Use of a collagen I matrix to enhance the potential of circulating angiogenic cells (CACs) for therapy

Aleksandra Ostojic

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Abstract

Acute myocardial infarction (MI) is the end result of many cardiovascular diseases and is one of the leading causes of death in the western world. Cell therapy, using circulating angiogenic cells (CACs) or $CD34^+$ cells from peripheral blood, is one approach under investigation for restoring blood flow and function to the ischemic heart. However, the numbers of CACs and $CD34^+$ circulating cells are inversely proportional to the severity of cardiovascular disease and age; therefore, there is a need to increase their numbers and/or function for therapy. One possibility is to enhance the therapeutic potential of the cells with the use of a biomaterial. In this study, we used a collagen matrix to culture human $CD34^+$ circulating cells, and evaluated the effect of the matrix on $CD34^+$ cell properties and function. The matrix was able to successfully increase proliferation, migration, $CD34^+$ phenotype and branching in an angiogenesis assay. These functional benefits may be associated with the sonic hedgehog (Shh) pathway.

The collagen matrix was previously shown to enhance the function of healthy CACs, but its ability to do the same for CACs from coronary artery disease patients is unknown. In this study, the matrix was shown to enhance the viability, proliferation and angiogenic potential of patient CACs. Furthermore, gene expression for integrins and Shh pathway components in the sub-population of $CD34^+$ cells was similar between patient and healthy donors when isolated from CACs. This work provides insight into the mechanisms for the observed matrix-enhanced function of therapeutic CACs and $CD34^+$ cells from both healthy and CAD patient donors.

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List of Abbreviations

AMI	acute myocardial infarction
ASTAMI trial	Autologous Stem-cell Transplantation in Acute Myocardial Infarction trial
BM	bone marrow
BMMNCs	bone marrow mononuclear cells
BOOST trial	BOne marrOw transfer to enhance ST-elevation infarct regeneration
BrdU	5-bromo-2-deoxyuridine
CACs	circulating angiogenic cells
CAD	coronary artery disease
CD	Cluster of differentiation
CVDs	cardiovascular diseases
DAPI	4',6-diamidino-2-phenylindole
dPBS	distilled phosphate buffered saline
ECFCs	endothelial colony forming cells
ECM	extracellular matrix
EPCs	endothelial progenitor cells
EGM-2	endothelial growth medium -2
FACS	fluorescence-activated cell sorting
FBS	fetal bovine serum
FOV	fields-of-view
G-CSF	granulocyte-colony stimulating factor

HSCs	hematopoietic stem cells
IGF-1	insulin-like growth factor 1
LV	left ventricular
LVEF	left ventricular ejection fraction
MACS	magnetic activated cell sorting
MI	myocardial infarction
MNCs	mononuclear cells
MSCs	mesenchymal stem cells
NO	nitric oxide
PBMNCs	peripheral blood mononuclear cells
PCI	percutaneous coronary intervention
PDGF	platelet-derived growth factor
PFA	paraformaldehyde
PI	propidium iodide
PTCH1	patched 1
REGENT trial	Myocardial Regeneration by Intracoronary Infusion of Selected Population of Stem Cells in Acute Myocardial Infarction Trial
REPAIR-AMI trial	Reinfusion of Enriched Progenitor cells And Infarct Remodeling in Acute Myocardial Infarction trial
RT	room temperature
SDF-1α	stromal cell-derived factor 1α
Shh	sonic hedgehog
STEMI	ST-segment elevation myocardial infarction

SWISS-AMI	Swiss Multicenter Intracoronary Stem Cells Study in Acute Myocardial Infarction
TAL1	T cell acute leukemia 1
VEGF	vascular endothelial growth factor

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Statement of Contribution

Drs. Erik Suuronen and Marc Ruel came up with the design and concept of the project. Dr. Brian McNeill contributed to the design and concept of the $CD34^+$ cell study. Suzanne Crowe helped with peripheral blood mononuclear cell (PBMNC) isolations and ran the flow cytometry. Dr. McNeill assisted with migration and phenotype assays, proliferation of healthy donor CACs assay, as well as the RT-qPCR for the $CD34^+$ cell after culture on matrix and fibronectin from the whole population.

1.0 Introduction

1.1 General Introduction

1.1.1 Myocardial Infarction

Cardiovascular diseases (CVDs) are the leading cause of death worldwide (World Health Organization, 2011). CVDs encompass a number of diseases that affect the heart and the vascular system. One type of common CVD is coronary artery disease (CAD), which is caused by the buildup of cholesterol and plaque in the inner walls of the vessels (atherosclerosis) leading to the myocardium. This impedes the heart's ability to receive oxygen and ultimately may result in a myocardial infarction (MI). During an MI, there is a decreased amount of blood flow reaching the heart, triggering an ischemic cascade. This cascade will include an inflammatory response (Deten, Volz et al. 2002), cardiomyocyte necrosis due to lack of oxygen (Weisman, Bush et al. 1988, Narula, Haider et al. 1996), and degradation of the left ventricular (LV) extracellular matrix (ECM) by matrix metalloproteinases (Tyagi, Campbell et al. 1996, Thomas, Coker et al. 1998). These events progress to infarct expansion, wall thinning (Hutchins and Bulkley 1978), collagen deposition resulting in scar tissue formation, LV dilation and an overall decrease in the function of the heart (Jeremy, Hackworthy et al. 1987). As a result, the myocardium is not able to pump blood throughout the body as efficiently anymore. While current therapies, such as defibrillators, beta-blockers, stents, bypass surgery and heart transplants have significantly improved patient survival, they do not directly address and reverse the biological deficiency in cardiac function following MI. As a result, cell therapy is coming out as a prospective treatment to limit and/or reverse the damage post-MI.

1.1.2 Endothelial Dysfunction

Endothelial dysfunction is a common underlying condition in CAD patients. Endothelial dysfunction is a term describing abnormal activity of the endothelium, which includes the expression of proinflammatory adhesion molecules and the deregulated production of messenger molecules (Le Brocq, Leslie et al. 2008). Endothelial dysfunction plays a key role in CAD; the endothelium lies between the blood stream and the body's tissues and plays an active role in vasodilation. The endothelium responds to chemical and physical stimuli in order to produce a variety of organic and inorganic messenger molecules that can affect the physiology of the neighboring tissue. The endothelium also responds to triggers from inflammation, which activate the expression of receptors and adhesion molecules, also affecting the physiology of the neighboring tissues (Le Brocq, Leslie et al. 2008).

A key factor in the vasodilation process is nitric oxide (NO). In normal human vascular physiology, the role of NO is to inhibit inflammation, proliferation of cells and thrombosis, thus preserving quiescence of the vascular wall. Endothelial dysfunction correlates with the switch between NO-mediated silencing of the cellular processes to redox signaling which leads to endothelial activation and further inflammation (host-defense response) (Deanfield, Halcox et al. 2007). This causes an imbalance in the endothelial equilibrium and affects the body's progenitor cell populations, their ability to respond to signals from the damaged tissue and to participate in tissue repair (e.g. reduced migratory activity) (Vasa, Fichtlscherer et al. 2001).

A number of conditions can contribute to endothelial dysfunction, such as high cholesterol (Pirro, Bagaglia et al. 2008), diabetes (Krankel, Adams et al. 2005), a history of smoking (Michaud, Dussault et al. 2006) and advanced age (Kushner, Van Guilder et

al. 2010). The recurrent exposure to cardiovascular disease risk factors can deplete the endothelial cells' protective endogenous anti-inflammatory abilities. At this point the cells are not only dysfunctional, but the cells can also lose their integrity, resulting in anoikis (Woywodt, Bahlmann et al. 2002). Exposure to risk factors has additionally shown to cause dysfunction to cellular properties *ex vivo*, such as adhesion, migration and the ability to form tubules (Vasa, Fichtlscherer et al. 2001). Patients who are at risk for cardiovascular disease and have one of the previously listed conditions have overall worse endothelial function, possibly speeding up the path to cardiovascular failure. In particular, patients at high risk for cardiovascular disease exhibit dysfunctional circulating angiogenic cells (CACs) (Ward, Thompson et al. 2011).

Endothelial dysfunction not only affects patients by contributing to their CAD, but it also affects the capacity in which these cells can be used for autologous cell therapy. Since these cells are already dysfunctional, simply injecting them into a targeted location may not be beneficial; the cells may need to be enhanced in order to have a positive therapeutic effect.

1.2 Cardiac Regenerative Therapies

1.2.1 Cell Therapy

Stem cells are being investigated extensively for their therapeutic potential for regenerative medicine for CAD patients. Post-MI, the blood flow to the myocardium is interrupted and there is a loss of functional cardiomyocytes. The heart has a limited regenerative capacity and is not able to replace the lost cardiomyocytes. One of the most critical factors in rescuing the myocardium post-MI is to restore the blood flow (Creager, Kaufman et al. 2012). Stem cells are capable of extensive proliferation and can

differentiate into multiple cell types. Therefore, stem cells may be able to differentiate into cardiac cells, incorporate into the injured myocardium and help restore the blood flow. Additionally, stem cells may be able to help the recovery process via paracrine effects.

Of the multiple reparative cell types available, bone marrow mononuclear cells (BMMNCs) are leading the way in clinical trials. The BMMNCs can be used for treatment as a whole population or they can be further separated into subpopulations such as mesenchymal stem cells (MSCs), hematopoietic stem cells (HSCs) and endothelial/progenitor cells (EPCs) (Dawn and Bolli 2005). The ability of BMMNCs to differentiate into non-hematopoietic cell types is a debated topic (Orlic, Kajstura et al. 2001, Wagers, Sherwood et al. 2002, Chien 2004, Kucia, Dawn et al. 2004, Murry, Soonpaa et al. 2004). The more agreed upon mechanism by which BMMNCs contribute to cardiac repair is through paracrine effects (Surder, Manka et al. 2013).

The first randomized controlled trial using BMMNCs for acute myocardial infarction (AMI) patients was the BOne marrOw transfer to enhance ST-elevation infarct regeneration (BOOST) trial. While the trial showed improvement in LVEF in the treatment group (6.7% increase versus 0.7% in control group) at 6 months (Wollert, Meyer et al. 2004), there was no significant difference between the two study groups at the 5-year follow-up (Meyer, Wollert et al. 2009). However, with an additional sub-group analysis it was discovered that patients with more severe disease did have significant improvements in LV and left ventricular ejection fraction (LVEF) after cell therapy compared to the control treatment group. Another important trial was the Reinfusion of Enriched Progenitor cells And Infarct Remodeling in Acute Myocardial

Infarction (REPAIR-AMI) trial. This study enrolled a total of 204 AMI patients to take part in the randomized controlled trial. There were significant improvements in LVEF with the treatment group at 4 months and, once again, the patients with the worse infarcts showed the greatest improvement (Schachinger, Erbs et al. 2006). When examined at 5 years, there was a significant improvement in the recurrence of MI, death and revascularization bestowed by the intracoronary infusion of cells. Overall, it was found that the placebo group experienced adverse outcomes while the treatment group experienced an association between the migratory capacity (basal and stromal cell-derived factor-1) of administered BMMNCs and survival without episodes (Assmus, Leistner et al. 2014). A smaller trial, Autologous Stem-cell Transplantation in Acute Myocardial Infarction (ASTAMI) had less encouraging results. While there was an improvement in exercise capacity at the 3-year follow up with the treatment patient, there was no significant difference in LVEF (Beitnes, Hopp et al. 2009). Overall, findings from clinical trials have shown modest effects on LV function over time (Abdel-Latif, Bolli et al. 2007, Lipinski, Biondi-Zoccai et al. 2007, Martin-Rendon, Brunskill et al. 2008, Zimmet, Porapakkham et al. 2012). A recent large meta-analysis study that included 33 trials with 1,765 participants has found reasonable improvements in LVEF at both short- (3.26%) and long-term (3.91%) follow-up (Clifford, Fisher et al. 2012). A common trend that is appearing within the clinical studies is that patients with baseline LVEF below the median respond better to BMMNC therapy (Jones, Choudry et al. 2012). This is encouraging for patients who have worse initial conditions, but more consistent results are necessary for the rest of the patient population.

Despite the advances made with BMMNCs for cell therapy post-MI, there are still a number of challenges that exist. One challenge is determining the best time to deliver the cells. The TIME-trial looked at the delivery of cells at days 3 and 7 in patients with ST-segment elevation myocardial infarction (STEMI) treated with successful primary percutaneous coronary intervention (PCI). The study found no significant differences between the placebo group and the treatment group on recovery of global or regional left ventricular function (Traverse, Henry et al. 2012). In the Late TIME-trial, BMMNCs were injected 2-3 weeks post-MI in patients who had undergone successful PCI and had a LVEF ≤45%. The study found that the injection of BMMNCs at this time points did not improve the global or regional functions at the 6 months follow-up (Traverse, Henry et al. 2011). The Swiss Multicenter Intracoronary Stem Cells Study in Acute Myocardial Infarction (SWISS-AMI) aimed to directly compare the effects of BMCs transplantation at early (5-7 days) versus late (3-4 weeks) time points post-AMI in patients with ST-segment elevation myocardial infarction and LV dysfunction who already had successful reperfusion. It was found that the intracoronary BMMNCs infusion at either time point did not improve LV function at the 4-month follow-up. However, sub-analysis indicated that revascularization therapy may be the most efficient at 4.5 hours after the onset of symptoms (Surder, Manka et al. 2013). Therefore, revascularization therapy directly post-MI remains to be thoroughly evaluated. The lack of improvements in LVEF with BMMNC therapy at both early and late time points bring up the question of whether LVEF is the proper endpoint to assess the efficiency of these clinical trials (Traverse, Henry et al. 2011).

Other challenges with cell therapy are determining the optimal cell dosage and optimal cell type. A recent meta-analysis found that LVEF improvement was prominent when less than 10^9 (9×10^6-10^9) BMMNCs were injected. However, no conclusions could be made regarding infusion of $>10^9$ cells since only two studies were conducted with such high numbers of BMMNCS, and therefore could not be used as a form of a reliable evaluation (Jiang, He et al. 2010). BMMNC populations contain only small numbers of progenitor cells. This progenitor cell population consists of mesenchymal stem cells (Toma, Pittenger et al. 2002), multipotent adult mesenchymal progenitors (Jiang, Jahagirdar et al. 2002), hematopoietic stem cells (van Ramshorst, Rodrigo et al. 2011), endothelial progenitor cells (Urbich and Dimmeler 2004) and a small amounts of embryonic-like stem cells (Wojakowski, Tendera et al. 2009). Since the stem cells hold the most potential to aid in the recovery process post-MI, it may prove to be more beneficial to focus on gathering larger concentrations of a subpopulation of progenitor cells and injecting those in the ischemic myocardium, rather than delivering the BMMNCs populations as a whole.

Despite only modest improvements and challenges presented with stem cell therapy, it is important to note that the clinical trials with BMMNCs appear safe without adverse effects compared to the control groups and thus are still promising cells for cell therapy (Abdel-Latif, Bolli et al. 2007, Lipinski, Biondi-Zoccai et al. 2007, Martin-Rendon, Brunskill et al. 2008). With improvements made to timing of delivery, cell dosage, optimal cell types and improvements in enhancing function, BMMNCs hold the potential to act as a regenerative therapy post-MI.

1.2.2 Cluster of differentiation 34^{+} (CD34^{+}) cells

CACs have also been referred to as early EPCs. They are derived from the bone marrow (BM) and can be isolated from adult peripheral blood (Asahara, Murohara et al. 1997) via differential centrifugation. They are believed to be able to home to sites of neovascularization (Asahara, Murohara et al. 1997) and contribute to postnatal neovascularization (Isner and Asahara 1999). CACs are also able to function in a paracrine manner through the secretion of angiogenic factors that signal for the formation of new blood vessels (Asahara, Kawamoto et al. 2011). Additionally, the secretion of chemokines may enable further recruitment of endogenous cell populations to the ischemic regions to contribute to the repair process and thus improve cardiac function. These features are both important following MI because the hypothesis is that rapid restoration of blood flow to the ischemic tissue will limit the amount of tissue remodeling and cell death (Creager, Kaufman et al. 2012).

