenic cells, or CACs were obtained from whole blood samples donated by patients undergoing clinical procedures at the University of Ottawa Heart Institute under a Research Ethics Board-approved protocol.[133] Isolation of mononuclear cells was achieved by centrifugation with a density-gradient (Histopaque 1077; Sigma-Aldrich), and isolated mononuclear cells were placed in culture for 4 to 6 days with endothelial basal medium 2 (EBM-2; Clonetics) and EGM-2-MV-SingleQuots supplements (Clonetics). Circulating angiogenic cells (CACs) were mechanically lifted for experiments within 7 days of initial blood processing. Commercial human umbilical vein derived endothelial cells (HUVECs) were cultured following the manufacturer's directions (Lonza).

3.2 Antigenic profiling

Flow cytometry (Guava easyCyte 8HT flow cytometer; Millipore) was performed in order to examine EDC phenotype using monoclonal antibodies and similarly conjugated isotype-matched controls directed against CD105, CD90, CD79, CD73 (FMC020, R&D Systems), CD166 (FAB6561P, R&D Systems), CD29 (FAB17781P, R&D Systems), CD44 (FAB4948P, R&D Systems), CD51 (FAB3050A, R&D Systems), CD31 (FAB3567F, R&D Systems), CD133 (130-090-826, Miltenyi Biotec), CD146 (FAB932F, R&D Systems), Nestin (IC1259F, R&D Systems), PDGFRα (FAB1264A, R&D Systems), Cadherin 11 (FAB17901G, R&D Systems), αSMA (ab66133, Abcam), DDR2 (ab63337, Abcam), abcg2 (FAB995P, R&D Systems), SSEA-1 (FAB2155A, R&D Systems), c-Kit (9816-11, Southern Biotech), and a cocktail of hematological markers (FMC020, R&D Systems). A minimum threshold of 20,000 cellular events was set after fluorescence compensation with unlabeled control cells. Positive cells were considered as the population percentage exceeding the 99th percentile of the mean fluorescence intensity of isotype control (FlowJo v. 10, TreeStar Inc.).

3.3 Conditioned media for angiogenesis, CAC migration and paracrine profiling

Conditioned media were obtained from EDCs following 48 hours of in hypoxia (1% oxygen) and low serum (Iscove's Modified Dulbecco's Medium with 1% fetal bovine serum, 100 ug/ml streptomycin, 100 U/ml penicillin G, 0.1 mmol/l 2-mercaptoethanol, and 2 mmol/l L-glutamine; Life Technologies) conditions. The paracrine output of EDCs was quantified with a multiplex assay (BioPlex, USA) for the 6 cytokines most abundantly secreted by EDCs.[133] The ability of EDCs to induce angiogenesis *in vitro* was assessed using HUVECs seeded on a matrigel depleted of growth factors (ECM625, Millipore). HUVECs were added to conditioned media samples (serum free or standard conditions) or 100 mM VEGF-supplemented serum-free DMEM (positive control) and left in culture for a period of 18 hours. At this time, each well was microscopically imaged and wells were reconstructed using software, to permit the cumulative measurement of tubular growth (NeuronJ software; National Institutes of Health).[65, 133] The capacity for endogenous progenitor cell recruitment was also assessed, by seeding 4,000 CACs in basal DMEM inside the top well insert of a trans-well dish coated with fibronectin (24 well dish, 3 millimeter pore size; Corning) and conditioned media from EDCs were added to the bottom of the well. Basal DMEM supplemented with 100 ng of VEGF served as a control which was used as a baseline of comparison for CAC migration. Following 24 hours in normoxic culture, migratory CACs were fixed using para-formaldehyde (4%) and cell nuclei were visualized using DAPI (4',6-diamidino-2-phenylindole; Sigma Aldrich). The average number of migrated CACs per field was quantified using fluorescence microscopy (10 times magnification; 6 random fields; Image J software, ICTN plugin, National Institutes of Health).[65, 133]

3.4 Cellular senescence and proliferation assays

To assess cellular senescence and proliferation, EDCs were seeded at 40% confluency on fibronectin-coated 12-well culture plates (Corning) and cultured for 48 hours. Senescence-

associated β-galactosidase (SA-β-gal) expression was quantified in EDCs after 5 days of culture in normoxic conditions. SA-β-gal+ EDCs were identified colorimetrically under phase-contrast microscopy to identify and count blue cells (N=4 cell lines, 5 random fields per cell line; Cellular Senescence Detection Kit, Millipore KAA002). Proliferating EDCs were identified by immunohistochemical staining for the cell cycle associated protein Ki67 (ab15580) in conjunction with the nuclear stain 4',6-diamidino-2-phenylindole (DAPI). Ki67+/DAPI+ nuclei were counted manually and expressed as a percentage of all visible (DAPI+) nuclei. N=4 cell lines, 5 random fields.

3.5 *In vitro* cardiogenicity assay

The effect of variable culture conditions on the ability of EDCs to adopt a cardiac phenotype was assessed by seeding 20,000 cells/cm^2 in cardiogenic medium (60% DMEM, 40% MCDB-201 (Sigma), 0.01% linoleum acid albumin, 0.01% ITS liquid media supplement, 0.1% 10mM L-ascorbic acid, 0.01% penicillin-streptomycin, 0.0002% 0.25 mM dexamethasone, 0.75% DMSO, 0.001% 2-mercaptoethanol, 10 ng/mL FGF-8b, 10 ng/mL rhDKK-1, 100 ng/mL FGF-4, 10 ng/mL BMP-2)[63, 67, 133, 135] and media were changed every 3 days. Following 7 days in culture, EDCs were harvested and used for flow cytometry (alpha smooth muscle actin (α-SMA; ab125266; Abcam), cardiac troponin T (cTnT, ab66133; Abcam) or von Willebrand's Factor (vWF; 11778-1-AP; ProteinTech)).

3.6 *In vivo* testing

Myocardial infarctions were performed experimentally in the hearts of male, severe combined immunodeficient, non-obese diabetic (NOD-SCID) mice via single suture left anterior descending (LAD) coronary artery ligation, under a University of Ottawa Heart Institute Animal

Care Service approved protocol.[63-65, 109, 133] Animals were given buprenorphine injections (0.05mg/kg subcutaneously) 1 hour before surgery and two times per day afterwards for 3 days. During the myocardial infarction surgeries, mice were intubated and anesthetized with isoflurane and physiological body temperatures were maintained using a heating pad. Animals were then closed and given 0.5cc of subcutaneous saline. After 7 days, 100,000 human EDCs were delivered into the injured myocardium within the infarct border and cardiac apex by trans-thoracic, echocardiographic guided (VisualSonics) injections. The left ventricular ejection fractions of mice were determined at 21 and 28 days following LAD ligation in order to quantify the functional improvements conferred by each cell therapy. Following the final echocardiographic assessment, each mouse was euthanized, and hearts were excised for histological sectioning or DNA extraction and analysis by quantitative polymerase chain reaction (qPCR). Long-term myocardial retention of injected cells was measured in a subset of animals (n=4/group) by qPCR for the non-coding human alu sequence repeats.[65, 133] Genomic DNA was extracted from left ventricles, and qPCR was carried out using transcript-specific primer probes. Remaining hearts were first fixed using 4% para-formaldehyde, then embedded within optimal cutting temperature (OCT) compound before being sectioned. Cardiac tissue viability within the infarcted zone was calculated in Masson's trichrome (Life Technologies) stained tissue sections by manual tracing of the infarcts. ImageJ software was used to calculate the percentage of viable myocardium within the infarcted area.[63-65, 109, 133] Transplanted cell engraftment was also confirmed by staining sections with human nuclear antigen (HNA; SAB4500768, Sigma-Aldrich), and EDC fate was examined by staining sections for co-segregation of HNA with cardiac troponin T (cTnT, ab66133; Abcam), alpha smooth muscle actin (α-SMA; ab125266; Abcam), or von Willebrand's Factor (vWF; 11778-1-AP; ProteinTech). Contributions of injected EDCs to infarct border-zone capillary density were assessed using isolectin B4 (B-1205, Vector

Laboratories) staining of tissue sections. All functional evaluations of cardiac function were conducted and data analyzed by investigators blinded to animals' treatment groups.

3.7 Teratoma study

Six SCID mice underwent anesthesia and received subcutaneous and intraperitoneal injections of 1 million expanded SF EDCs in 40 μL of chilled 1:1 matrigel in PBS. Fifteen minutes following injections, the animals were allowed to wake up, and were monitored for a period of 2 months for the formation of teratomas at the sites of injection. At this time, animals were sacrificed and surgically examined for the presence of formed masses. Any findings were excised and fixed for H/E staining and other histological analysis.

3.8 EDC stability and delivery testing

An 11Fr (3.7 mm) TREK Coronary Dilation Catheter (Abbott Vascular) and an 8Fr (2.7 mm) NOGA MyoStar Intramyocardial Injection Catheter (Biosense Webster) were used to assess the impact of catheter delivery on cell viability. After coating the internal channels of both catheters with 25% human albumin (A2153, Sigma), harvested EDCs were suspended in Plasmalyte A (2B2544X, Baxter) with 2.5% human albumin for catheter delivery. Viability of the 2 million cells injected through the internal channels was followed by viability testing (Trypan Blue) and delivery counts. The long-term stability of EDCs for transport between institutions was established using EDCs drawn up into BD Luer-Lok syringes and stored at 4 degrees Celsius for 18 hours. Cell viability using Trypan Blue (H7901, Sigma) exclusion was determined at time 0 (prior to loading the syringes) and after 18 hours.

3.9 Statistical analyses

All data are expressed as mean ± standard error unless otherwise stated. To determine whether or not statistically significant differences existed between groups, a one-way ANOVA was performed. If any such difference(s) were found, a Bonferroni's corrected t-test was run to determine the group(s) and difference(s) (Prism 6.01, GraphPad). A Chi Square test was used to detect any differences in categorical measures. A final threshold value of $p \leq 0.05$ was considered to be statistically significant for all analyses.

4. RESULTS

4.1 Baseline patient characteristics

Atrial appendages were obtained from eleven patients (Table 4.1; 63% male; age 67±3 years; BMI 29±2) enrolled in this study undergoing clinically indicated cardiac surgery. All patients had a history of stable cardiac disease with cardiovascular risk factors including diabetes (63%), hypertension (82%), dyslipidemia (82%), thyroid disease (10%), and peripheral vascular disease (20%). The majority of patients had a history of coronary artery disease (82%), history of myocardial infarction (55%), valvular heart disease (36%), or congestive heart failure (18%). All patients were on stable medication regimens including angiotensin-converting enzyme inhibitors and/or angiotensin receptor blockers (100%), anti-platelet therapy (100%), beta-blockers (73%) and statins (91%) for at least six months prior to surgery. The majority had normal renal function (creatinine 94±18 μmol/L) and evidence of well controlled blood glucose (Hemoglobin A1c 6.6±0.5 %).

Ventricular biopsy specimens were obtained from six patients (67% male; age 52±7 years; BMI 29±1) enrolled in this study undergoing post-transplant surveillance. Fewer of these patients had a history of stable cardiac disease, with cardiovascular risk factors including diabetes (17%), hypertension (17%), dyslipidemia (33%), and thyroid disease (33%). Only 17% of patients had a history of coronary artery disease or myocardial infarction, while half had a history of valvular heart disease. All patients were on stable medication regimens including angiotensin-converting enzyme inhibitors and/or angiotensin receptor blockers (67%), anti-platelet therapy (100%), beta-blockers (50%) and statins (83%). The majority of patients had normal renal function (creatinine 92±14 μmol/L) and evidence of well controlled blood glucose (Hemoglobin A1c 6.5±0.5 %).

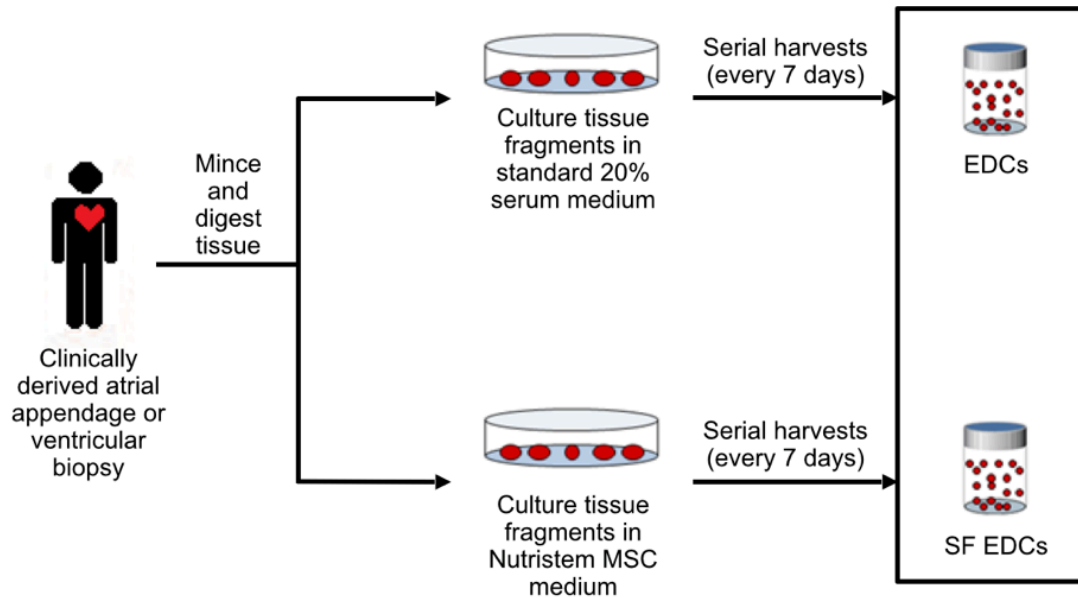
Cardiac specimens were collected at the time of biopsy and processed within one hour of collection. In order to control for variability in baseline demographics, primary EDC cultures were established by plating half of each atrial appendage specimen or ventricular biopsy in standard serum-supplemented media and half in SF medium (50:50 split by mass; Figure 4.1).

Table 4.1. Baseline characteristics of enrolled patients.

	Atrial Appendage donors (n=11)	**Ventricular Biopsy donors (n=6)**
Age (yrs)	67±3	52±7
BMI (kg/m2)	29±2	29±1
Gender (%male)	63%	67%
Diabetes	63%	17%
Hypertension	82%	17%
Dyslipidemia	82%	33%
Ongoing smoking	0%	0%
Thyroid disease	10%	33%
Peripheral vascular disease	20%	0%
Coronary artery disease	82%	17%
History of MI	55%	17%
Valvular heart disease	36%	50%
Congestive heart failure	18%	0%
NYHA class	1.7±0.4	–
LV ejection fraction (%)	45±6	–
CCS class	2.2±0.6	–
Creatinine (umol/L)	94±18	92±14
Hemoglobin A1c	6.6±0.5	6.5±0.5
Medications:		
Anti-platelet therapy	100%	100%
Beta-blocker	73%	50%
Statins	91%	83%
ACEI or ARB	100%	67%

BMI = Body Mass Index; MI = Myocardial infarction; NYHA = New York Heart Association; LV = Left ventricle; CCS = Canadian Cardiovascular Society; ACEI = Angiotensin-converting enzyme inhibitor; ARB = Angiotensin receptor blocker.

A



B

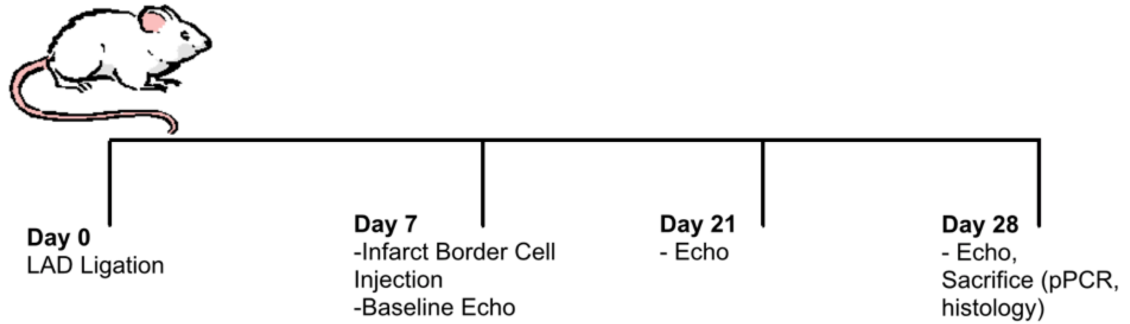


Figure 4.1. Experimental outline. (A) Schematic representation of culture protocol for obtaining standard and SF EDCs and (B) Timeline of animal surgeries, EDC injections and endpoints.

4.2 SF GMP compatible culture yields and phenotype

4.2.1 SF EDC size and homogeneity

Brightfield images (Figure 4.2 A-F) suggested that EDCs that spontaneously emerged from tissue plated in the SF medium were smaller and more uniform in size. Analysis of these images (Figure 4.2 G) confirmed a drastically reduced adherent cell length in SF cultures (99±9 versus 200±12 μm for cells cultured in CEM, $p<0.0001$). This finding was further confirmed through flow cytometric analysis of the forward (a correlate of cell surface area or size) and side (a correlate of granularity or internal complexity) scatter within harvested cells (Figure 4.2 H). EDCs cultured in SF medium demonstrated both a reduced forward scatter and reduced areas of 95% elliptical containment (46±6 versus 103±7 square units for cells cultured in CEM, arbitrary units; $p=0.002$); suggesting that EDCs cultured in SF medium are smaller and more homogeneous than those cultured in standard serum-dependent media.