As heterogeneous bone marrow/peripheral blood cell populations have yielded modest results in clinical trials, there has been a shift to looking at individual cell types for therapeutic purposes. Of particular interest is the CD34^{+} cell population due to its pro-angiogenic properties (Asahara, Murohara et al. 1997). A lot is not understood about the mechanism by which CD34^{+} cells operate in tissue repair. The two most common beliefs are that CD34^{+} cells promote angiogenesis through direct incorporation into the newly developing vasculature or through paracrine effects. Some evidence, albeit conflicting, supports the ability of CD34^{+} cells to transdifferentiate into cardiomyocytes (Yeh, Zhang et al. 2003, Balsam, Wagers et al. 2004, Murry, Soonpaa et al. 2004, Iwasaki, Kawamoto et al. 2006) or to fuse with existing cardiomyocytes (Zhang, Wang

et al. 2004, Zhang, Shpall et al. 2007), thus making $CD34^+$ cells promising candidates for cell therapy in MI patients.

A number of animal studies have determined that $CD34^+$ cells are a good cell source for inducing recovery post-MI. In one study, $CD34^+$ cells were shown to have a therapeutic effect post-MI in athymic nude rats. Animals that received systematic injection of human-isolated $CD34^+$ cells exhibited a reduction in cardiomyocyte apoptosis, in collagen deposition and in infarct size (Kocher, Schuster et al. 2001), presumably due to increased blood flow to ischemic areas. Another study injected $CD34^+$ cells directly into ischemic tissue post-MI and through histologic analysis showed that the cells had incorporated into the peri-infarct zone and expressed lectin, demonstrating the $CD34^+$ cells' ability to differentiate to vascular cells (Kawamoto, Tkebuchava et al. 2003). Another report showed that the greatest attenuation of structural changes happened upon insertion of solely $CD34^+$ cells, even in comparison to a group of total mononuclear cells (MNCs) that had the equivalent number of $CD34^+$ cells present (Kawamoto, Iwasaki et al. 2006). This further suggested that a purified $CD34^+$ cell population may yield greater therapeutic outcomes than a mixed MNC population. A comparable trend was seen in clinical trials. The REGENT trial compared intracoronary infusion of $CD34^+CXCR4^+$ cells versus unselected MNCs in patients with AMI. The study found that patients with a worse baseline LVEF < median (37%) had a significant improvement in LVEF at 6 months. Additionally, the authors found that the same beneficial endpoints could be achieved whether using total MNCs or using 100-fold less $CD34^+CXCR4^+$ cells (Tendera, Wojakowski et al. 2009). The finding in the REGENT clinical study complements the results seen in rats (Kawamoto, Iwasaki et al.

2006) and strengthens the argument that cells selected for $CD34^+$ expression in comparison to total cells have a greater therapeutic potential for treatment post-MI.

In the past decade there have been a few clinical trials that have been centered on cell therapy using $CD34^+$ cells. For example, $CD34^+$ cells have been used for a phase I/IIa intractable angina clinical trial. Results showed an overall improvement in angina frequency, decreased NTG tablet use, increased exercise capacity and a reduction in the CCS class. Additionally, on the Seattle Angina Questionnaire, physical limitations, angina stability, angina frequency, disease perception and treatment satisfaction were more favourable for the $CD34^+$ cells treatment group at 3 months, with consistent results at 6 months, except for angina stability (Losordo, Schatz et al. 2007). $CD34^+$ cell treatment has also been used for refractory angina. This study additionally looked at the dosage concentration that is ideal for the most favourable effects. It was found that frequency of angina decreased and exercise improved at 6 and 12 months in the lower dosage treatments ($1x10^7$ cells/kg versus $5x10^7$ cells/kg) (Losordo, Henry et al. 2011). This data suggests that there is an optimal concentration for therapeutic purposes and that more is not necessarily better, at least under the administration protocol used in this study. $CD34^+$ cell transplantation has also been tested in non-ischemic dilated cardiomyopathy patients. At the 5 year follow-up, there was an increase in LVEF, and an increase in the 6 minute walk distance. It was also found that there were fewer deaths in the treated population. This finding is primarily attributable to the deaths that occurred with pump failure mortality (Vrtovec, Poglajen et al. 2013). As a whole, the results from clinical trials using $CD34^+$ cells have been promising.

It is important to note that $CD34^+$ cells are not a homogenous population; for example, CD34 expression is found on endothelial progenitor cells and on fully differentiated endothelial cells (although less intensely). This heterogeneity may mean that not all cells within the population are contributing equally to the therapeutic effects. A challenge presented with using $CD34^+$ cells as an autologous treatment for CAD patients is that the numbers of $CD34^+$ circulating cells are inversely proportional to the severity of cardiovascular disease and age (Vasa, Fichtlscherer et al. 2001, Taguchi, Matsuyama et al. 2004, Valgimigli, Rigolin et al. 2004). One way to overcome this problem is to treat patients with granulocyte-colony stimulating factor (G-CSF) as a form of mobilizing the cells into circulation. Despite G-CSF's ability to mobilize EPCs, including $CD34^+$ cells into circulation, it does not improve myocardial perfusion or angina in chronic ischemic heart disease when administered alone (Chih, Macdonald et al. 2012), suggesting that localization/targeting of the $CD34^+$ cell population to the myocardium may be required. $CD34^+$ cells only represent 0.5-5% of the bone marrow cell population (Pala, Mumcuoglu et al. 2013). Therefore, due to the reduced number and function of $CD34^+$ cells, strategies are needed to increase their numbers and ability to engraft and promote regeneration.

1.2.3 Cell Preconditioning

While stem cell therapy is a promising prospect for post-MI therapy, there are still a number of concerns that exist. One of these is the survival of stem cells once they are transplanted into the ischemic myocardium due to the hypoxic conditions, minimal blood supply, lack of nutrition, inflammation and oxidative stress (Lu, Li et al. 2012).

Fortunately, there a number of methods currently being investigated to increase the survival of stem cells post-transplantation.

One method is to precondition the cells pharmacologically as a means to activate the cells' survival pathways. For example, a common drug used is diazoxide. Diazoxide opens up the mitochondrial ATP-dependent potassium channel as a means of inhibiting apoptosis (Kis, Nagy et al. 2004). Studies have shown that treating mesenchymal stem cells with diazoxide resulted in increased cell survival (Afzal, Haider et al. 2010). Pre-conditioning cells also increases the release growth factors and cytokines that contribute to angiogenesis and cytoprotection, thus once again increasing cell survival as well as blood vessel growth (Lu, Li et al. 2012).

Another form of stem cell preconditioning is through the direct application of growth factors. Growth factors contribute to a number of biological processes, including angiogenesis and repair mechanisms via paracrine effects. One growth factor that has been tested is SDF-1. The pre-conditioning of EPCs with SDF-1 was able to inhibit apoptosis, while having a positive impact on vascular density and cell attachment via the SDF-1/CXCR4 signaling pathway (Zemani, Silvestre et al. 2008). A growth factor that has been used for the preconditioning of $CD34^+$ cells is VEGF2, due to its ability to reduce apoptosis through the activation of the AKT pathway. Post-MI treatment with VEGF2-treated $CD34^+$ cells in rats yielded an increase in capillary density, an improvement in fractional shortening and overall infarct size reduction (Shintani, Kusano et al. 2006). Additionally, growth factors can be used for preconditioning as a method of promoting cell differentiation (Bartunek, Croissant et al. 2007). The treatment

can be used to guide cells towards cardiomyocyte differentiation for example, and thus enhance the potential of the cells to contribute to regenerative therapy.

Transgenic over-expression and recombinant growth factors can also be used as a form of stem cell preconditioning (Dzau, Gnecchi et al. 2005). The benefit of over-expression of growth factors is that there is a continued release of said growth factors that are able to activate downstream pathways via paracrine, endocrine and autocrine mechanisms. An example is the over-expression of insulin-like growth factor 1(IGF-1). When mesenchymal stem cells were transfected with IGF-1 (via viral vectors), the cells were able to secrete 200× more IGF than control cells for a time period of 12 days and as a result was able to promote myocardial repair (Haider, Jiang et al. 2008). In another study, SDF-1α was non-virally transfected into skeletal myoblasts. The end result was an increase in stem and progenitor cell ($CD31^+$, $C\text{-}kit^+$, $CD34^+$ cells) migration to the myocardium, increased angiogenesis and activated cell survival signaling in the myocardium post-MI (Elmadbouh, Haider et al. 2007). Additionally, it is possible to combine the over-expression of multiple growth factors in order to promote greater survival and impact for therapy post-MI.

A popular form of cell preconditioning is environmental preconditioning where the cells are exposed to environments they may face upon transplantation. The concept, termed hormesis, is the short exposure of cells/tissues to stresses that result in quicker recovery post-acute stress (Calabrese, Bachmann et al. 2007); such strategies include oxidative, hypoxic and heat shock preconditioning. Hypoxic preconditioning exposes the cells to an ischemic-like environment and gives the cells an opportunity to adapt. For example, culturing MSCs in hypoxia resulted in increased survival, reduced infarct size

and enhanced angiogenesis in rat MI models (Hu, Yu et al. 2008). Additionally, short-term exposure to oxidative stresses at low concentrations and heat shock treatments enhanced the survival of stem cells (Sart, Ma et al. 2014).

Studies have shown that preconditioning stem cells as aggregates promotes ECM secretion. The culture of cell aggregates results in a gradient of diffusion of oxygen and nutrients that resembles an ischemic environment (Van Winkle, Gates et al. 2012). The cell-to-cell contact promotes the secretion of endogenous ECM proteins thus preventing anoikis (Bartosh, Ylostalo et al. 2010). Additionally, a number of other factors are secreted that promote cell proliferation, integrin signaling and survival (Sart, Ma et al. 2014).

The encapsulation of stem cells has the potential to lower invasiveness, while increasing the cell survival in *in situ* delivery (Ye, Zhou et al. 2011). There are two main methods being investigated for cell encapsulation. One method is having a liquid core that surrounds the cells, that is itself surrounded by a semi-solid membrane; the second method is to embed single cells or aggregates within hydrogels (Radhakrishnan, Krishnan et al. 2014). The hydrogels protect the cells and limit the inflammation and immune responses to the cells upon transplantation, and thus are capable of improving cardiac function (Sart, Ma et al. 2014).

In a similar fashion, stem cells can also be cultured on biomaterials as a form of preconditioning. The exposure of cells to components of the ECM may activate pathways that contribute to regeneration and cell survival post-MI. Once preconditioned, the cells can then be inserted into the ischemic myocardium with or without a delivery biomaterial. Our group has shown that CACs cultured on a thermosensitive collagen

type I matrix had improved proliferation, greater angiogenic and migration potential, enriched endothelial and CAC phenotypes *in vitro*, and they were able to restore blood flow in a hindlimb ischemia mouse model (Kuraitis, Hou et al. 2011).

1.3 Pathways

1.3.1 Integrins

Integrin receptors are a family of heterodimeric transmembrane proteins that are comprised of an alpha and beta subunit. There are 18 different alpha and 8 different beta subunits that can come together to form a total of 24 different integrin receptors (Brakebusch, Bouvard et al. 2002, Hynes 2002). Integrin-mediated cell adhesion to the ECM triggers a number of signaling pathways including those involved in migration, cell survival, differentiation, growth, tissue repair and inflammatory response amongst others (Niu and Chen 2011). Integrins are able to communicate in an outside-in and an inside-out signaling manner through the plasma membrane (Hartmans, Jansen et al. 1991). Integrins bind to a number of ECM components, and out of 24 integrin receptors, only 4 are known high affinity collagen-binding integrins, $\alpha1\beta1$, $\alpha2\beta1$, $\alpha10\beta1$ and $\alpha11\beta1$ (Leitinger 2011).

A number of integrins have been identified to play an important role in angiogenesis and/or the health of the myocardium. Manipulating these integrins within the ischemic myocardium could prove to be beneficial in improving vascularity and perfusion, resulting in decreased necrosis. For example, one study showed that $\alpha4$, which is expressed on hematopoietic stem cells was necessary for survival in mice (Williams, Rios et al. 1991). Specifically, the loss of $\alpha4$ resulted in defects in the formation of the epicardium and in coronary artery development. This, in combination

with the defect present in the placentation process, leads to embryonic lethality demonstrating the importance of α4 (Yang, Rayburn et al. 1995). Additionally, integrin α4β1is able to selectively stimulate the homing of circulating progenitor cells to neovascular tissue (Jin, Aiyer et al. 2006). Another study showed that tumours grown in α1β1-deficient mice had less angiogenesis occurring, suggesting that α1β1 is implicated in angiogenesis (Pozzi, Moberg et al. 2000). Integrin α5β1 was shown to be induced in response to angiogenic stimuli (Boudreau and Varner 2004), and to play a role in promoting endothelial cell migration (Kim, Harris et al. 2000). Integrin αVβ3 has also been associated with angiogenesis (Brooks, Clark et al. 1994). In endothelial cells, integrin αVβ3 is involved in cell survival and migration during the angiogenesis process (Brooks, Clark et al. 1994, Brooks, Montgomery et al. 1994). αVβ5 (Friedlander, Brooks et al. 1995), α6 (Lee, Seng et al. 2006) and α9β1 (Vlahakis, Young et al. 2007) have also been shown to be involved in angiogenesis.

In a study that came from our lab, it was observed that the culture of CACs on the collagen matrix resulted in an altered integrin profile (Ahmadi, McNeill et al. 2014). Of particular interest, it was found that integrin α2β1 was involved in the proliferation, adhesion and paracrine properties of matrix-cultured CACs, while integrin α5 played a role in the CACs' angiogenic properties *in vitro*. These results suggest that the matrix is able to enhance the CAC function via the α2β1and α5 signaling pathways (Ahmadi, McNeill et al. 2014). These studies used healthy donor CACs, but the ability of the matrix to enhance the function of CACs from CAD patients is yet to be explored.

1.3.2 TAL1

The T cell acute lymphoblastic leukemia 1(TAL1) gene, also referred to as stem cell leukemia (SCL), is necessary for the establishment of all blood lineages and endothelial development (Robb, Lyons et al. 1995, Shivdasani, Mayer et al. 1995, Visvader, Fujiwara et al. 1998), and also for early endocardial morphogenesis (Bussmann, Bakkers et al. 2007). Recently, it has been discovered that TAL1 plays an essential role in the vascular repair potential of human endothelial colony forming cells (ECFCs) (Palii, Vulesevic et al. 2014). An *in vivo* mouse ischemia model was used to demonstrate that TAL1 serves an angiogenic purpose in ECFCs; when TAL1 was knocked down in ECFCs, there was a lack of engraftment to the ischemic tissue and blood flow recovery was not achieved. TAL1 was also found to activate a transcriptional program for adhesion and migration in ECFCs (Palii, Vulesevic et al. 2014). This complements the work done with TAL1/SCL mutant embryos that showed defective migration causing aggregation of the endocardial cells in the ventricular pole of the myocardium (Bussmann, Bakkers et al. 2007). Thus TAL1 plays an important role in migration in prenatal and postnatal cells. Migration is necessary in order for cells to be able to respond to the host environment, and in the case of myocardial infarction, to migrate to the ischemic regions and participate in the revascularization process.

1.3.3 Sonic Hedgehog (Shh) Pathway

The hedgehog gene family was discovered over 3 decades ago and was found to play a critical role in cell growth and differentiation (Shahi, Afzal et al. 2010). For the Shh pathway to be activated, a Shh ligand needs to bind to Patched at the cell membrane to switch on the pathway. At this point the Patched receptor releases its hold on the G-

coupled transmembrane Smoothened protein, which allows Smoothened to freely incorporate into the primary cilium membrane. In the primary cilium, Smoothened can relieve Gli from microtubule association, allowing translocation of the Gli activators (Gli1 and Gli2) into the nucleus, resulting in activation of a number of target genes. In the off state, Patched maintains it association with Smoothened and the Gli activators are degraded or the Gli repressor (Gli3) is produced, thus leading to the repression of the target genes (Beachy, Hymowitz et al. 2010).

Shh is an angiogenic morphogen (Pola, Ling et al. 2001) that plays a role in heart development in mammals by promoting angiogenesis and cardiomyoblast proliferation throughout the secondary heart field development (Lavine, White et al. 2006, Dyer and Kirby 2009, Dyer, Makadia et al. 2010). Reports have shown a close relationship between the hedgehog signaling pathway and coronary artery diseases. There is an up-regulation of Shh in the ischemic myocardium, in both acute and chronic MI; and preservation of left ventricular (LV) function can be obtained with Shh gene transfer (Kusano, Pola et al. 2005). In adult mice, the absence of Shh signaling leads to the loss of coronary vasculature, causing tissue hypoxia, cardiomyocyte cell death and heart failure, ultimately leading to lethality (Lavine, Kovacs et al. 2008). Alongside these examples, Shh partakes in the recovery process in postnatal ischemic injuries (Pola, Ling et al. 2003, Kusano, Pola et al. 2005, Straface, Aprahamian et al. 2009). Overall, these examples showcase the importance of Shh signaling in promoting angiogenesis and its potential to aid revascularization of the ischemic myocardium. Additionally, Mackie, Klyachko et al., showed that transfecting Shh into the angiogenic $CD34^+$ cell population produced cells that were capable of preserving heart function when transplanted to the

myocardium post-MI (Mackie, Klyachko et al. 2012). Furthermore, the authors of this work reported increased retention of the Shh $CD34^+$ cells at 24h, but not at 5 days compared to the control $CD34^+$ cells. Therefore, given the observed functional enhancements (e.g. migration, proliferation, angiogenesis, retention) seen in CACs interacting with our matrix (Kuraitis, Hou et al. 2011; Ahmadi, McNeill et al. 2014), the Shh pathway may be involved.

1.4 Research Plan

1.4.1 Rationale

Cells from CAD patients are dysfunctional; therefore strategies are needed to improve the therapeutic potential of these cells if they are to succeed in autologous cell therapies. The use of biomaterials is a promising approach to achieve this objective. Type 1 collagen is the most abundant component of the ECM and the most abundant protein within vertebrates (San Antonio, Iozzo et al. 2006). Culturing CACs on a collagen I matrix has been shown to improve their migratory potential, differentiation and survival *in vitro* and matrix-cultured CACs are superior in their ability to restore blood flow in a hindlimb ischemia mouse model (Kuraitis, Hou et al. 2011). The enhanced function of healthy CACs exposed to the collagen matrix depends on their interaction with the matrix through specific integrin receptors (Ahmadi, McNeill et al. 2014). In the present study we examined whether the collagen matrix can also improve the function of the $CD34^+$ cell fraction of CACs, since they are considered to be the most pro-angiogenic sub-population. Furthermore, the ability of the matrix to restore or improve the function of CACs obtained from CAD patients was investigated.

1.4.2 Aims and Objectives

Given the above rationale, two main aims were pursued. Aim 1 was to determine the effects of a collagen I matrix upon the isolated pro-angiogenic $CD34^+$ cell population within CACs. Aim 2 was to determine the ability of the matrix to restore/improve the function of CACs from CAD patients. Specifically the objectives for aim #1 were to:

1. Determine if the matrix could enhance the function of $CD34^+$ cells, including proliferation, viability, angiogenesis, adhesion and migration.
2. Determine some of the potential pathway(s) involved in the observed functional benefits.

The objectives for aim #2 were to:

1. Investigate the effects of culturing patient CACs on collagen I matrix by evaluating changes in function including angiogenesis, viability, proliferation, and adhesion.
2. Determine which integrins are differentially expressed in CACs and $CD34^+$ cells between healthy donor and heart disease patients, and how these are affected by different culture conditions.

1.4.3 Hypotheses

For aim #1, it was hypothesized that culturing $CD34^+$ cells on a collagen I biomaterial would enhance their function through the activation of the Shh pathway.

For aim #2, it was hypothesized that the matrix would enhance the function of CAD patient CACs, in part through the restoration of the cells' integrin profile.

2.0 Materials and Methods

In order to determine the effect our collagen I matrix had on the $CD34^+$ cell population we conducted a number of functional assays including adhesion, proliferation, viability, maintenance of phenotype and migration. After seeing the beneficial effects of culture on matrix, we investigated if the Shh pathway was affected using RT-qPCR. Furthermore, we investigated whether culture of $CD34^+$ cells on their own or with other CACs had a greater affect on the Shh pathway. Finally we investigated if the secretome from the $CD34^+$cells on matrix had any effects on angiogenesis.

Upon the confirmation that the matrix can enhance the $CD34^+$ cell population, complementing previous results that the matrix can enhance the total CAC population from healthy donors (Kuraitis, Hou et al. 2011), we investigated if the matrix was capable of enhancing the dysfunctional CAD patient CAC population. Firstly we conducted adhesion, angiogenesis, proliferation and viability assays on our cells to determine the effect matrix had on the cells. Afterwards we investigated the gene expression of a number of integrins and TAL1 on PBMNCs, CACs and after culture on matrix and fibronectin to identify any differences between CAD patient and healthy donor CACs. We also investigated the differences in gene expression between healthy donor and CAD patient $CD34^+$ cells isolated from PBMNCs and CACs in order to investigate any differences between the populations and to hypothesize the outcome of culturing $CD34^+$ cells from CAD patients on matrix.

2.1 CAC Isolation & Culture

Methods were approved by the Human Research Ethics Board of the University of Ottawa Heart Institute. Approximately 50 ml of peripheral blood was collected from informed CAD patients and approximately 100 ml was collected from informed healthy donors into EDTA vacutainer tubes. See Table 1 for inclusion/exclusion criteria for CAD patients enrolled in the study and Table 2 for recruited patient characteristics. Peripheral blood mononuclear cells (PBMNCs) were isolated by density-gradient centrifugation using Histopaque 1077 (Sigma). Following centrifugation, PBMNCs contained within the buffy coat were removed with a pipette, and washed twice with distilled phosphate buffered saline (dPBS). Cells were plated on fibronectin-coated tissue culture polysterene (TCPS; 20 ug/ml) with 10ml of Endothelial Growth Medium - 2 (EGM-2; Clonetics; FBS, hEGF, R^3-IGF-1 and VEGF supplements added). After 4 days in culture, the supernatant and non-adherent cells were aspirated, and the adherent cells were lifted with dPBS and considered to be CACs.

Table 1 - Inclusion/Exclusion Criteria for CAD Patient Study

Criteria	**Inclusion**	**Exclusion**
Sex	Male	Female
Age	>50	<50
CABG	Yes	No stenosis of vessels
EP	Yes- end stage failure, require defibrillator	N/A
Control Patient	Healthy male	Sick or female

CABG: Coronary Artery Bypass Graft; EP: electrophysiology

Table 2 - Patient Recruitment Characteristics

Number Recruited	42
Mean Age	63.7
CABG Patients	35
EP Patients	7
Number of Diabetics	10
Number of Smokers	9 (2 undocumented)
Number of Diabetic Smokers	1

CABG: Coronary Artery Bypass Graft; EP: electrophysiology

2.2 Biomatrix Preparation

The collagen matrix was prepared as previously described with some modifications (Kuraitis, Hou et al. 2011). Porcine collagen type I (3.3 ml of 0.375%; Nippon) was mixed with 500 µl of buffer (28 ml buffer made from: 9 ml 10× HEPES, 9 ml 10× DMEM, 10 ml FBS and a 50 ul of gentamycin (50mg/ml)) in a glass tube on ice. Chondroitin sulfate-C (CS-C; 40% w/v; final concentration of 11.49 mg/ml; Wako) was then added. Glutaraldehyde (1.5%) in 1× DMEM was added to cross-link the mixture and left on ice for 45 min. Glycine (20%) was added to react with excess aldehydes and incubated on ice for at least 45 min. The matrix solution was applied to plates (10cm tissue culture dish) and wells (12-, 24-, and 48- well plates) and left at 37°C for at least 1 hour to solidify. Upon gelation, the gels were washed twice with PBS before use.

2.3 CD34$^+$ Isolation

The EasySep^{TM} Human CD34 Positive Selection Kit (StemCell Technologies) was used to isolate CD34$^+$ cells according to the manufacturer's protocol. Briefly, CACs were lifted from culture plates and incubated with a CD34$^+$ antibody for 15 minutes at room temperature (RT). Magnetic beads targeting the CD34$^+$ antibody were added and cells were incubated for another 10 minutes at RT. Approximately 2.5 ml of EGM-2

supplemented with growth factors (Lonza) was added to the cells and they were put in a EasySep magnet (StemCell Technologies); following a 5 minute incubation in the magnet, unbound cells were collected and remaining cells and beads were washed 2× with EGM-2. Each wash consisted of a 5 minute incubation at room temperate followed by a 5 minute incubation in the EasySep magnet. Cells left in the tube were considered $CD34^+$ cells and the unbound cells were considered to be $CD34^+$ cells. FACS analysis showed that over 80% of the cells isolated in the bound fraction were $CD34^+$ cells.

2.4 Cell Phenotype Analysis

After 4 days on fibronectin, CACs were lifted with dPBS and isolated for $CD34^+$ cells. In a 12-well plate, $CD34^+$ cells were cultured on either fibronectin or matrix at a concentration of 2×10^5 – 2.5×10^5 cells. After 4 days, the cells were lifted and 100,000 cells were stained for CD34 for 30 minutes with a PE-CD34 antibody (10ul/million cells; Cell Signaling) at 4^oC in dPBS, then washed and resuspended in 0.5% BSA in dPBS and analyzed by flow cytometry (FACSAria™, BD Biosciences).

2.5 Proliferation Assay

Cells were lifted from fibronectin plates, counted and then 4×10^4 cells were plated on fibronectin or matrix coated plates (48-well). After 24 hours, the cells were then treated with 5-bromo-2-deoxyuridine (BrdU; 4ug/ul). BrdU is a thymidine analog and is taken up by the cells in lieu of thymidine, thus indicating proliferation. After 48h, cells were fixed with 4% paraformaldehyde (PFA) and followed with 3 washes with dPBS. Cells were permeabilized with 0.5% Triton X-100 in dPBS for 10 minutes, followed by 3

washes with dPBS. Cells were blocked with 10% fetal bovine serum (FBS) for 1 hour at RT. Cells were treated with DNase 1 (1.8U/ul) for 1 hour at 37°C and then washed 3× with dPBS. Cells were stained for Ki67 (rabbit-derived antibody; 1:500) and BrdU (mouse-derived antibody; 1:500) overnight at 4°C. The following day, cells were washed 3× with dPBS and then secondary antibodies Alexafluor488 (anti rabbit; 1:600) or Alexafluor 546 (anti mouse; 1:600) for 1 hour at RT. Cells were washed 3× with dPBS. Cells were stained with 4',6-diamidino-2-phenylindol (DAPI) for 5-10 minutes at RT, followed by 1 wash with dPBS and then were imaged at 40× magnification using a Zeiss Z1 fluorescence microscope. Experiments were done in triplicate and 3 random fields-of-view (FOV) were imaged for each well.

2.6 Live/Dead Assay

CACs and CD34^{+} cells were lifted from fibronectin plates and $2×10^{5}$ cells were plated per well in 12-well plates coated with fibronectin or matrix. Cells were placed in hypoxic conditions in EGM-2 without serum or growth factors for 48h. Cells were then stained using the Live/Dead Cell Double Staining Kit (Sigma-Aldrich). Briefly, cells were washed with dPBS, and then stained with 0.2% Calcein-AM and 0.05% Propidium Iodide (PI) in dPBS for 30 min at RT, to identify live and dead cells, respectively. Cells were rinsed with dPBS and imaged with a Zeiss Z1 fluorescence microscope. Both Calcein-AM and PI can be excited with 490nm and therefore could be observed simultaneously at the same excitation wavelength. Live and dead cells were counted in 5 random FOV.

2.7 Adhesion Assay

After 4 days on fibronectin, cells were lifted and replated for 4 days on fibronectin or matrix coated plates. A total of 1×10^4 cells were resuspended in 1 ml EGM-2 and seeded in 24-well dishes coated with fibronectin or matrix. After 1 h, media was aspirated and the adherent cells were fixed with 4% PFA. Wells are washed twice with dPBS and DAPI was used to stain the nuclei. Three random FOV were imaged using a Zeiss Z1 fluorescence microscope, and DAPI positive cells were counted to determine the number of adherent cells

2.8 Angiogenesis Assay

After 4 days on fibronectin, cells were lifted and replated for an additional 4 days on fibronectin or matrix coated plates. ECMatrix™ (Millipore) was prepared and 15 μl was added to μ-Slide Angiogenesis (Ibidi) slides and incubated for 1 h at 37°C to allow gelation. DAPI-stained CACs (5×10^3 cells from fibronectin or matrix culture) were then added with 5×10^3 of human umbilical vein endothelial cells (HUVECs) to each μ-Slide well containing the ECMatrix and cultured for 16-18 hours. DAPI positive cells that incorporated into the capillary network formation were counted in 3 random FOV captured with a Zeiss Z1 fluorescence microscope. Total tubule branching was also calculated for some experiments. Prior to their use in the angiogenesis assay, the HUVECs were maintained in HUVEC media (Life Technologies; M200 medium supplemented with FBS, hydrocortisone, hEGF, bFGF and heparin).

2.9 Angiogenesis Assay - Tubule Branching with Hypoxic Media

After 4 days on fibronectin, $CD34^+$ cells were isolated from the CAC cultures. $CD34^+$ cells (2×10^5) were plated on either fibronectin or matrix coated 12-well plates. The plates were maintained in normoxic conditions for 3 days. On day 3, the complete EGM-2 media (Lonza) was removed and replaced with EGM-2 without any supplements. Cultures were then placed in a hypoxic incubator (1% O_2, 5% CO_2) for 24 h. After 24 h, the media was removed and used in an angiogenesis assay. Briefly, the angiogenesis assay was conducted as described above, with the exception that 1×10^4 HUVECs were plated per well and were resuspended in the hypoxic media instead of M200 medium. The cells were visualized using a Zeiss Z1 fluorescence microscope, and branching was quantified in 3 random FOV.

2.10 Migration Assay

After 4 days on fibronectin, cells were lifted and replated on fibronectin or matrix coated plates for an additional 4 days. Using a modified Boyden Chamber migration assay, 5×10^3cells from the fibronectin or matrix coated plates were stained with DAPI and added to the upper chamber in serum-free media. The lower chamber was supplemented with 50ng/ml of VEGF. After 48h, cells that migrated to the lower chamber were counted using a hemocytometer.

2.11 RNA extraction, cDNA synthesis and quantitative PCR

Total RNA extraction from cells was performed using a High Pure RNA Isolation Kit (Roche) or TRI Reagent (Zymo Research), following the manufacturers' protocols.

Briefly, for the High Pure RNA Isolation Kit, the cells were lysed and the RNA was bound to the filter. The filter was washed 3 times and the RNA was eluted into a clean centrifuge tube. For the TRI Reagent protocol, TRI Reagent was added to the cells. Chloroform (200 µl) was then added to each centrifuge tube and the samples were incubated for 5-10 minutes at RT. Samples were spun at 12000g, and the top phase was transferred to 500 µl of isopropanol in clean centrifuge tubes. Samples were inverted and incubated overnight at 4^oC. The next day, samples were spun at 12000g, the supernatant was removed, 750 µl of 70% cold ethanol was added and the samples were spun for 5 minutes at 7500g. The supernatant was removed and samples were left at RT ≈5 minutes to dry. Nuclease free water (30 µl) was added to the samples and they were heated at 65^oC for 5 minutes to yield the final solution of RNA. First strand cDNA synthesis was performed using approximately 0.3mg of total RNA and SMARTScribe™ Reverse Transcriptase (Clontech). The samples were prepared for qPCR with LightCycler® 480 SYBR Green I Master (Roche) using the primer sequences in Table 2. The reactions were conducted and analyzed on a LightCycler® 480 (Roche) using LightCycler® 480 data software (Roche). The comparative Ct method was used to calculate relative gene expression (Pfaffl 2001). Results were normalized to 18S.