This observed difference in cell size was not attenuated when SF EDCs were transitioned to CEM or when those from standard serum-dependent cultures were transitioned to the SF formulation for a period of 96 hours.

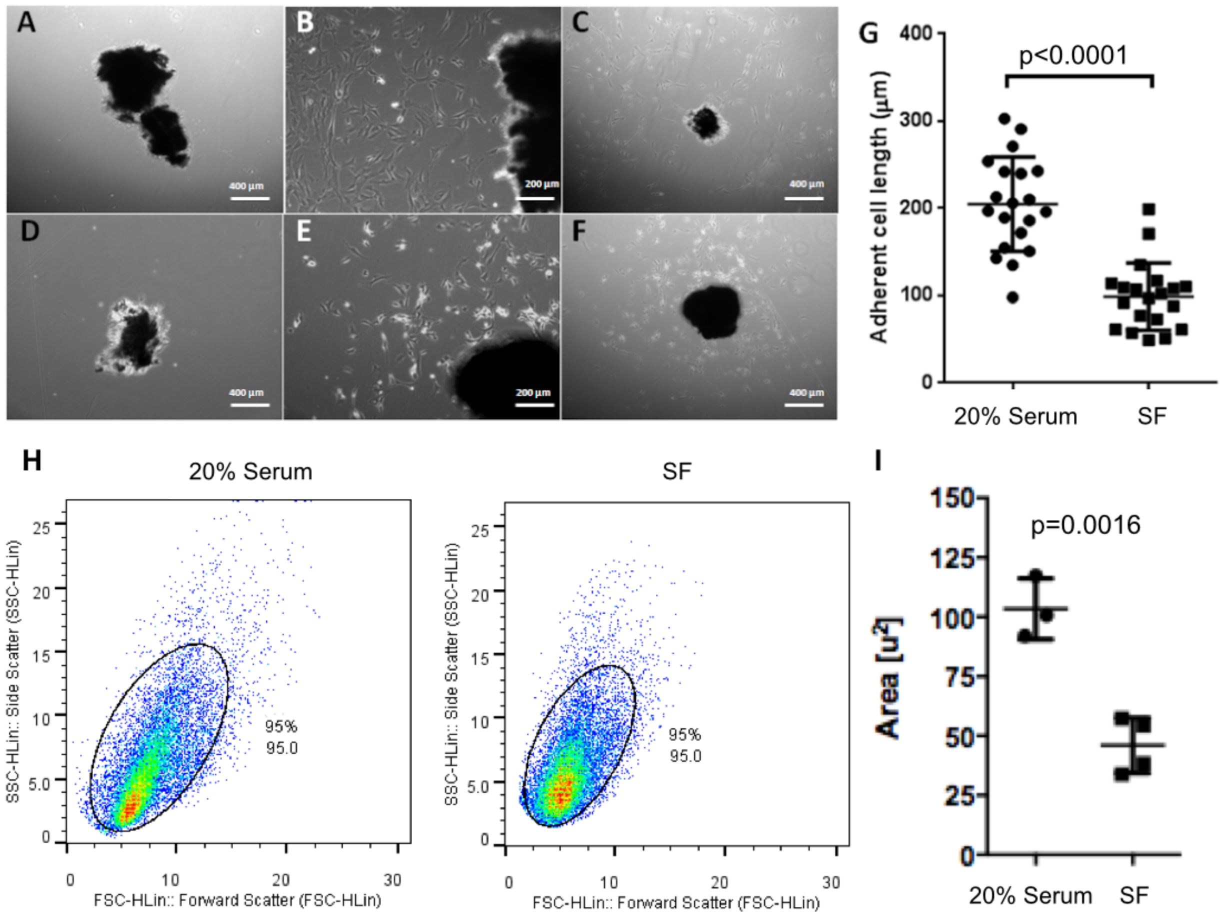


Figure 4.2. Comparison of EDC size under standard serum-dependent conditions versus SF GMP compatible conditions. Representative brightfield images of plated cardiac tissue fragments and EDC outgrowth under 20% serum conditions (A-C) and serum-free conditions (D-F). (G) Total adherent cell length from ImageJ analysis. (H, I) Flow cytometry demonstrating that the cells cultured in serum-free medium were smaller and more homogenous than cells cultured in standard 20% serum supplemented media conditions (mean ± SEM, n=4 explant cultures).

4.2.2 SF GMP compatible culture yields

In contrast to previous work demonstrating divergent culture practices having profound effects on EDC biology,[55, 130, 131] transitioning tissue explant culture from commercial grade collagenase IV to GMP compliant collagenase I/II did not significantly influence either overall cell culture yields (200±54 vs. 208±56 x10^5 cells per mg tissue plated, respectively; p=0.92) or the major sub-population content at each serial harvest from the plated tissue (Figure 4.3). Culture of atrial appendage biopsies within SF conditions did not alter the overall cell culture yields as compared to standard serum culture (190±30 vs. 220±40 x105 cells per mg tissue plated, respectively; p=0.57).

Tissue source did not alter the proliferative capacity of EDCs from plated tissue as culture yields from ventricular biopsies were maintained in the SF medium (1.7±0.3 vs. 1.2±0.5 million cells cultured per biopsy sample; p=0.45 vs. culture in media with serum).

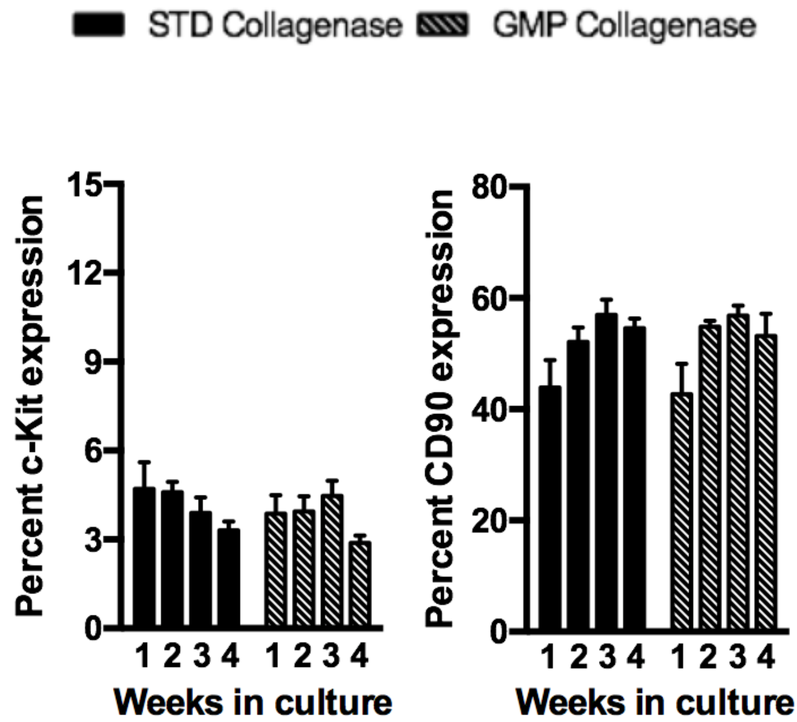


Figure 4.3. Effect of transitioning explant digestion from standard (STD) laboratory grade to GMP compliant collagenase on c-Kit and CD90 content of serial weekly harvests from explant culture conditions (mean ± SEM, n=5 explant cultures).

4.2.3 Profiling of EDC proliferation and senescence

Given the commonly encountered issues surrounding proliferation and cellular senescence when transitioning cells to serum-free media, these parameters were assessed in the present study. While a small percentage of EDCs were found to express senescence-associated β-galactosidase, no statistically significant differences were observed between SF, exSF, and serum cultured EDCs (Figure 4.4 A).

In order to assess cellular proliferation (Figure 4.4 B), EDCs were stained with Ki67. Both SF EDCs ($p=0.0076$ versus serum EDCs) and exSF EDCs ($p=0.0007$ versus serum EDCs) demonstrated increased levels of actively proliferating cells.