2.12 Statistical Analysis

Due to donor variability, most results were reported as a mean fold-change between the treatment and control conditions ± standard error mean, or as a mean percentage of treatment/control ± standard error mean. Statistical analysis was performed using paired *t*-tests. Additionally, RT-qPCR results comparing CAD patient cells to healthy cells

were reported as a fold-change between healthy and donor cells ± standard error mean and were analyzed using a student *t*-test. P<0.05 was considered to be statistically significant.

Table 3 - qPCR Primer Sequences

Gene	FORWARD PRIMER, 5' to 3	REVERSE PRIMER, 5' to 3'
18S	CGGCTACCACATCCAAGG	CTGGAATTACCGCGGCT
Alpha 1	CGTGGATAGACTGGCCAAA	CATTTATCATGGAAGTGGCAAG
Alpha 2	GCCGAGCTTCCATAAAATTG	ACCTGATGAGAAAGCCGAAG
Alpha 4	CATGAACAGTCAGCTTAACCTCA	TTAGCTTTCTCCTGGATGTGAG
Alpha 5	CTGGAGGCTTGAGCTGAGT	CAAGGCAGAAGGCAGCTATG
Alpha V	TCTGACTGCTGGTGCACACT	GCCAGGTGGTATGTGACCTT
Beta 1	TAGCTTTAAAACCTGTGTGCCA	AACTGTCAACACTCTTGTCTTCCA
VEGFA	CCTTGCTGCTCTACCTCCAC	ATGATTCTGCCCTCCTCCTT
Ptch1	TAGCCCTGTGGTTCTTGTCC	TGTGGTCATCCTGATTGCAT
Cyclin D1	TTCAACCCACAGCTACTTGG	ACAGCGCTATTTCCTACACC
Cyclin E	AGGGGACTTAAACGCCACTT	CCTCCAAAGTTGCACCAGTT
Hes 1	GGCTAAGGTGTTTGGAGGCT	GGTGGGTTGGGGAGTTTAGG
TAL1	ACCAAAGTTGTGCGGCGTAT	AGGCCCCGTTCACATTCTG
Shh	CCAATTACAACCCCGACATC	CTCTGAGTGGTGGCCATCT

3.0 Results

3.1 Aim 1.

The first aim of the project was to determine the beneficial effects the matrix on the pro-angiogenic $CD34^+$ cell population within CACs. Several *in vitro* assays were performed as presented below to identify how the matrix can enhance the function of $CD34^+$ cells isolated from healthy donor blood.

3.1.1 $CD34^+$ Cell Migration

Angiogenic cells from CAD patients usually exhibit abnormal migratory capacity (Vasa, Fichtlscherer et al. 2001). Therefore, increasing the migration potential of therapeutic cells from these patients may lead to their increased participation in the revascularization process. The matrix was investigated for its potential to increase the migration potential of $CD34^+$ cells. $CD34^+$ cells cultured on matrix and fibronectin were added to serum-free media in the upper chamber of a modified Boyden Chamber, while the lower chamber was supplemented with VEGF for 48 hours. Cells that migrated to the lower chamber were counted with a hemocytometer and it was found that $CD34^+$ cells from matrix had a 2.5-fold increase in migration towards VEGF over cells cultured on fibronectin (Fig. 1; $p<0.01$).

3.1.2 $CD34^+$ Cell Proliferation

$CD34^+$ cells represent a small percentage of the total peripheral blood cell population (Pala, Mumcuoglu et al. 2013). Therefore a method to increase their proliferation would result in a greater number of cells for use in therapy. The ability of the matrix to enhance proliferation was investigated. After 48 hours of culture, $CD34^+$ cells from the matrix

had a 2-fold increase in proliferation compared to fibronectin-cultured cells (Fig. 2A; $p=0.01$), as determined by Ki67 staining. This was confirmed by BrdU incorporation experiments, which showed a 1.8-fold increase in proliferation for matrix- versus fibronectin- cultured CD34$^+$ cells (Fig. 2A; $p=0.01$). When looking at cells on the substrates after 48 hours, CD34$^+$ cells on matrix appeared to cluster and have more proliferation within the clusters (Fig. 2B), whereas the CD34$^+$ cells cultured on fibronectin were more dispersed and less proliferative (Fig. 2C).

3.1.3 CD34$^+$ Cell Phenotype

In addition to proliferation, the maintenance of the CD34$^+$ phenotype may be an important factor in determining the cells' therapeutic potential. The number of CD34$^+$ cells that can be obtained from donor blood samples is low, therefore, the ability of the matrix to expand and maintain the CD34$^+$ cell phenotype was investigated. After 4 days of culture on matrix or fibronectin, there was a 2.8-fold increase in the number of CD34$^+$ cells on the matrix compared to fibronectin (Fig. 3; $p<0.01$).

3.1.4 CD34$^+$ Cell Viability

Therapeutic cells have trouble surviving the harsh environment post-transplantation in MI subjects (Lu, Li et al. 2012). The matrix was evaluated for its ability to enhance the viability of CD34$^+$ cells in hypoxia and serum-deprived conditions. CD34$^+$ cells were cultured on collagen and fibronectin in hypoxic conditions for 48 hours in serum-free media without any growth factors. After 48 hours, cells were stained with Calcein-AM for live cells and Ethidium homodimer-1 for dead cells. The CD34$^+$ cells cultured on

matrix had a trend towards increased survival, 8% greater than cells cultured on fibronectin (Fig. 4; p=0.07).

3.1.5 CD34$^+$ Cell Angiogenesis

Since therapeutic cells from CAD patients typically have a reduced efficacy in promoting vascularization, a method to enhance their angiogenic potential is needed. The collagen I matrix was investigated for its ability to increase the angiogenic potential of CD34$^+$ cells. CD34$^+$ cells were cultured on matrix and fibronectin for 4 days. Following the cultures, 5,000 CD34$^+$ cells were plated with 5,000 HUVECs overnight on Matrigel. There were no significant differences in the number of CD34$^+$ cells incorporated in the HUVEC capillary-like structures formed (Fig. 5A). However, the HUVECs co-cultured with CD34$^+$ cells from the matrix had a 1.6-fold increase in branching compared to HUVECs co-cultured with CD34$^+$ cells from fibronectin (Fig. 5B; p=0.01).

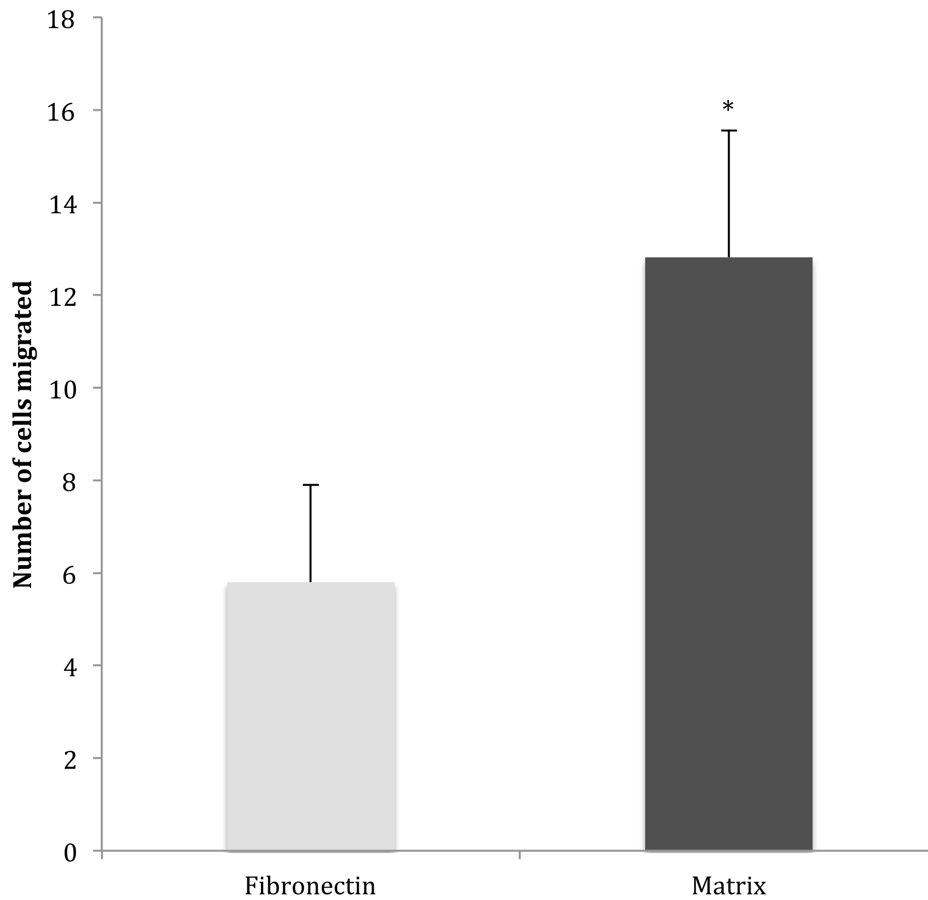
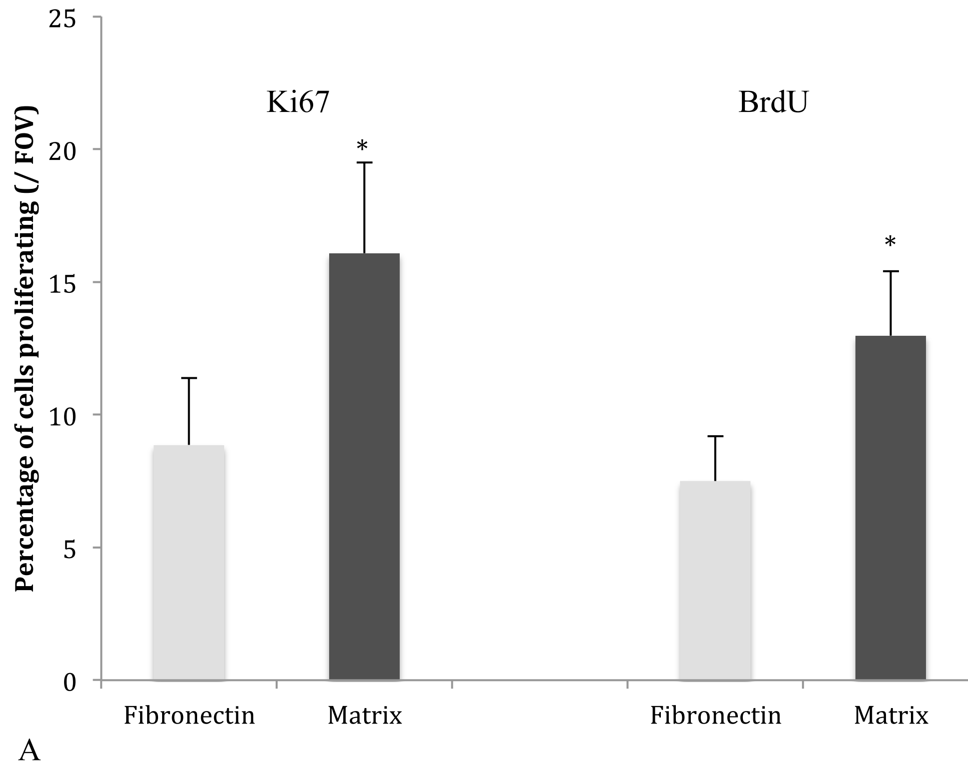


Figure 1. Matrix culture increases the migration of $CD34^+$ cells towards VEGF. PBMNCs were isolated and cultured on fibronectin for 4 days. The adherent CACs were lifted, $CD34^+$ cells were isolated and then re-plated on matrix or fibronectin for 4 days. $CD34^+$ cells were then lifted and 5,000 cells were placed per Boyden Chamber for 24 hours. n=5; *p<0.01.



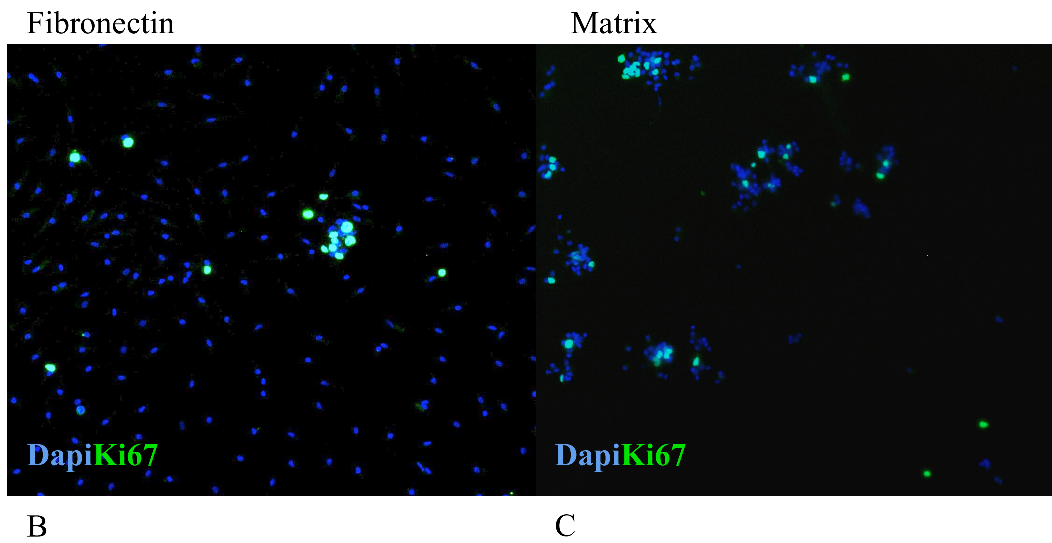


Figure 2. Matrix culture increases proliferation of CD34$^+$ cells.
PBMNCs were isolated and cultured on fibronectin for 4 days. CACs were lifted, CD34$^+$ cells were isolated and re-plated on matrix or fibronectin for 48 hours. A) Proliferation was analyzed by Ki67 stain and BrdU incorporation. n=6; *p=0.01 B) CD34$^+$ cells were dispersed evenly on fibronectin and C) clusters were observed on matrix.

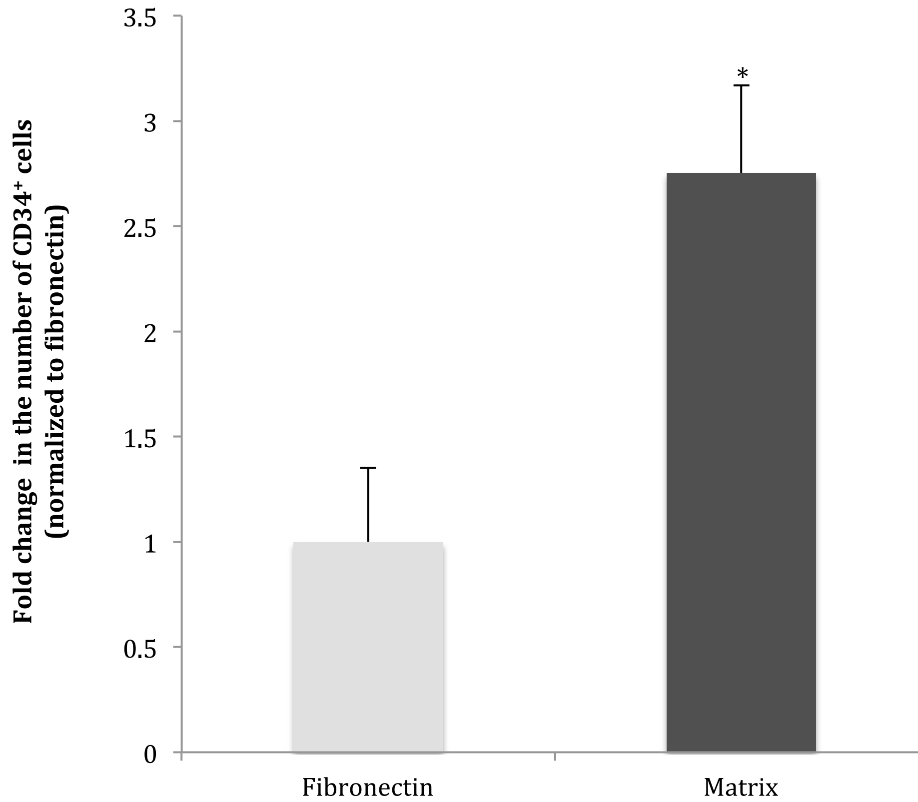


Figure 3. Matrix culture increases the number of $CD34^+$ cells.
PBMNCs were isolated and cultured on fibronectin for 4 days. CACs were lifted, $CD34^+$ cells were isolated and re-plated on matrix or fibronectin for 4 days. Cells were then lifted and analyzed by flow cytometry for CD34 expression. n=5; *p<0.01.