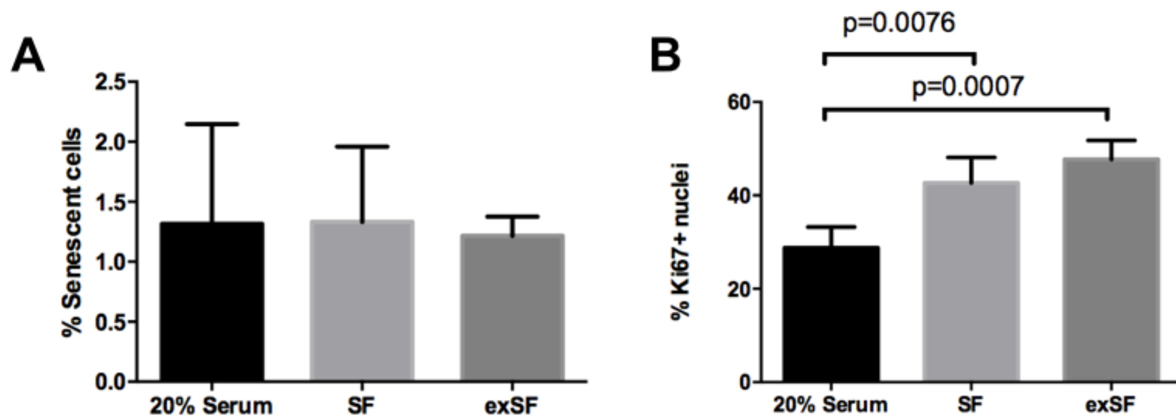


Figure 4.4. Quantification of cellular senescence and proliferation. (A) Senescence-associated β-galactosidase (SA-β-gal) expression was quantified in explant-derived cardiac stem cells (EDCs) after 5 days of culture in standard 5% O2 conditions. SA-β-gal+ EDCs were identified colorimetrically under phase-contrast microscopy by the presence of intracellular hydrolyzed X-gal; N=4 cell lines, 5 random fields. (B) Proliferating EDCs were identified by immunohistochemical staining for the cell cycle associated protein Ki67 in conjunction with the nuclear stain 4',6-diamidino-2-phenylindole (DAPI). Ki67+/DAPI+ nuclei were counted manually and expressed as a percentage of all visible (DAPI+) nuclei. N=4 cell lines, 5 random fields.

4.2.4 Flow cytometry profiling of EDC phenotype

The effects of SF GMP conditions on the phenotypic signature of EDCs was investigated using a custom flow cytometry panel to evaluate expression of cardiac, endothelial, hematopoietic, mesenchymal and stem cell identity. As depicted in Figure 4.5, SF conditions had only minor effects on the CD29, CD44, CD31 and Nestin content of EDCs.

Given that EDC culture is inherently limited by a constant culture output return proportional to the scale of production,[67] the influence of straightforward EDC sub-culture within adherent cell cultures was investigated as a means of attaining clinically meaningful cell "doses" (exSF group). Plating of EDCs within SF media provided a 5.5±1.1-fold increase in cell numbers over 7 days with a population doubling time of 73±11 hours. With the exception of a minor decline in the proportion of SSEA-1+ cells (Δ1.0±0.1%, $p=0.01$ vs SF culture), static expansion within SF media had negligible effects on the antigenic profile of EDCs.

Taken together, these data suggest that serum-free xenogen-free culture conditions support *ex vivo* proliferation of EDCs from multiple tissue sources with negligible effects on the phenotypic signature of cells expanded to clinically relevant doses.

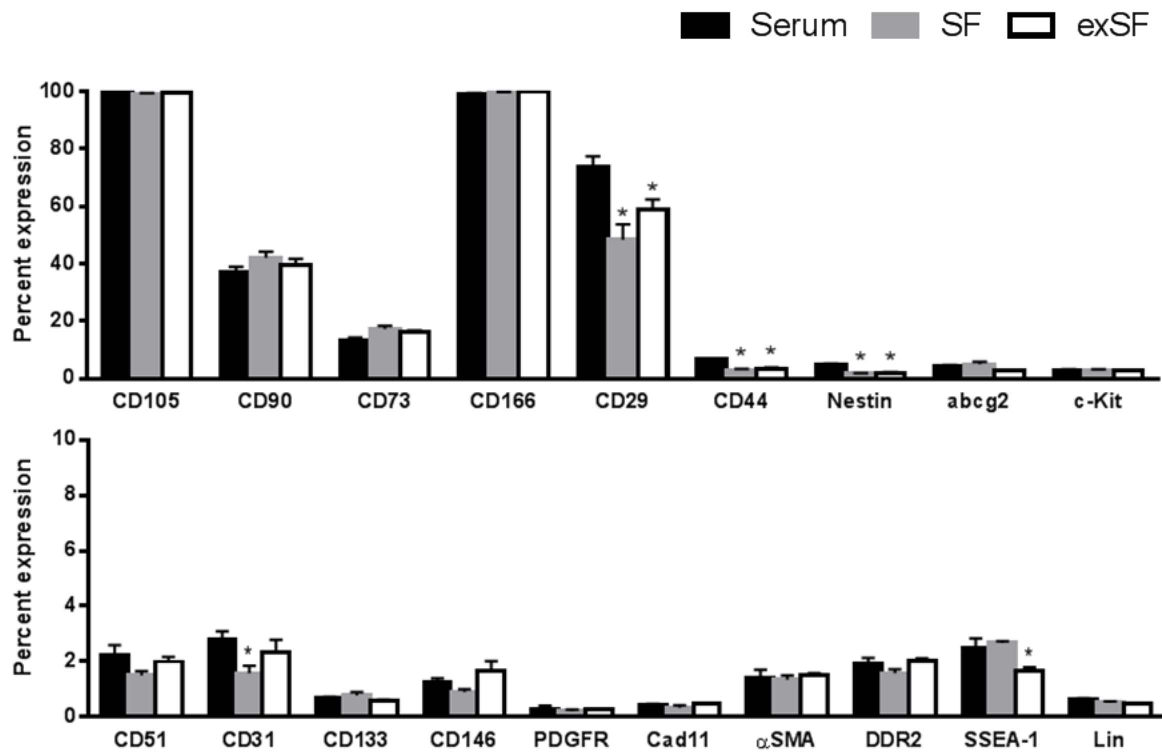


Figure 4.5. Effect of initial and expanded serum free culture conditions on the phenotypic composition of explant-derived cardiac stem cells (EDCs) (mean ± SEM, *p<0.05 vs. standard 20% serum culture; n=5 explant cultures).

Table 4.2. List and details of antibodies used in flow cytometry panel

Abbr.	Full/Alternate Antigen Name	Function	Lineage Association
abcg2	ATP-binding cassette sub-family G member 2	Xenobiotic transporter, implicated in multi-drug resistance[136]	embryonic
αSMA	Alpha smooth muscle actin	Cell motility and structure[137, 138]	myofibroblast
Cad11	Cadherin 11	Calcium-dependent cell-cell adhesion[139]	osteoblast
CD29	Integrin beta-1; Fibronectin receptor	Cell adhesion, recognition; linking of actin cytoskeleton to ECM[140]	–
CD31	Platelet endothelial cell adhesion molecule	Intercellular junctions, implicated in angiogenesis, integrin activation[141]	endothelial
CD44	Homing cell adhesion molecule; Pgp-1	Cell adhesion, hyaluronic acid receptor[142-144]	mesenchymal
CD51	Integrin alpha-V	Cell adhesion, signal transduction, receptor for ECM proteins[145, 146]	–
CD73	5'-nucleotidase	Cell surface enzyme implicated in cell adhesion, migration[147, 148]	–
CD90	Thy-1	Cell-cell and cell-matrix interactions[149]	mesenchymal
CD105	Endoglin	Angiogenesis, TGF-β signaling[150, 151]	mesenchymal
CD133	Prominin-1	Implicated in self-renewal, metastasis, metabolism, regeneration[152]	endothelial progenitor
CD146	Melanoma cell adhesion molecule	Angiogenesis, receptor for laminin alpha-4[153, 154]	endothelial, mesenchymal
CD166	Activated leukocyte cell adhesion molecule	Mediator of cell adhesion, migration[155]	cardiomyocyte
c-Kit	Tyrosine receptor-kinase Kit, SCF receptor	Cytokine receptor, implicated in cell survival, proliferation[156, 157]	cardiac progenitor
DDR2	Discoidin domain-containing receptor 2	Regulation of cell growth, differentiation and metabolism[158]	fibroblast
Lin	Cocktail of hematological markers	–	hematological
Nestin	Neuroectodermal stem cell marker	Regulation of intermediate filament assembly[159]	neural stem cell
PDGFR	Platelet derived growth factor receptor	Regulation of proliferation, differentiation, and growth[160, 161]	mesenchymal
SSEA-1	Stage-specific embryonic antigen 1	Mediator of cell adhesion and migration[162]	pluripotent stem cell

ATP = adenosine triphosphate; ECM = extracellular matrix; Pgp-1 = phagocytic glycoprotein 1; TGF-β = transforming growth factor beta; SCF = stem cell factor

4.3 Paracrine signature and differentiation of serum-free cardiac stem cells

4.3.1 Multiplex cytokine profiling of conditioned media

The effects of GMP SF compatible conditions on the regenerative potency of the initial and expanded EDC cultures were investigated using established in vitro measures of indirect and direct cardiac repair.[65, 76, 133] As shown in Figure 4.6 A, multiplex profiling of the cytokine content within initial or serum free EDC conditioned media demonstrated equivalent secretion of stromal cell derived factor 1α (SDF-1α) and stem cell factor (SCF) with reduced production of hepatic growth factor (HGF; 82±2% less; p=0.003), interleukin 6 (IL-6; 96±2% less; p=0.001) and vascular endothelial growth factor A (VEGF-A; 50±5% less; p=0.02) by SF cells. Similar to effects seen on the phenotypic profile of EDCs following expansion to clinically relevant cell “doses”, expansion within serum free media had no detectable effects on the paracrine signature of EDCs.

4.3.2 *In vitro* measures of paracrine regenerative capacity

The capacity of human EDCs to promote blood vessel formation was assessed using a surrogate assay for angiogenesis. Despite notable effects by SF conditions on the cytokine profile of EDCs, application of conditioned media to a HUVEC cytokine depleted matrigel cord-formation assay or CAC transwell cultures demonstrated that altered EDC culture conditions had negligible effects on the formation of capillary-like networks or on CAC recruitment (Figure 4.6 B, C).

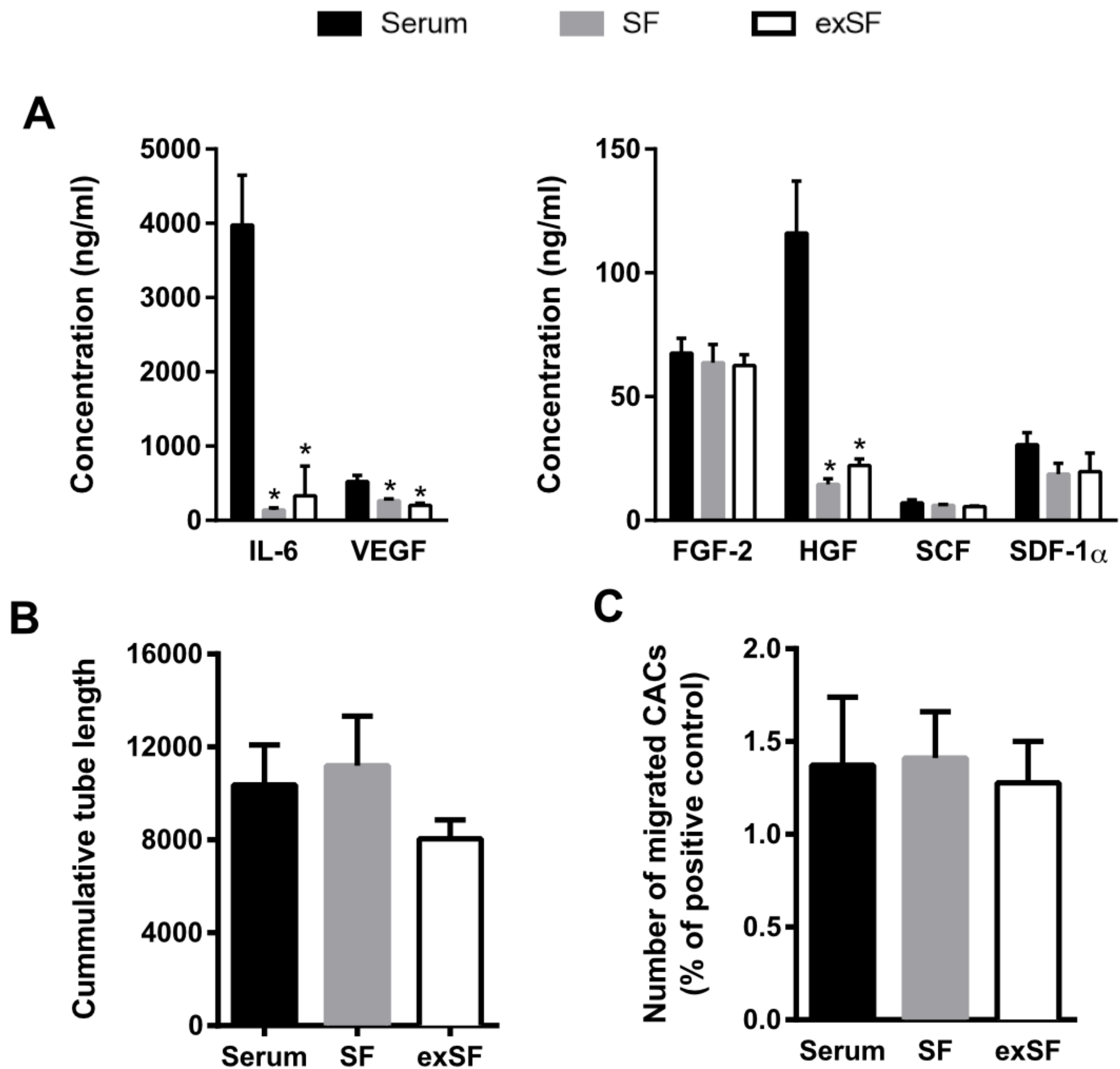


Figure 4.6. Effects of SF culture on the paracrine output of explant-derived cardiac stem cells (EDCs). (A) Multiplex cytokine profiling of conditioned media from standard 20% serum, serum free media and expanded serum free EDCs (mean ± SEM, *p<0.05 vs. standard 20% serum culture; n=4 explant cultures). (B) Conditioned media from serum free and expanded serum free EDCs had negligible effects on the cumulative tubule length of HUVECs after 18 hours in culture (mean ± SEM, n=4 explant cultures performed in duplicate with 6 random fields per sample assayed). (C) Conditioned media from serum free and expanded serum free EDCs had negligible effects on the number CACs attracted through a transwell assay (mean ± SEM, n=4 explant cultures performed in duplicate with 6 random fields per sample assayed and using a single donor sourced CAC line).

4.3.3 *In vitro* measures of EDC differentiation capacity

The influence of serum free culture on the ability of EDCs to adopt a cardiac phenotype was investigated after 7 days of culture in conditions known to favor a cardiac identity.[67, 135] Flow cytometry revealed an equivalent propensity for SF cells to adopt a cardiomyocyte lineage (cTnT+; 20±1% of cells, p=0.55 vs. standard EDCs) and endothelial lineage (vWF+; 4.3±0.5% of cells, p=0.22 vs. standard EDCs) but a reduced tendency toward smooth muscle differentiation (αSMA+, 4.3±0.1% of cells, p=0.017 vs. standard EDCs, Figure 4.7). Expansion of SF cultured cells did not influence the expression of cTNT, vWF or αSMA after culture in cardiogenic conditions (p=ns vs. EDCs from SF conditions).

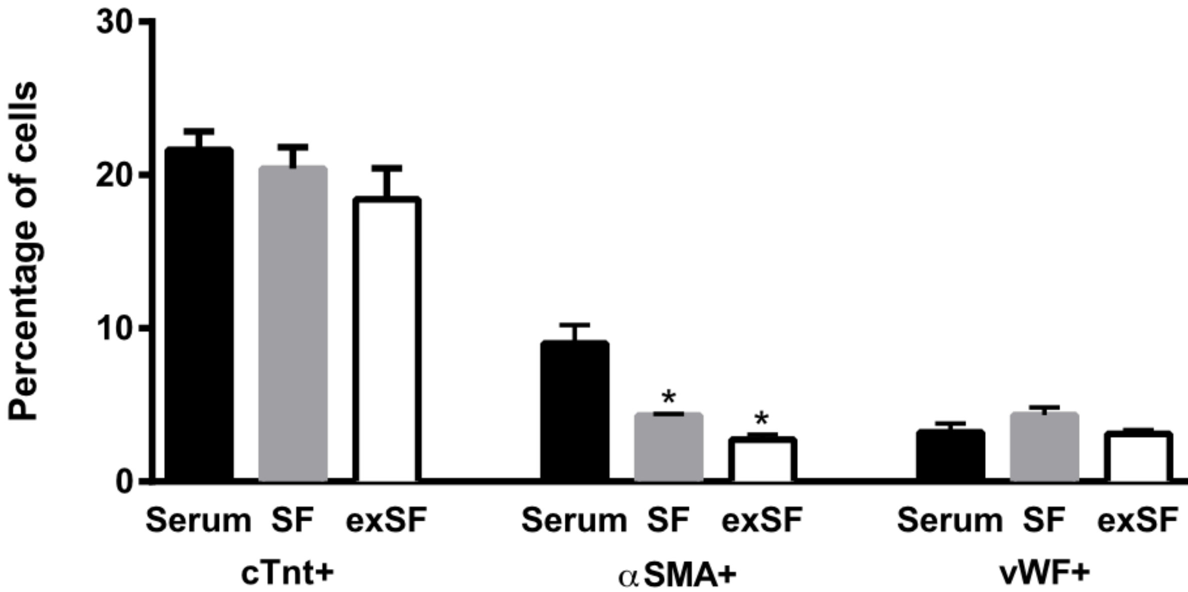


Figure 4.7. Effects of serum-free (SF) culture on the *in vitro* differentiation capacity of explant-derived cardiac stem cells (EDCs). EDCs cultured and expanded in serum free conditions demonstrated a reduced ability to adopt a smooth muscle fate (alpha smooth muscle actin, αSMA) relative to standard 20% serum cultured EDCs. In contrast, serum free culture and expansion of EDCs had negligible effects on the ability of cells to adopt a myogenic (cardiac troponin T, cTNT) or endothelial (von Willebrand Factor, vWF) identity. Data are shown as mean ± SEM with 4 explant cultures (*$p<0.05$ vs. standard 20% serum culture).