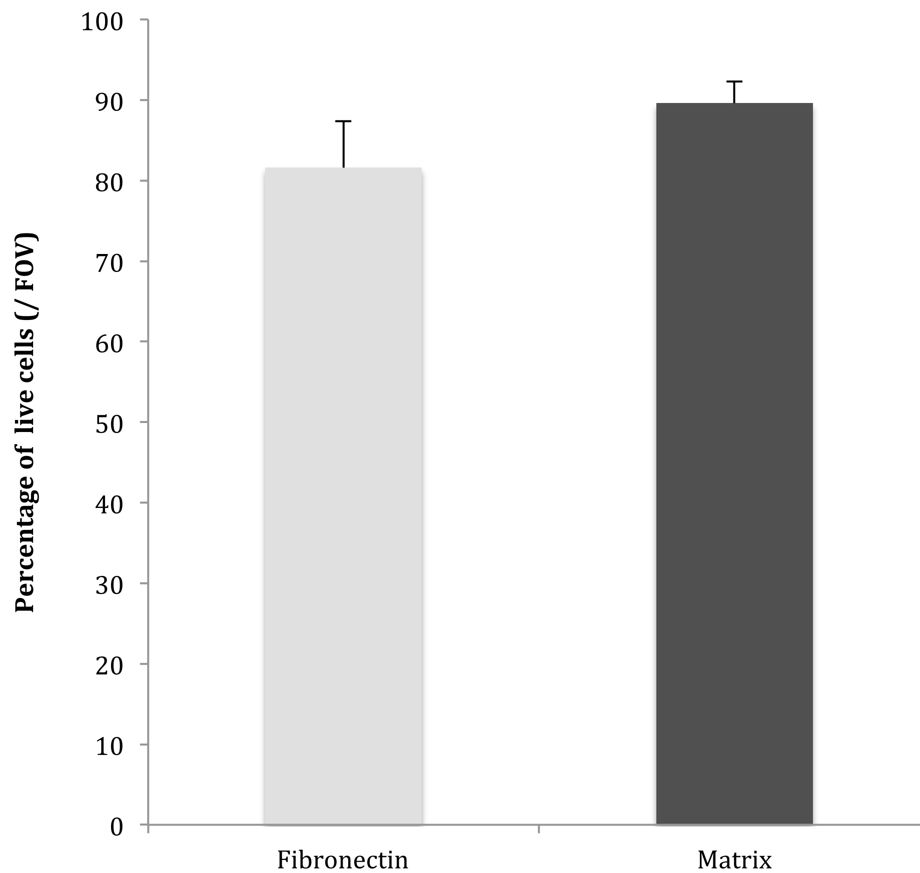
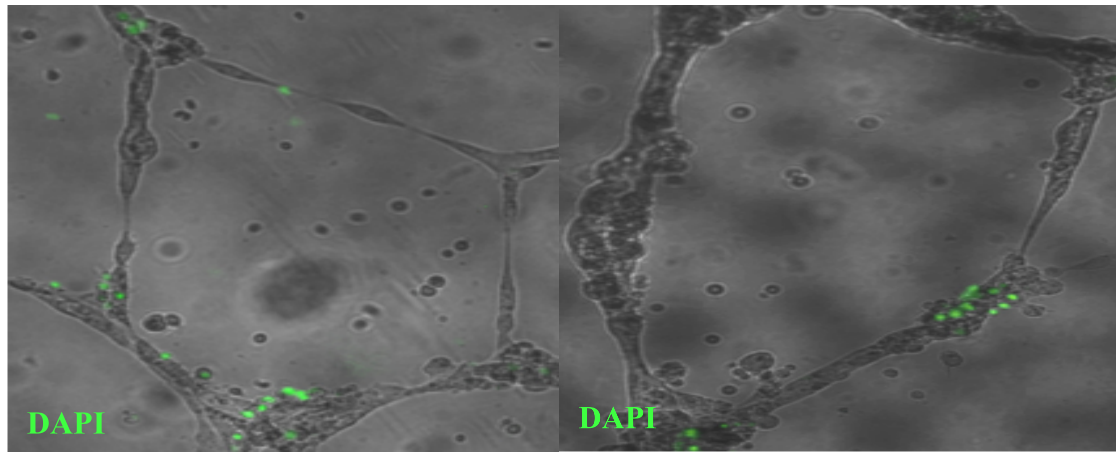
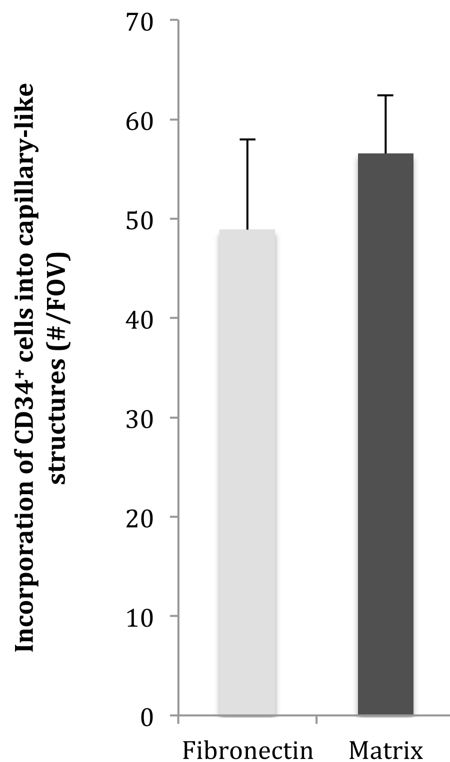


Figure 4. CD34$^+$ cells cultured on matrix show a trend towards increased viability. PBMNCs were isolated and cultured on fibronectin for 4 days. CACs were lifted, CD34$^+$ cells were isolated and re-plated on matrix or fibronectin for 48h on matrix or fibronectin in hypoxic conditions. A viability assay was conducted: live cells were stained with Calcein-AM (green) and dead cells were stained with Ethidium homodimer-1 (red). n=5; p=0.07.





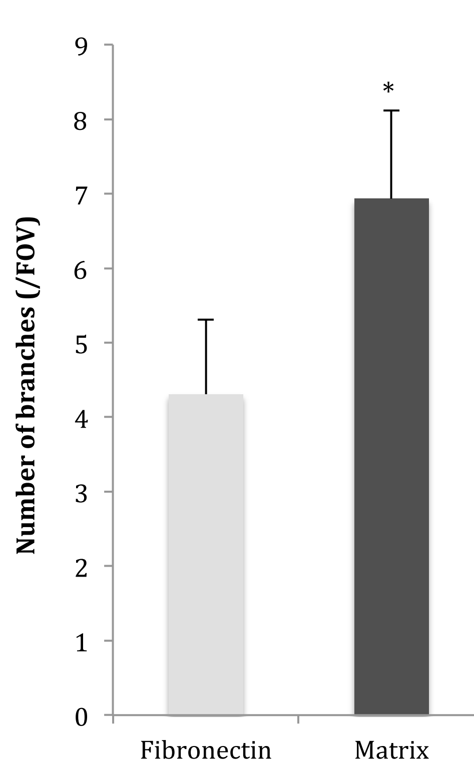


Figure 5. Branching is increased in an angiogenesis assay with matrix-cultured CD34$^+$ cells.

PBMNCs were isolated and cultured on fibronectin for 4 days. CACs were lifted, CD34$^+$ cells were isolated and re-plated on matrix or fibronectin for 4 days. CD34$^+$ cells were then used in an angiogenesis assay. 5,000 HUVECs and 5,000 CD34$^+$ cells were plated together on ECMatrix overnight. Angiogenesis was analyzed the following morning.

A) Angiogenesis assay using CD34$^+$ cells cultured on matrix. B) Angiogenesis assay using CD34$^+$ cells cultured on fibronectin. C) Cell incorporation into capillary-like structures. D) Number of branches. **p*=0.01. n= 5 for both.

3.1.6 $CD34^+$ Cell Adhesion

The ability of transplanted cells to adhere and engraft within the ischemic tissue is an important factor in determining the extent (magnitude and duration) of their therapeutic effects. The matrix was evaluated for its ability to enhance the adhesion potential of $CD34^+$ cells. $CD34^+$ cells were cultured on matrix and fibronectin for 4 days. Following the cultures, the $CD34^+$ cells were plated on matrix and fibronectin for 1 hour. There were no significant differences in adhesion properties (Fig. 6).

3.1.7 Gene expression of Shh pathway members in $CD34^+$ cells isolated from the total CAC population after culture

The activation of certain pathways may prove to be beneficial to the survival and therapeutic effects of the $CD34^+$ cells. Shh increases proliferation, is up-regulated post-MI and is involved in angiogenesis and hence constituted a pathway of interest (Pola, Ling et al. 2003, Kusano, Pola et al. 2005). The ability of matrix to enhance the Shh pathway in $CD34^+$ cells when cultured within the entire CAC population was evaluated (Pola, Ling et al. 2003). CACs were cultured on matrix and fibronectin for 4 days. At the end of the culture period, $CD34^+$ cells were separated, and RNA was isolated and down-stream target genes of Shh were analyzed by RT-qPCR. $CD34^+$ cells cultured on matrix had a 3.8-fold increase in expression of Hes1, a 13.8-fold increase in expression of Gli1 and a 7.3-fold increase in Cyclin D expression compared to $CD34^+$ cells cultured on fibronectin (Fig. 7, $p<0.05$ for all).

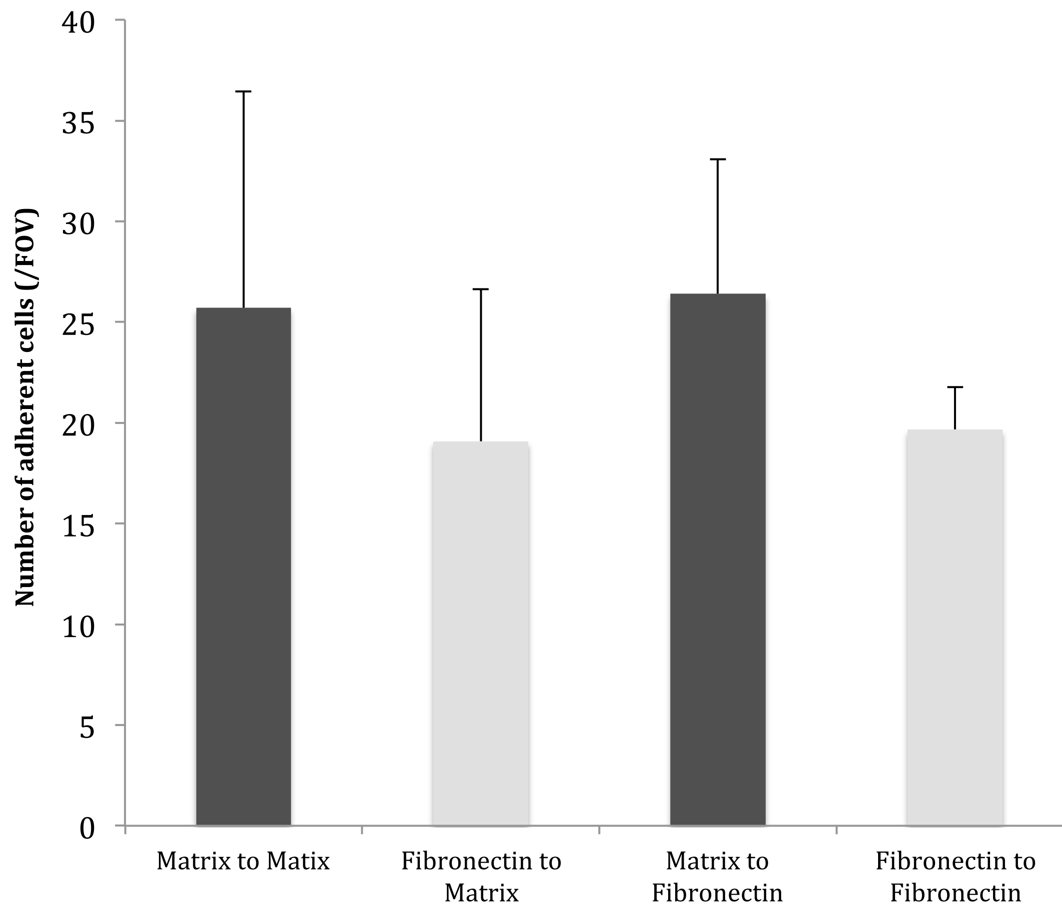


Figure 6. Adhesion of $CD34^+$ cells cultured on matrix and fibronectin is similar. PBMNCs were isolated and cultured on fibronectin for 4 days. CACs were lifted, $CD34^+$ cells were isolated and re-plated on fibronectin or matrix for 4 days. $CD34^+$ cells were then lifted and re-plated at a density of 5,000 cells/well for 1 hour on fibronectin and matrix. n=5.

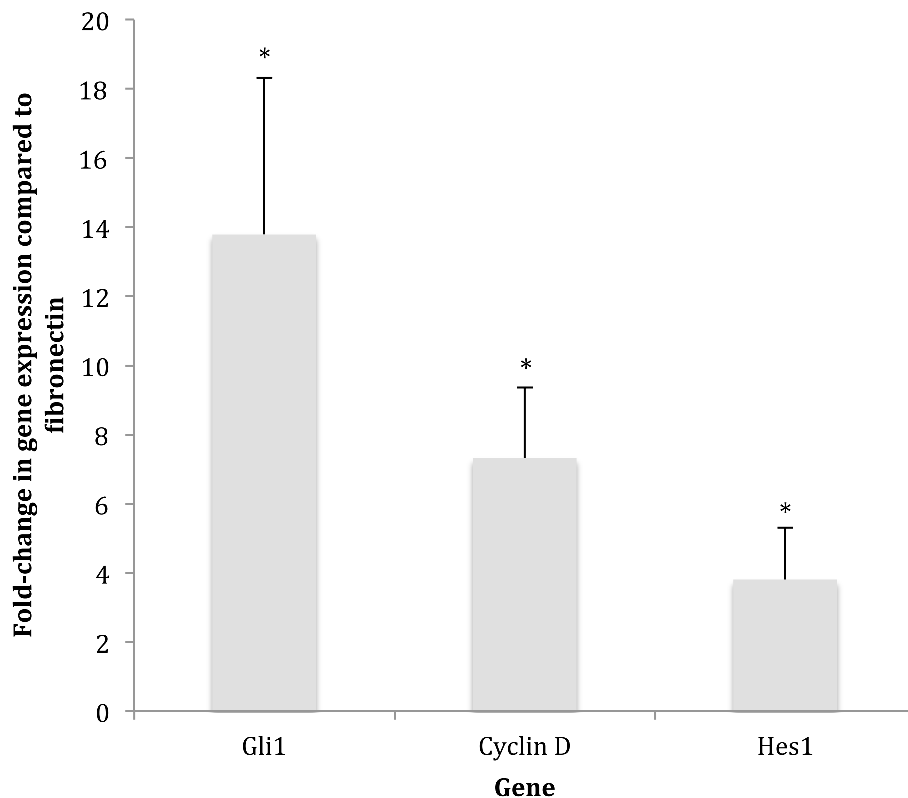


Figure 7. Fold-change in mRNA expression of Shh pathway genes for CD34$^+$ cells isolated from the total CAC population after culture.