4.4 Serum-free culture conditions enhance cell-mediated cardiac repair

4.4.1 Transplantation of stem cells improves cardiac function

The influence of SF culture and EDC expansion on therapeutic cardiac repair was investigated in a series of immunodeficient mice randomized to echocardiographic guided injection of serum, SF or expanded SF EDCs 1 week after LAD ligation. As shown in Table 2, all animals had equivalent ejection fractions, chamber dimensions and stroke volumes 7 days post LAD ligation. Animals treated with SF EDCs showed superior improvements in echocardiographic ejection fraction 3 weeks after cell treatment compared to animals receiving traditional serum cultured EDCs (48±3 vs. 40±2%, respectively; $p=0.046$, Figure 4.8 A and Table 2). The regenerative advantage conferred by administering cells cultured in SF conditions was reduced in animals that received equivalent “cell doses” of expanded SF EDCs (41±2%; $p \leq 0.05$ vs. SF EDCs) to an extent that was comparable to animals who received cells cultured in standard serum conditions.

4.4.2 Serum-free cells provide equivalent long-term engraftment

Long term retention of EDCs was determined by performing qPCR for retained human Alu sequences in the left ventricles of a subset of treated animals 4 weeks post-infarction (Figure 4.8 B). As treatment with SF cultured EDCs provided no detectable influence on modest long-term cell retention, the superior functional benefits seen in SF EDC treated animals cannot be attributed to improved long-term cell engraftment.

Table 4.3. Echocardiographic measurements of left ventricular function 7 and 28 days after left coronary ligation.*p≤0.05 vs. serum cultured EDCs, **p≤0.05 vs. expanded serum free cultured EDCs.

	Left ventricular end diastolic volume (µL)	Left ventricular end systolic volume (µL)	Stroke Volume (µL)	Ejection Fraction (%)	Fractional Area Shortening (%)
7 days post LC ligation					
Serum (n=13)	52.6±3.9	34.8±3	17.8±1.7	33.7±2.3	19.4±1.8
SF (n=10)	51.6±2.7	33.6±2.2	18±1.2	35.1±1.7	21.6±1.4
exSF (n=12)	56.1±4.4	37±3.4	19.1±1.8	34.4±2.2	20.9±1.7
28 days post LC ligation					
Serum (n=13)	54.1±2.5	32.6±2.5	21.4±0.6	40.8±2.3	25±1.5
SF (n=10)	49.9±3.1	26.5±2.7	23.4±1.1	47.9±2.7*,**	30.2±1.9*
exSF (n=12)	58.9±4.4	35.1±3.8	23.8±1	41.4±2.4	26±1.7

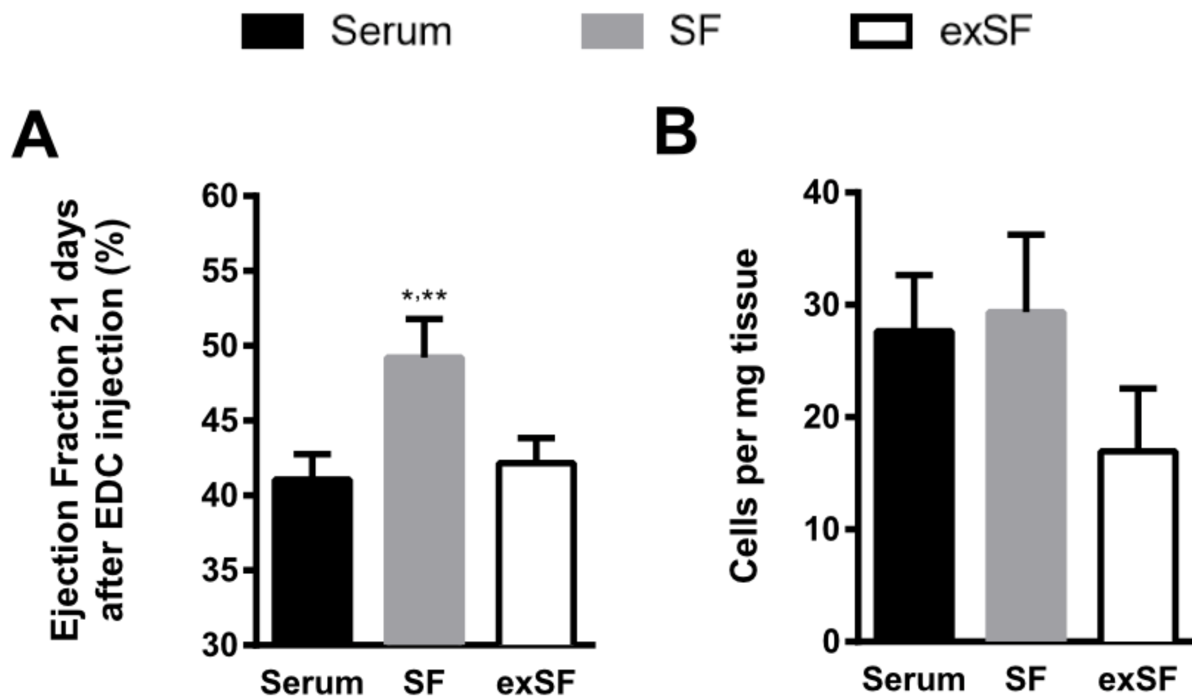


Figure 4.8. Transplant of explant-derived cardiac stem cells (EDCs) from serum free culture conditions. (A) Transplant of 100,000 serum free EDCs into immunodeficient mice 1 week after left coronary ligation provided marked increases in myocardial function 21 days after cell injection as compared to equivalent numbers of EDCs cultured in 20% serum or expanded serum free EDCs (mean ± SEM, *p≤0.05 vs. 20% serum cultured EDCs, ** p≤0.05 vs. expanded serum free cultured EDCs; n=10-13 mice per group). (B) Effects of serum free EDC transplantation on engraftment at 4 weeks as determined using qPCR for retained human alu sequences (mean ± SEM, n=6 mice per group).

4.4.3 Transplantation of serum-free cells increases viable myocardium

Despite clear improvements in myocardial function, administration of SF EDCs had no effect on overall scar burden (Figure 4.9) or capillary densities (Figure 4.10). The functional echocardiographic benefits seen after administration of SF cultured EDCs were attributable to increases in viable myocardium within the infarct itself- hinting that SF cultured EDCs either promote larger degrees of newly formed myocardium or attenuate myocardial losses within the treatment zone.

4.4.4 Serum-free culture influences transplanted cell fate

To provide insight into the final fate of transplanted cells, immunohistochemistry was performed to identify cells positive for human nuclear antigen (HNA) in conjunction with markers indicative of cardiomyocyte (cTnT), smooth muscle (αSMA) or endothelial vascular (vWF) lineages. As depicted in Figure 4.11, localized clusters human cells positive for cTnT and vWF were seen in all groups while relatively fewer human cells of smooth muscle identity were observed.

4.4.5 Expanded serum-free cells do not undergo malignant transformation

Two months following subcutaneous and intramuscular injection of 1 million exSF EDCs, no masses were present in any of the 6 NOD-SCID mice investigated.

4.4.6 Summary of *in vivo* findings

Taken as a whole, these results suggest that administration of SF cultured cells provides an enhanced cell product that boosts recovery within the infarcted tissue without influencing the final scar burden, vascularity or fate of transplanted cells. Furthermore, expansion within SF media provides greater amounts of cells to be delivered but attenuates the ability of cells to promote post-infarct cardiac repair.

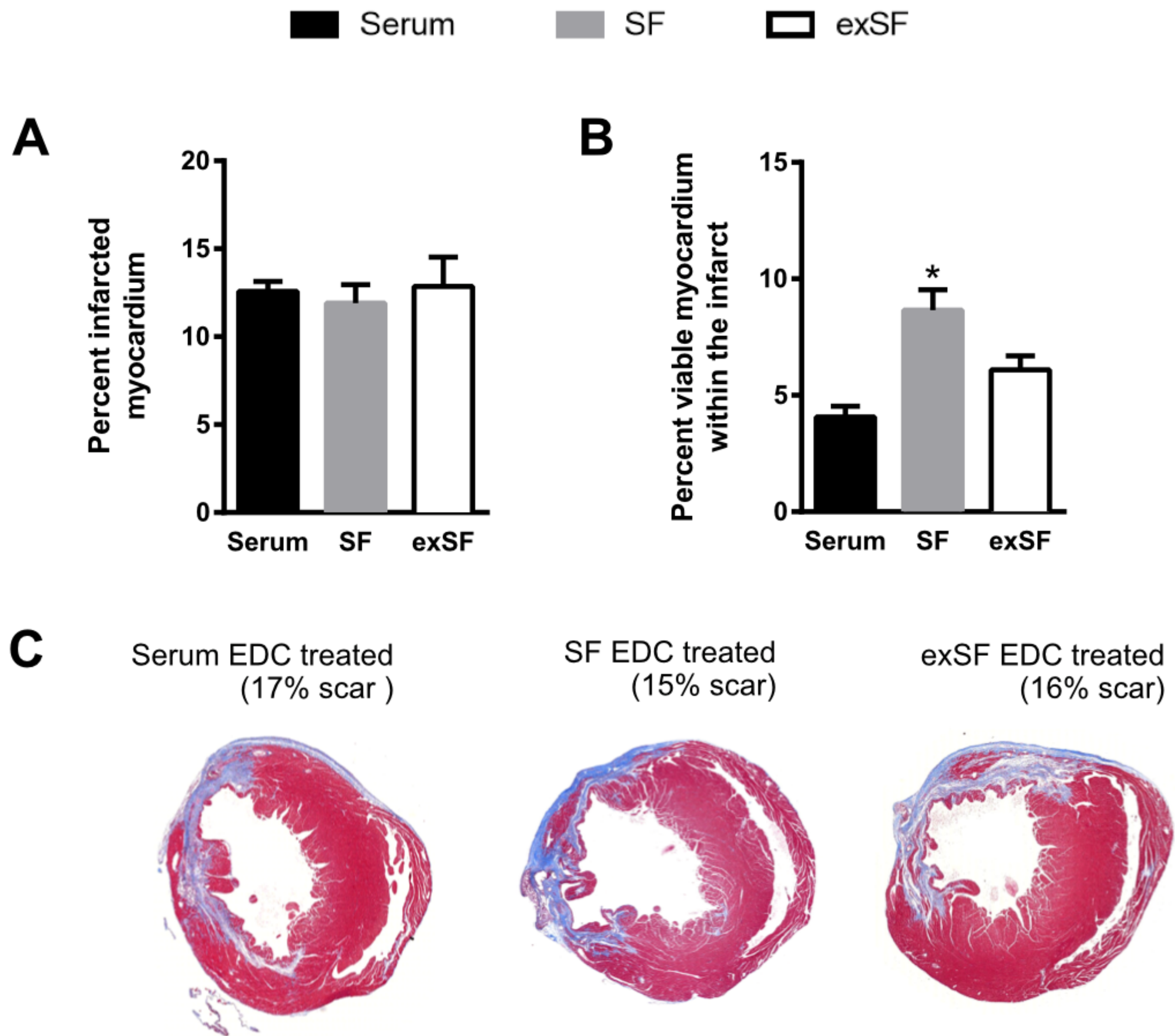


Figure 4.9. Effects of serum-free (SF) explant-derived cardiac stem cell (EDC) treatment on myocardial scar burden. (A) Effects of serum free EDC transplantation on the overall percentage infarcted myocardium as determined from Masson's trichrome staining (mean ± SEM, n=5 mice per group with 3 adjacent sections averaged per mouse). (B) Effects of serum free EDC transplantation on the percentage of viable myocardium within the infarct zone (mean ± SEM, *p≤0.05 vs. 20% serum cultured EDCs; n=5 mice per group with 3 adjacent sections averaged per mouse). (C) Representative short axis sections from animals randomized to transplant of serum, serum-free or expanded serum free cultured EDCs. These typical sections were stained with Masson's trichrome prior to imaging and scar (blue) was manually traced and quantified using Image J. Also shown is the measure of scar size obtained for each image.

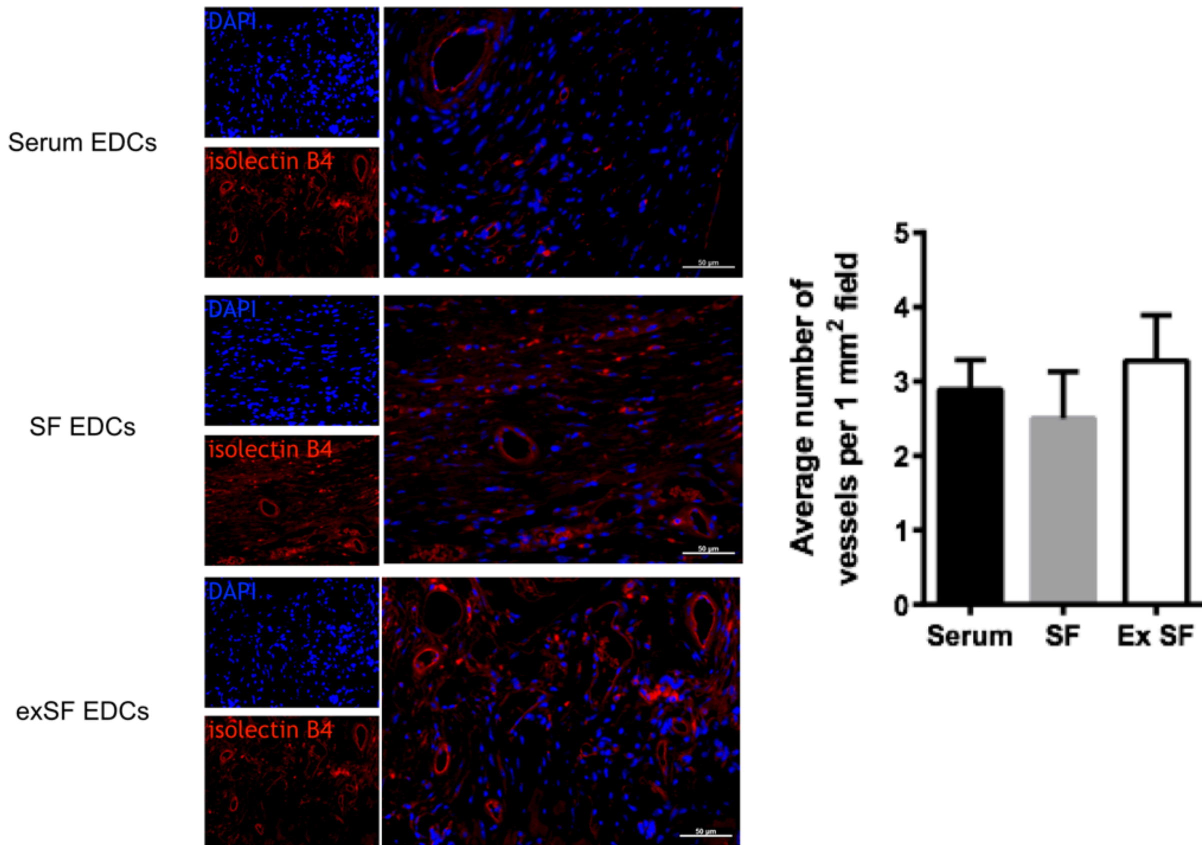


Figure 4.10. Effects of explant-derived cardiac stem cell (EDC) treatment on infarct border-zone vascularity. Representative peri-infarct images (left) and quantification (right) demonstrating capillary density in sections obtained from animals randomized to transplant of serum, serum-free or expanded serum free cultured EDCs. Immunohistochemical single and merged images of peri-infarct sections demonstrating nuclei (DAPI) and myocardial vessels (isolectin B4). Scale = 50 um.