PBMNCs were isolated and cultured on fibronectin for 4 days. CACs were lifted and re-plated on fibronectin or matrix for 4 days. CD34$^+$ cells were then isolated and RT-qPCR analysis of mRNA was performed with values normalized to 18S. Expression values were then calculated as a fold-change for matrix versus fibronectin cultures. n≥5; *p<0.05.

3.1.8 Expression of Shh pathway genes in $CD34^+$ cells cultured alone

For comparison with the previous mixed cell culture work, the expression of Shh target genes was evaluated in $CD34^+$ cells cultured alone to determine the effects of the matrix on these cells in the absence of interactions with other CACs. $CD34^+$ cells alone were cultured on matrix and fibronectin for 4 days and RT-qPCR was conducted. $CD34^+$ cells cultured on matrix had a 2-fold increase in the expression of Hes1 (p=0.03), and there was a trend for increased Gli1 and Cyclin D expression (by 4.2- and 9-fold, respectively; p=0.3 and p=0.1 respectively) compared to fibronectin-cultured $CD34^+$ cells (Fig. 8).

3.1.9 Angiogenesis assay with the supernatant from hypoxic $CD34^+$ cell cultures

$CD34^+$ cells have been shown to promote vascularization via paracrine effects (Asahara, Kawamoto et al. 2011, Mackie, Klyachko et al. 2012). The matrix was investigated for its ability to enhance the secretion of pro-angiogenic cytokines/exosomes. HUVECS (10,000 cells) were cultured on Matrigel with the supernatant from $CD34^+$ cells cultured in hypoxia on matrix and fibronectin. There was a trend towards increased branching of capillary-like structures for HUVECs in the supernatant from matrix-cultured $CD34^+$ cells (1.2-fold increase on matrix versus fibronectin; Fig. 9, p=0.08).

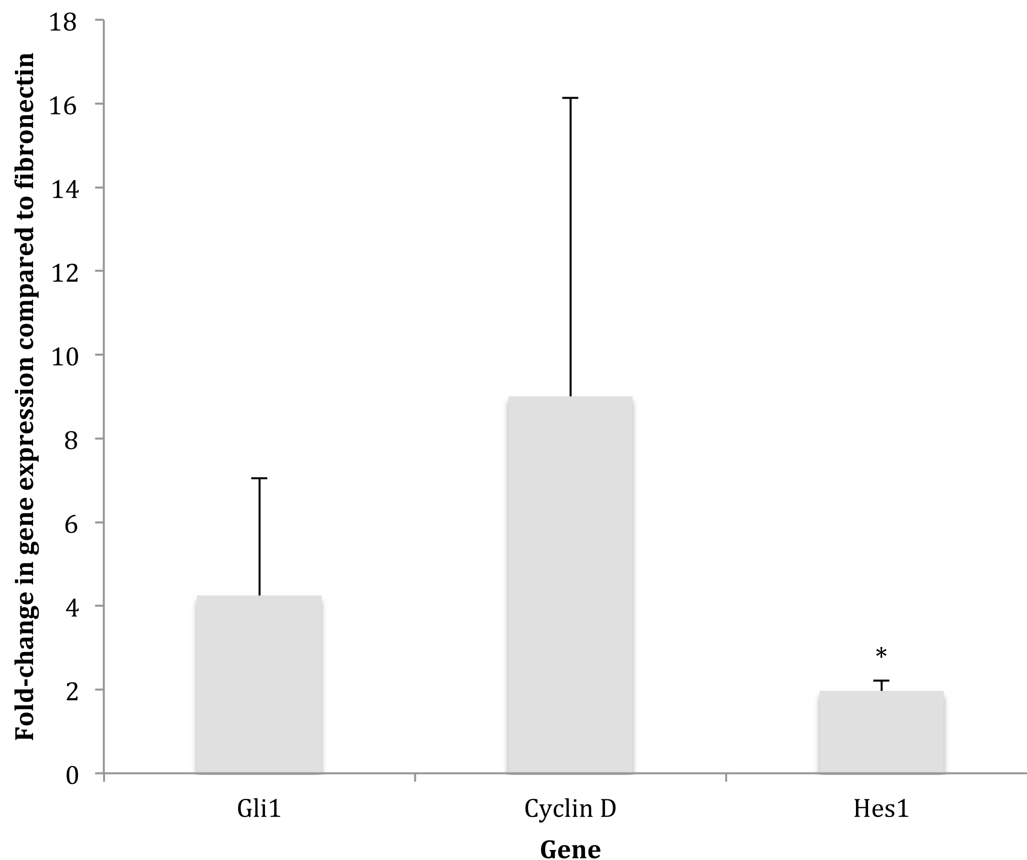


Figure 8. Fold-change in mRNA expression for Shh pathway genes in $CD34^+$ cells cultured alone.

PBMNCs were isolated and cultured on fibronectin for 4 days. CACs were lifted, $CD34^+$ cells were isolated and re-plated on fibronectin or matrix for 4 days. RT-qPCR analysis of mRNA was performed with values normalized to 18S. Expression values were then calculated as a fold-change for matrix versus fibronectin cultures. n≥3; *p=0.03 for Hes1.

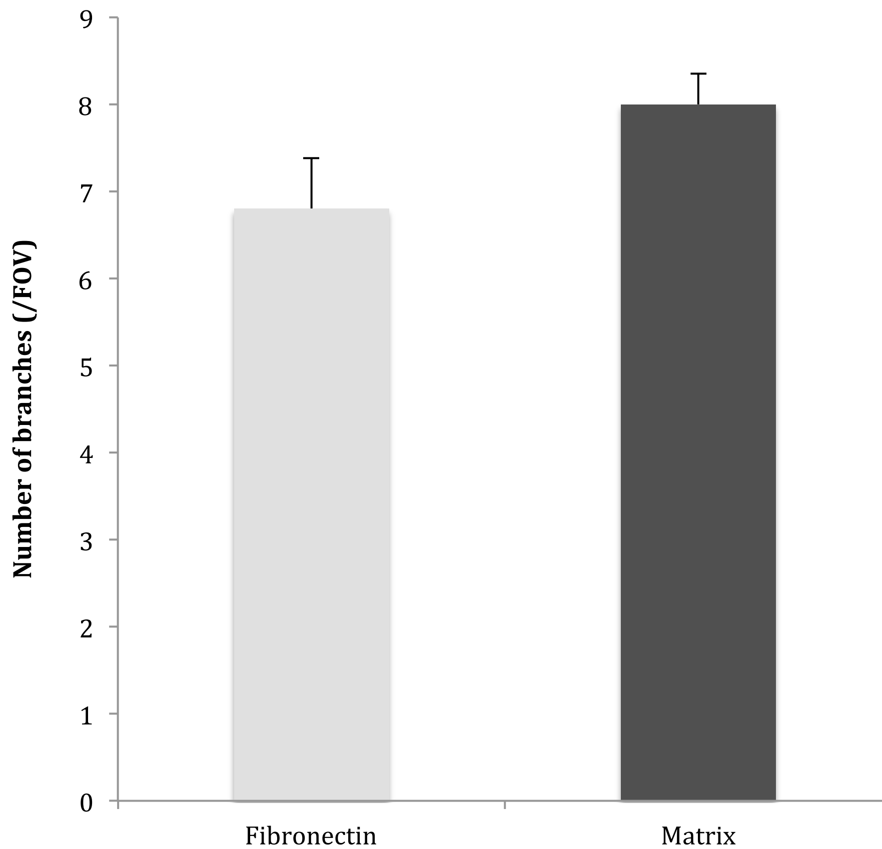


Figure 9. Trend towards increased branching in an angiogenesis assay with hypoxic media from $CD34^+$ cells cultured on matrix.

PBMNCs were isolated and cultured on fibronectin for 4 days. CACs were lifted, $CD34^+$ cells were isolated and re-plated on matrix or fibronectin for 4 days. Cells were in normoxia for 3 days, followed by hypoxic conditions for 24 hours. The hypoxic media was removed and used in an angiogenesis assay with 10,000 HUVECs on ECMatrix overnight. n=6; p=0.08.

3.2 Aim 2.

The results of Aim 1 showed that the matrix is able to enhance the function of $CD34^+$ cells isolated from healthy donors. In previous work, Kuraitis et al. showed that the collagen I matrix was able to enhance the regenerative potential of healthy CACs. To further investigate the therapeutic potential of the matrix in a more clinically relevant population, Aim 2 of my thesis was to determine if the matrix could be used to restore/enhance the function of cells from CAD patients.

3.2.1 Effects of collagen I matrix culture on angiogenesis of patient CACs

CAD patient CACs were obtained and cultured on either fibronectin or collagen matrix. CACs were then lifted and co-cultured with HUVECs in an *in vitro* angiogenesis assay. The matrix enhanced healthy CAC incorporation into capillary-like structures by 2.3-fold (Fig. 10; $p<0.01$) and patient CAC incorporation into capillary-like structures by 1.7-fold (Fig. 10; $p=0.02$) compared to CACs cultured on fibronectin. Therefore, the matrix was almost as effective at enhancing the angiogenic potential of patient CACs, as it was for CACs from healthy donors.

3.2.2 Effects of collagen I matrix culture on viability of patient CACs

The ability of the matrix to enhance the viability of patient CACs was investigated. CACs were cultured on matrix and fibronectin in hypoxic conditions for 48 hours in serum-free media without any growth factors. After 48 hours, healthy CACs had increased viability, up by 1.3-fold on matrix compared to fibronectin (Fig. 11; $p=0.03$). Patient CACs also exhibited an increase in viability, up by 1.2-fold when cultured on matrix compared to fibronectin. (Fig. 11; $p=0.02$).

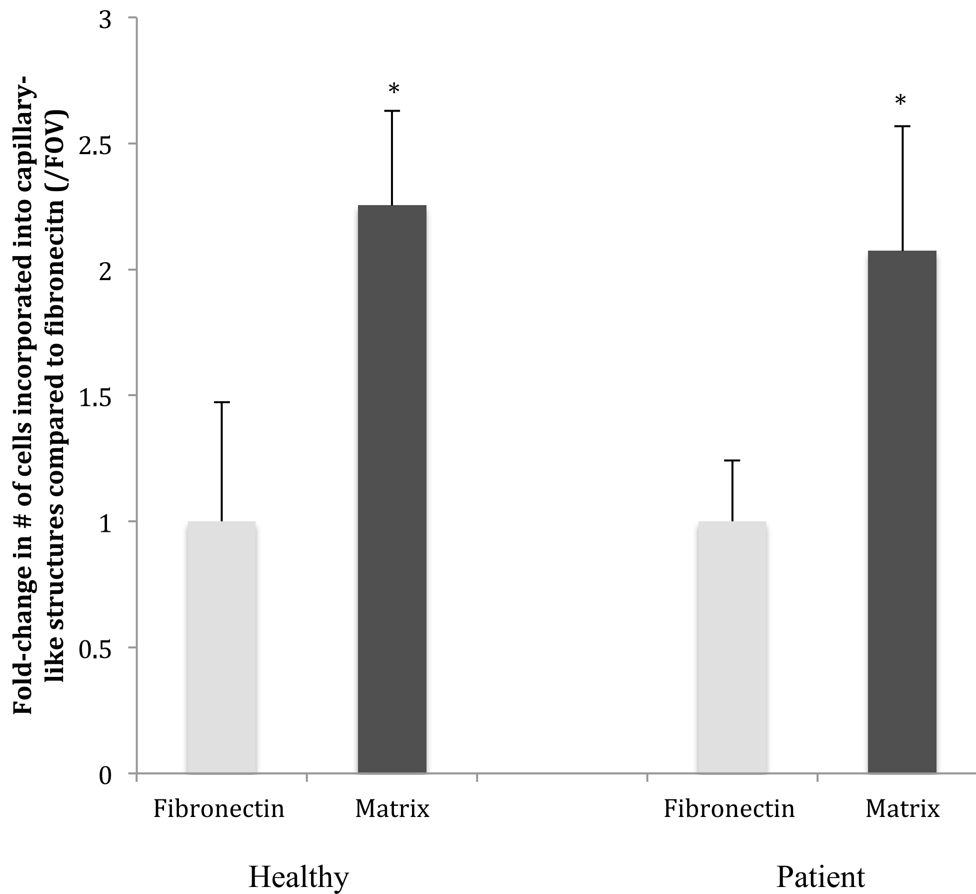


Figure 10. Increased angiogenesis of patient CACs when cultured on matrix. PBMNCs were isolated and cultured on fibronectin for 4 days. CACs were lifted and re-plated on matrix or fibronectin for 4 days. An angiogenesis assay was conducted overnight, plating 5,000 HUVECs and 5,000 CACs on ECMatrix. n=6; $p \leq 0.02$ for matrix versus fibronectin within each group.

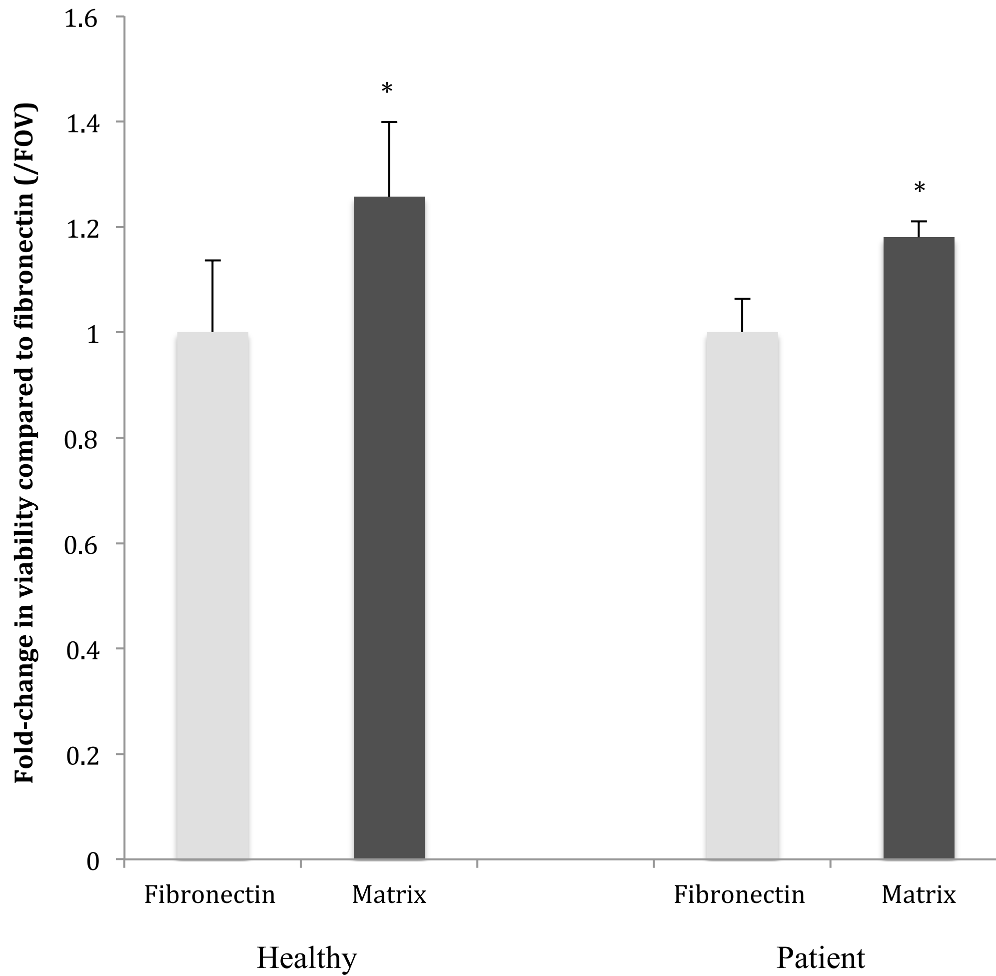


Figure 11. Matrix culture increases the viability of patient CACs.
PBMNCs were isolated and cultured on fibronectin for 4 days. CACs were lifted and re-plated for 48h on collagen matrix or fibronectin in hypoxic conditions. A viability assay was conducted. Live cells were stained with Calcein-AM. n=6; $p \leq 0.03$ for matrix versus fibronectin within each group.

3.2.3 Effects of collagen I matrix culture on the proliferation of patient CACs

The ability to expand the CAC population in culture would provide greater numbers of these cells for use in therapy. The matrix was evaluated for its potential to increase the proliferation of patient CACs. After 48 hours of culture, 5.5% of healthy CACs were proliferating on the matrix compared to 1.5% proliferation on fibronectin, as determined by Ki67 staining (Fig. 12; p=0.04). Patient CACs had 3.3% proliferation on matrix versus 0.7% on fibronectin (Fig. 12; p=0.05).

3.2.4 Adhesion of patient CACs when cultured on matrix

The ability of the matrix to enhance patient CAC adhesion was investigated. CACs from healthy and patient donors were cultured on matrix and fibronectin for 4 days. Following the cultures, CACs were lifted and re-plated on matrix or fibronectin for 1 hour. There were no significant differences observed in the adhesion properties of CACs from healthy or patient donors (Fig. 13).

3.2.5 mRNA expression for freshly isolated PBMNCs and CACs from CAD patients in comparison to healthy donors

It was hypothesized that the interaction of CACs with the collagen I matrix would confer functional benefits through the activation of integrins and/or TAL1. Freshly isolated PBMNCs and CACs from patients and healthy donors were first investigated to identify any differences in integrin and TAL1 expression that existed between the two groups before culture. RNA was isolated from patient and healthy donor PBMNCs and CACs. RT-qPCR was performed to look at the expression of a number of integrins and TAL1. No significant differences were seen in the expression of genes between the healthy and

patient PBMNCs (Fig. 14). After a 4-day culture on fibronectin, RT-qPCR was conducted for the generated CAC populations. There was a statistically significant difference in the expression of integrin α1; integrin α1 mRNA levels were reduced 3.3-fold in CAD patient CACs compared to healthy CACs (Fig. 14; p=0.03). Additionally, there was a 5.7-fold decrease in TAL1 expression, when comparing patient versus healthy CACs compared to patient versus healthy PBMNCs (Fig. 14; p=0.02).

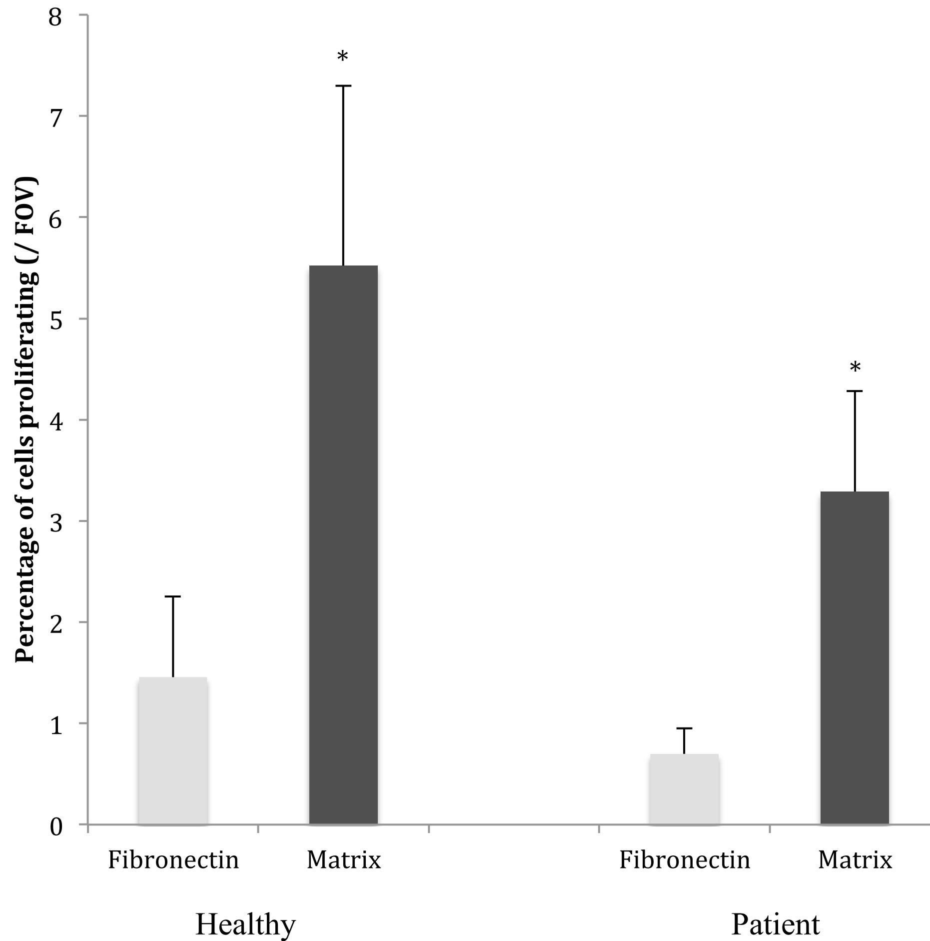


Figure 12. Increased proliferation of CACs on matrix compared to fibronectin. PBMNCs were isolated and cultured on fibronectin for 4 days. CACs were lifted and re-plated for 48h on collagen matrix or fibronectin in normoxia. Proliferation was measured with Ki67 staining. n=5; $p \leq 0.04$ for matrix versus fibronectin within each group.

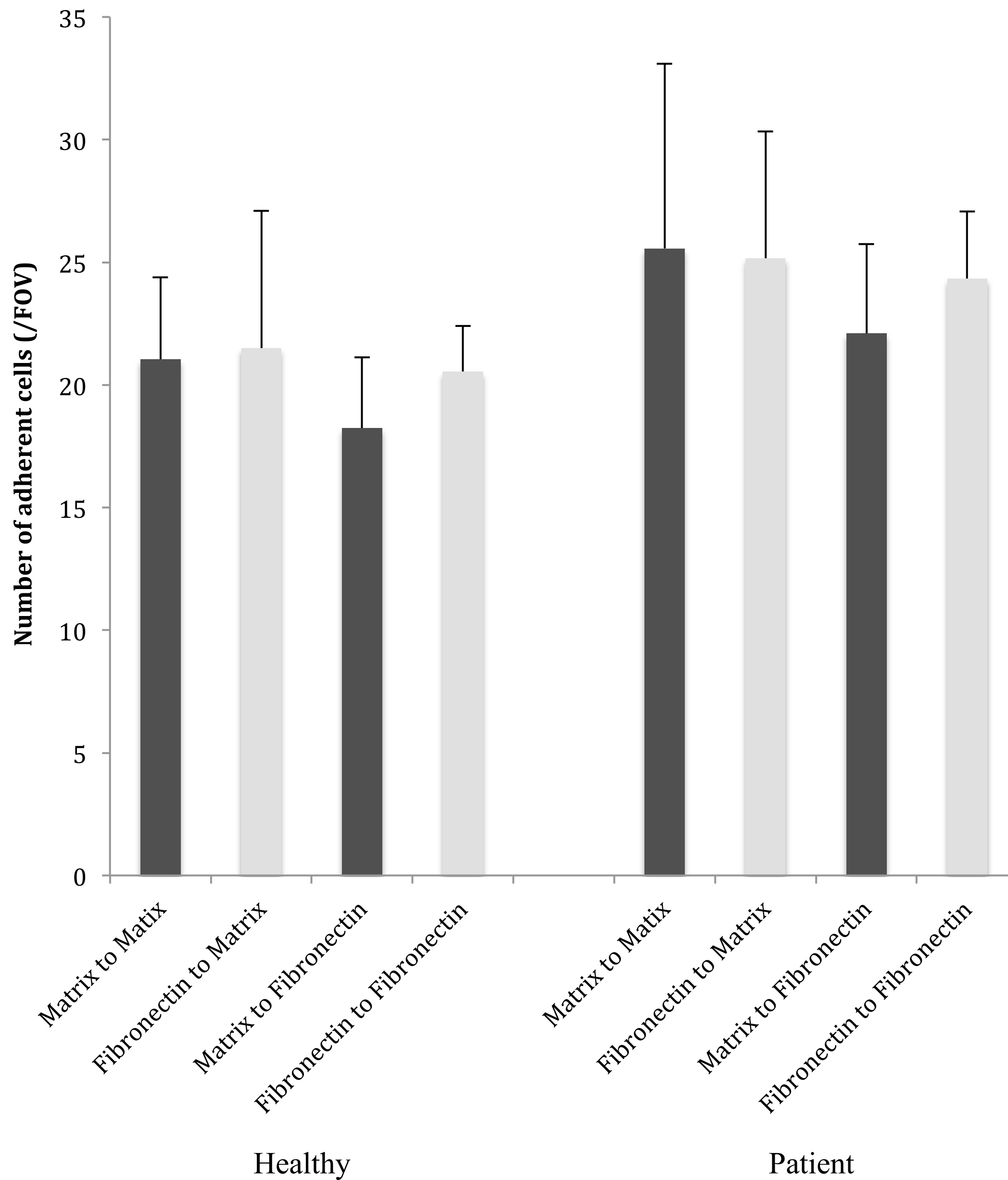


Figure 13. Adhesion of patient CACs is not altered by matrix culture.
PBMNCs were isolated and cultured on fibronectin for 4 days. CACs were lifted and re-plated on fibronectin or on matrix for 4 days. CACs were then isolated and re-plated at 10,000 cells/well for 1 hour on fibronectin and matrix. Healthy, n=5; patient, n=9.

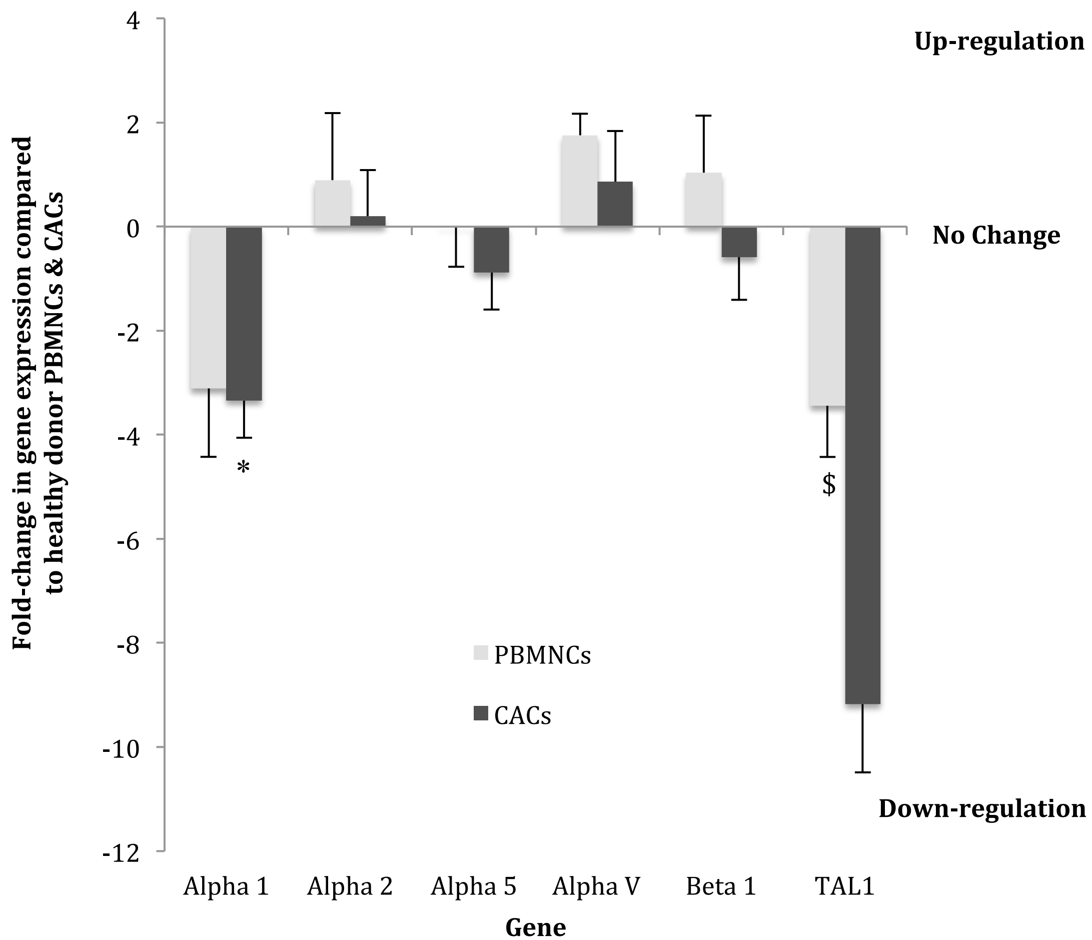


Figure 14. Fold-change in mRNA expression of patient and healthy PBMNCs and CACs.
RNA was isolated from patient and healthy donor PBMNCs and CACs. cDNA was made and RT-qPCR analysis of mRNA was performed with values normalized to 18S. Expression values were then calculated as a fold-change for patient versus healthy samples. n≥4; **p*=0.03 for alpha 1 in patient CACs versus healthy donor CACs; $^{\$}$*p*=0.02 for CACs versus PBMNCs.

3.2.6 mRNA expression of CAD patient and healthy CACs after matrix and fibronectin culture

The expression of integrins and TAL1 was investigated in patient CACs after culture on matrix versus fibronectin to see if the matrix could restore any of the expression differences observed above, and hence possibly contribute to the improved function of patient CACs that was observed with matrix culture. After a 4-day culture on matrix or fibronectin, CACs were lifted and the RNA was isolated. The cDNA was synthesized and the samples were analyzed by RT-qPCR to look at a number of integrins and TAL1. Gene expression on matrix was compared to that on fibronectin and these values were then compared between CAD patients and healthy donors. Integrin α1 expression in patient CACs cultured on matrix compared to fibronectin was reduced by 5-fold compared to healthy CACs cultured on matrix compared to fibronectin (Fig. 15; $p<0.02$). There were no statistical differences in expression between any of the other genes for CAD patient CACs after culture versus healthy donor CACs after culture.

3.2.7 mRNA expression for $CD34^+$ cells isolated from healthy and CAD patient donor PBMNCs

As described above, $CD34^+$ cells are a highly pro-angiogenic sub-population of CACs (Asahara, Murohara et al. 1997). The expression of various integrins and Shh pathway genes in $CD34^+$ cells freshly isolated from patient and healthy PBMNCS were compared to determine if the $CD34^+$ cells from patients were exhibited any dysfunction in these important pathways. $CD34^+$ cells were isolated directly from the PBMNCs obtained from CAD patient and healthy donors. RNA was isolated, cDNA was synthesized, and

the samples were analyzed by RT-qPCR to look at a number of integrins, genes involved in the Shh pathway, and VEGF. Integrins α2 (p=0.02), α5 (p=0.05), and αV (p=0.05), Cyclin E (p=0.03), VEGF (p<0.02) and Hes1 (p<0.02) were all significantly down-regulated in patient $CD34^+$ cells compared to healthy $CD34^+$ cells. There was a trend for down-regulation of integrin α4 (p=0.06) and Cyclin D (p=0.09) in CAD patient $CD34^+$ cells (Fig.16).

3.2.8 mRNA expression of $CD34^+$ cells isolated from healthy and CAD patient donor CACs

As reported in 3.2.7, the expression of a number of integrins and Shh pathway genes were down-regulated in patient $CD34^+$ cells isolated from PBMNCs compared to those isolated from healthy donors. The expression of the same genes was investigated in $CD34^+$ cells after their isolation from patient and healthy donor CAC cultures. This would elucidate if culturing on fibronectin or matrix could restore expression levels in the patient group. After a 4-day culture on fibronectin, CACs were generated. $CD34^+$ cells were separated from these CACs and RNA was isolated. cDNA was made and analyzed by RT-qPCR for the same genes as mentioned above (Fig. 16). There were no significant differences in gene expression in $CD34^+$ cells obtained from CACs of CAD patient versus healthy donors (Fig. 17).