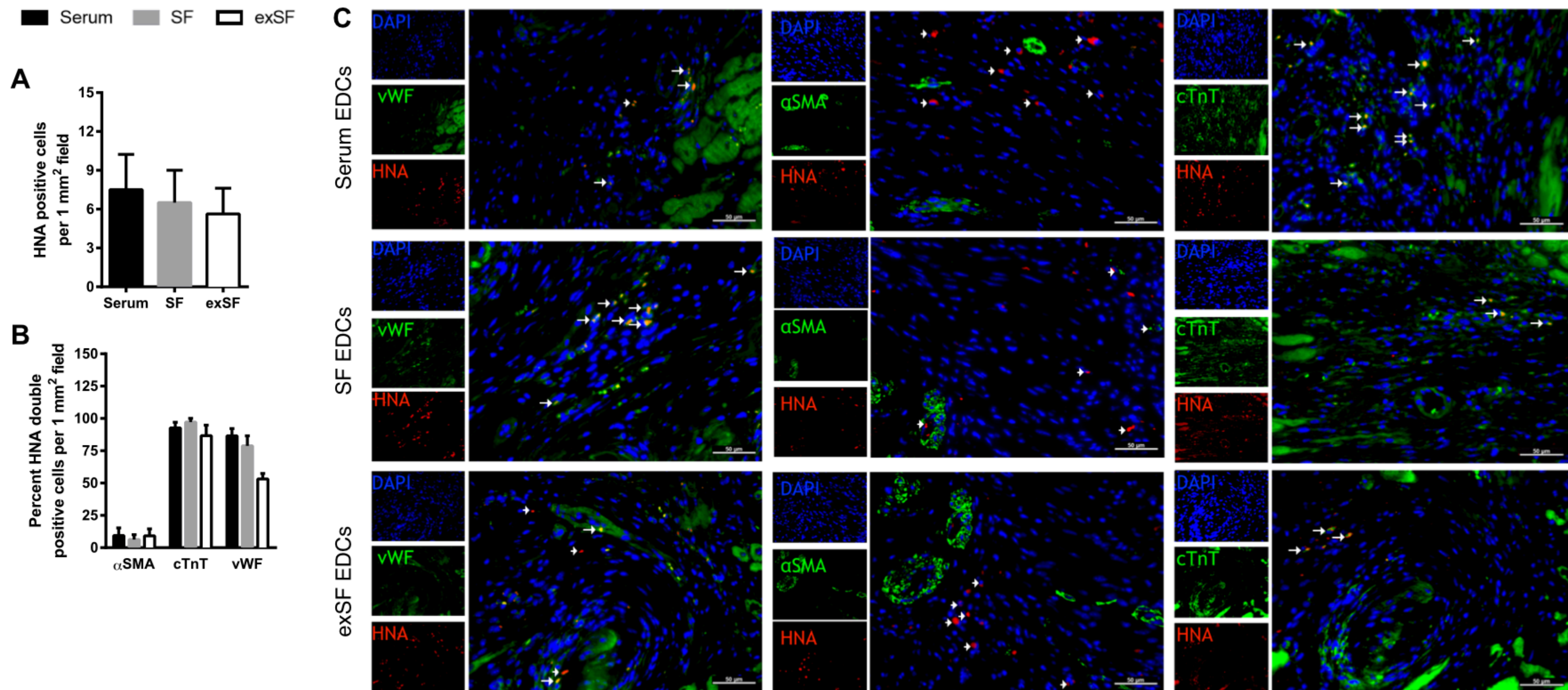


Figure 4.11. Transplanted explant-derived cardiac stem cell (EDC) fate. Effects of serum free EDC transplantation on (A) engraftment as determined using random field counts of human nuclear antigen (HNA) positive cells (mean ± SEM, n=5 mice per group with 3 random fields sampled within 3 adjacent sections per mouse) and (B) fate of engrafted EDCs within the peri-infarct zone (mean ± SEM, n=3 mice per group with 3 adjacent sections averaged per mouse). (C) Representative peri-infarct images demonstrating cell of human origin expressing markers of endothelial (vWF+), smooth muscle (αSMA+), and cardiomyocyte (cTnT+) fate in sections obtained from animals randomized to transplant of serum, serum-free or expanded serum free cultured EDCs. Immunohistochemical single and merged images of peri-infarct sections demonstrating nuclei (DAPI), transplanted cell origin (human nuclear antigen; HNA) and lineage marker. Arrows indicate single cell co-segregation of markers. Scale = 50 um.

4.5 Effects of clinical delivery and brief suspension storage

To enable the straightforward clinical implementation of cultured EDCs, the impact of catheter delivery via clinical intra-coronary and transendocardial routes was evaluated. Counts of viable cells before and after intra-coronary catheter delivery demonstrated successful delivery of 96±2% of EDCs with 95±2% of cells remaining viable after delivery. Similarly, delivery of cells through a NOGA transendocardial catheter demonstrated successful delivery of 94±1% of cells with 97±1% of cells remaining viable after delivery. Tests of EDC stability revealed negligible changes in viable cell counts after 18 hours in suspension at 4 degrees Celsius (96±2% $p = 0.07$ vs. baseline viability) with no attrition attributable to serum free culture conditions ($p=0.58$ vs. serum cultured EDCs).

5. DISCUSSION

5.1 Optimization of cell culture protocols for clinical translation

Although cardiac stem cell therapy has progressed from bench to bedside over the past decade,[61, 80] further straightforward clinical translation will be hampered by reliance on traditional culture conditions which often include ill-defined or xenobiotic components. Overcoming these barriers represents a critical next step in the translation of cardiac-based cell therapies into clinical use. In this study, cultures of primary outgrowth of plated cardiac tissue (EDCs) were undertaken to investigate the effects of a serum-free, xeno-free culture system on proliferation and cell product identity. EDCs were chosen as the cell type to study as they represent the initial cell product used prior to antigenic selection[52] and/or prolonged inductive culture.[53] Previous work has shown that EDCs provide a complimentary admixture of cell types that provide degrees of myocardial repair equivalent to CDCs while retaining a 1000-fold greater capacity to adopt a cardiac fate,[67] making EDCs a valuable tool to detect the early effects of divergent cell culture practices.[55]

5.2 Culture outcomes using GMP compliant medium

In order for a serum-free culture platform to be considered suitable for clinical translation, it must support cell growth sufficient to reach target therapeutic “doses” while maintaining product identity. The cell culture outcomes outlined above suggest that serum free conditions yield a cell product morphologically similar to standard cardiac explant conditions, while maintaining primary culture yields similar to those obtained in the traditional culture medium. Interestingly, the smaller and more homogeneous cell product derived using SF xenogen-free conditions likely results from stable consistent recombinant cytokines found in the defined media. These

differences in cell-size were not attenuated in SF EDCs transitioned to CEM or in standard serum-dependent EDCs transitioned to the SF formulation for a period of 96 hours.

Profiling of cell surface marker expression demonstrated that this media formulation maintains an overall antigenic profile consistent with traditional cultures, resulting in only minor declines in the expression of endothelial/mesenchymal markers CD29, CD44, CD31, and the neural stem cell marker Nestin. Also encouraging is the finding that, unlike EDCs cultured in autologous or commercial human serum formulations,[129] EDCs cultured in the serum-free xenogen-free medium exhibited negligible cellular senescence, proliferating at greater levels that EDCs cultured in FBS even after an additional week of adherent expansion. Notably, the use of recombinant serum-free media also avoids exposing human cells to bovine-sourced exosomes – a component of traditional media that remains poorly defined.

5.3 Effects of serum-free culture on cell therapeutic potency

Interestingly, eliminating ill-defined bovine sourced cytokines or exosomes had marked effects on the cytokine profile of EDCs, reducing secreted levels of IL-6, HGF, and VEGF. This suggests that serum free recombinant cytokine conditions permit human EDC to retain a more "human" or "natural" identity. Despite these differences in cytokine secretion, conditioned media collected from serum-free EDCs did not exhibit biologically-significant impairments in the ability to promote HUVEC tube formation or the migration of circulating angiogenic cells. Further, *in vitro* measures of cardiogenic potential revealed an equivalent propensity for SF EDCs to adopt cardiomyocyte and endothelial fates, with a slightly reduced tendency toward smooth muscle differentiation.

Differences in the paracrine output of SF EDCs very likely altered the manner in which EDCs mediated post infarct healing as reduced production of key cytokines (such as IL-6 or VEGF) can influence the manner in which transplanted cells interact with the damaged host. These effects remain to be defined but, as shown above, delivery of a more homogenous cell-product provided greater healing within scarred tissue and superior functional gains.

5.4 Study limitations and future directions

The media formulation chosen for this work was based on pre-clinical work comparing different SF media formulations for allogeneic bone marrow derived mesenchymal stromal cell culture (Cellular Immunotherapy for Septic Shock: A Phase I Trial (CISS), NCT02421484). These data suggested that Nutristem XF media provided the most consistent culture outcomes, rationalizing its use for the CISS trial. While extension of other SF media formulations towards EDC culture is uncertain, future studies are needed to understand if important differences exist between current commercial products.

In order to control for interpatient variability in this study, cardiac biopsies were split by mass and cultured in either standard 20% serum or the serum-free formulation. However, to permit the unbiased comparison of EDC paracrine effects, conditioned media were collected using IMDM supplemented with 1% fetal bovine serum, as unsupplemented IMDM was insufficient to support EDC growth. While the transition of standard EDCs to hypoxic, 1% serum conditions is an established method for the collection of conditioned media,[63-65, 109, 133] the effects of transitioning serum-free EDCs to these conditions on the paracrine output of these cells are unknown.

Previous work by our group has identified that EDCs express important receptors (such as insulin-like growth factor 1 and SDF-1α) that may influence proliferation.[63, 64] Thus, it follows that supplementation of commercial SF media formulations may be used in the future to formulate media tailored to EDC culture outcomes.

6. CONCLUSIONS

This study provides the first evidence that defined, serum-free xeno-free culture conditions can provide a more uniform and functionally superior EDC product ready for clinical delivery. While straightforward expansion of serum-free EDCs to clinically relevant “doses” attenuates the ability of cells to promote post-infarct cardiac repair, this approach also provides greater amounts of cells to be delivered while avoiding risks of non-cardiac or malignant transformation. As efforts to boost cell product efficacy are undertaken, it is equally important to eliminate product variability to ensure clinical compliance of these therapies. Establishing serum-free and GMP compliant culture methods represents a critical next step in the development of superior and clinically compliant EDC therapies for phase III trials and for the clinic.

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