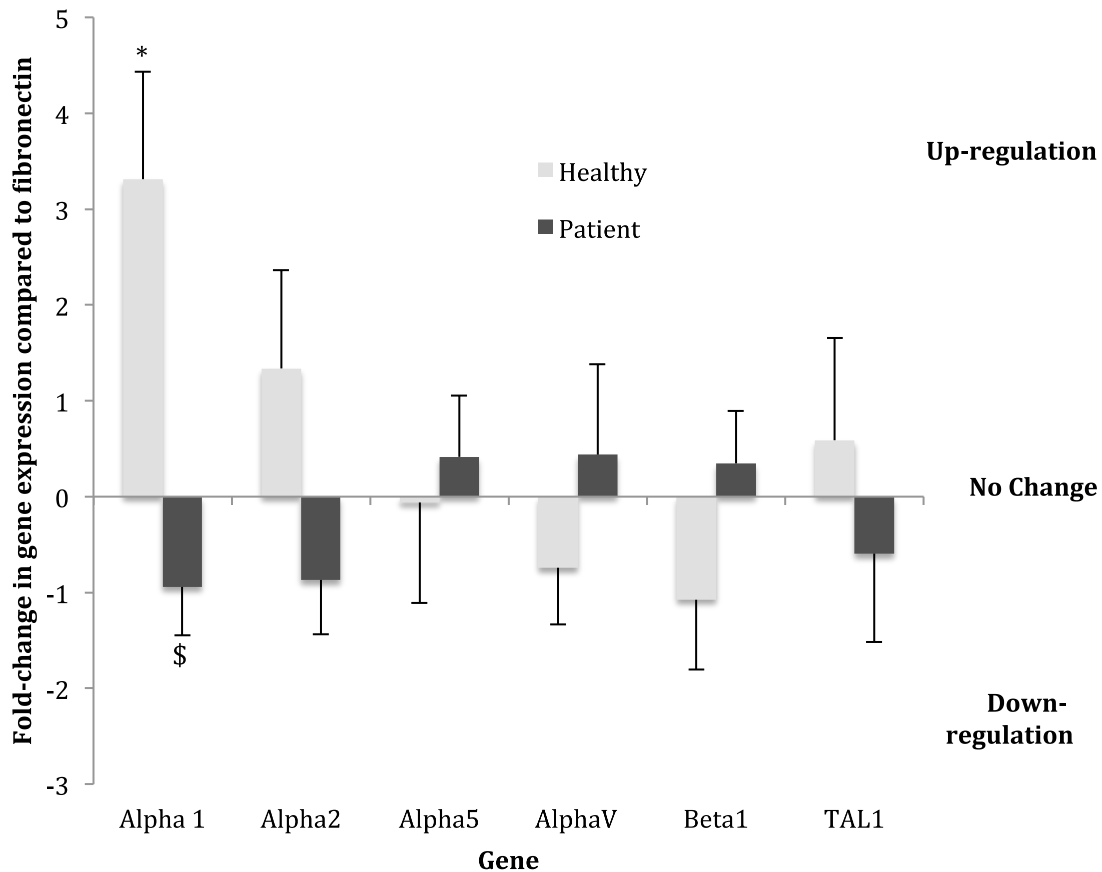


Figure 15. Fold-change in mRNA expression of patient and healthy CACs cultured on matrix and fibronectin.

PBMNCs were isolated and cultured on fibronectin for 4 days. CACs were lifted and re-plated on matrix or fibronectin for 4 days. CACs were lifted and RT-qPCR analysis of mRNA was performed with values normalized to 18S. Expression values were then calculated as a fold-change for matrix versus fibronectin. n≥4; *p=0.03 for matrix versus fibronectin; $^{\$}p<0.01$ for alpha1 in patient versus alpha 1 in healthy donors.

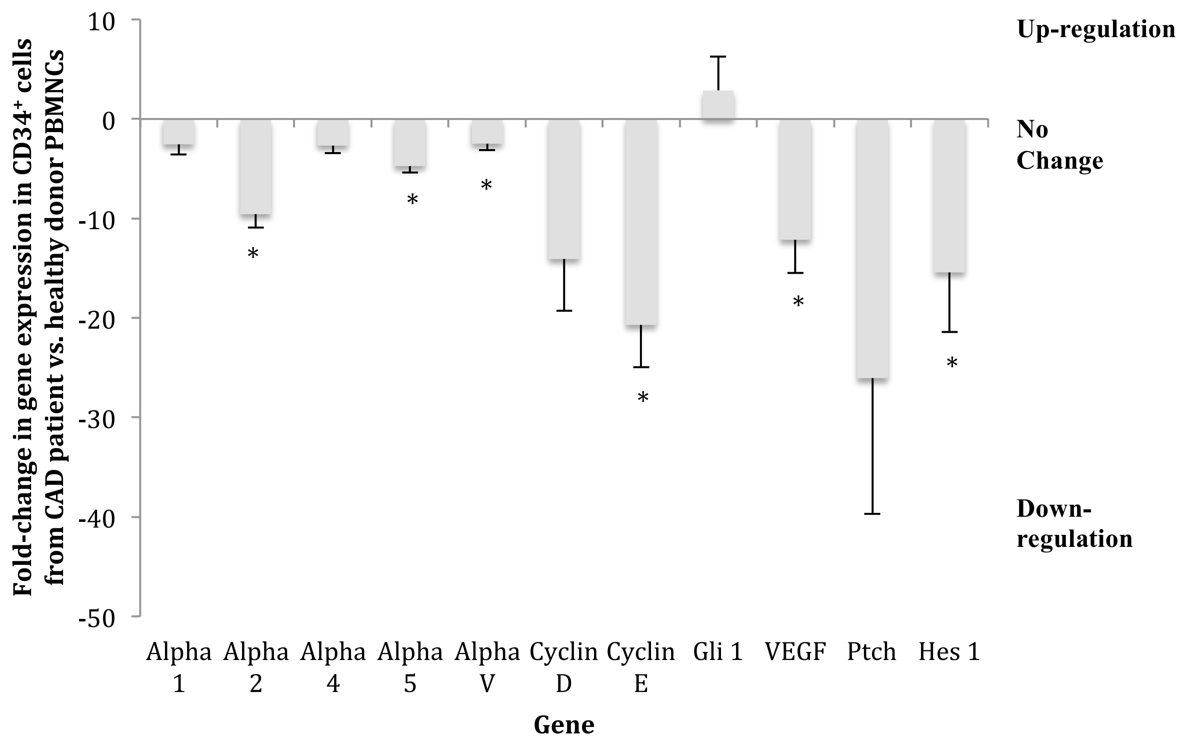


Figure 16. Fold-change in mRNA expression for CD34^{+} cells isolated from the PBMNCs of CAD patients versus healthy donors.

CD34^{+} cells were isolated from PBMNCs and RT-qPCR analysis of mRNA was performed with values normalized to 18S. Expression values were then calculated as a fold-change for patient versus healthy donors. The expression for most of the genes is down-regulated in patient CD34^{+} cells. n=4; *p<0.05.

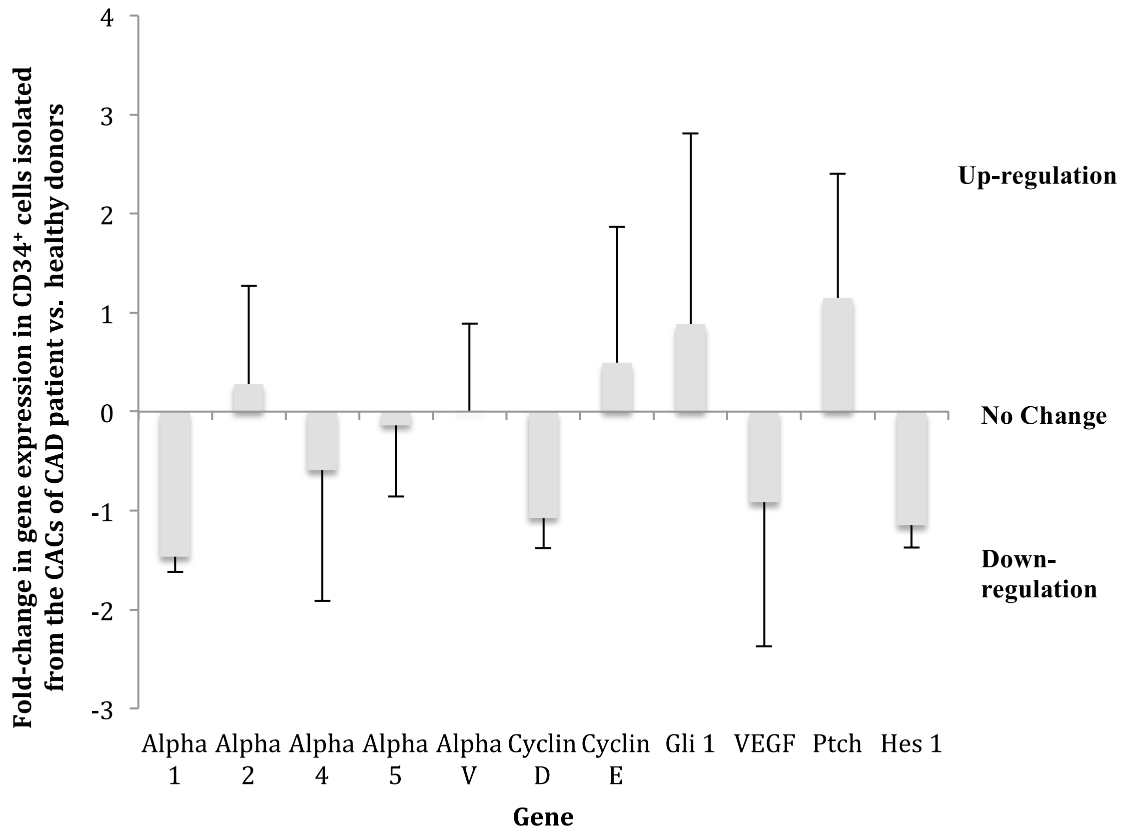


Figure 17. Fold-change in mRNA expression for CD34$^+$ cells isolated from CACs of CAD patients versus healthy donors.

PBMNCs were isolated and cultured on fibronectin for 4 days. The generated CACs were lifted, CD34$^+$ cells were separated and RT-qPCR analysis of mRNA was performed with values normalized to 18S. Expression values were then calculated as a fold-change for patient versus healthy donors. No differences in gene expression were observed between patient and healthy CD34$^+$ cells. n=3.

4.0 Discussion

One aim of this study focused on determining if a collagen I biomaterial would be able to enhance the function of the pro-angiogenic $CD34^+$ cell population within CACs. The matrix conferred beneficial effects on the $CD34^+$ population in terms of migration, proliferation, phenotype, and angiogenesis, as well as up-regulated genes associated within the Shh pathway.

Fibronectin was used for the control cultures in this work since it is considered the golden standard for isolating CACs using an adhesion-selective approach (Kalka, Masuda et al. 2000). Variable lengths of culture can promote either early or late-outgrowth stages. For the purpose of this study, the early CACs, and more specifically, the $CD34^+$ cells, were sought out in order to investigate a progenitor phenotype for cell therapy purposes as opposed to the fully differentiated late-growth cells.

Despite the ability of fibronectin to adhere CACs, it does not promote proliferation of the early CAC population (Hristov and Weber 2004). The present study indicated that while fibronectin can promote proliferation within the $CD34^+$ population specifically, it still could not match the proliferation induced by the culture of the cells on matrix. Additionally, culture on the matrix induced characteristic clusters of cells. As observed by immunohistochemistry, it is within these clusters that most of the proliferation is occurring, suggesting that cell-to-cell communication plays a role in inducing/maintaining proliferation (Nelson and Chen 2002). Since $CD34^+$ cells represent such a small fraction of the total CAC population, this presents a constraint for their use in therapy; however, the collagen I matrix proves to be a promising option for the culture and expansion of the $CD34^+$ subpopulation of CACs.

The matrix was able to enhance the migration potential of the $CD34^+$ cells towards a VEGF stimulus. In CAD patients, cells usually exhibit abnormal migratory capacity, and as a result cannot contribute to the revascularization process as efficiently (Vasa, Fichtlscherer et al. 2001). Migration plays an important role in the recovery process post-MI. In animal models, it was discovered that there was a relationship present between induced migratory capacity and cell retention within the hindlimb ischemic tissue and thus the neovascularization capacity (Heeschen, Lehmann et al. 2004, Walter, Haendeler et al. 2005, Aicher, Heeschen et al. 2006, Seeger, Rasper et al. 2009). Similarly, the REPAIR-AMI trial found that patients administered BMMNCs with a high migratory capacity had improved event-free survival (Assmus, Leistner et al. 2014), as well as greater LVEF improvement at 4 months, compared to patients with low migratory BMMNCs (Assmus, Tonn et al. 2010). It has also been shown that the SDF-1-induced migratory capacity of BMMNCs used for clinical studies is correlated with reduced infarct size (Britten, Abolmaali et al. 2003). All these findings suggest that an improvement in migration capabilities would result in superior therapeutic benefits post-MI.

According to a study by de Wynter, Coutinho et al., it was determined that FACS and magnetic activated cell sorting (MACS) were the most ideal methods for isolating $CD34^+$ cells because they yielded >70% purity (de Wynter, Coutinho et al. 1995). The MACS method was used in the present study for the isolation of $CD34^+$ cells, and the purity was confirmed with FACS; the $CD34^+$ cell isolation procedure yielded >80% purity. Confident with the purity of the cells, the amount of the cell population expressing CD34 and maintaining their "progenitor" phenotype was compared between

cells cultured on matrix versus fibronectin. The culture on matrix resulted in a 2.8-fold increase of cells with $CD34^+$ expression compared to fibronectin. It has been shown that $CD34^+$ cells on their own had a greater therapeutic effect post-MI in animal studies and in clinical trials, therefore increasing their numbers for transplantation is deemed a promising approach to improving the outcome of cell therapy using bone marrow derived cell populations (Kawamoto, Iwasaki et al. 2006, Tendera, Wojakowski et al. 2009). Only about 1.5% of low-density human marrow mononuclear cells express CD34, therefore it would be ideal to maintain the $CD34^+$ "progenitor" phenotype until the cells are inserted into the ischemic myocardium where they can respond to the host environment and differentiate and/or exert their therapeutic function accordingly (Civin, Strauss et al. 1990).

Due to previous results where the matrix was able to enhance CAC incorporation in an angiogenesis assay (Kuraitis, Hou et al. 2011), it was expected the same outcome would be observed with the $CD34^+$ cells. However, the number of cells incorporating into capillary-like structures in the angiogenesis assays was roughly the same for $CD34^+$ cells cultured on matrix versus fibronectin. Despite similar incorporation rates, it was discovered that the matrix-cultured $CD34^+$ cells induced a greater amount of network branching. This suggests that the superior function of matrix-cultured $CD34^+$ cells may be, at least in part, associated with the cytokines they secrete; thus paracrine effects over direct incorporation of matrix-cultured $CD34^+$ cells in the angiogenic process. Additionally, the adhesion assay showed no differences in regards to adhesion capabilities of $CD34^+$ cells cultured on either matrix or fibronectin, thus further encouraging the theory that the $CD34^+$ cells confer benefits via paracrine effects.

Since the matrix was able to enhance the function of the $CD34^+$ cell population in a number of ways, the investigation sought out an intracellular signaling pathway that could be involved in mediating the observed effects. The Shh pathway is activated in the ischemic myocardium post-MI and has been shown to have a role in angiogenic processes (Kusano, Pola et al. 2005); hence it served as an initial pathway of interest. The expression of select genes of the Shh pathway, consisting of Gli 1, Cyclin D and Hes 1 were examined in $CD34^+$ cells at the end of their culture (matrix versus fibronectin). It was found that Shh pathway genes were activated in matrix-cultured $CD34^+$ cells, whether they were cultured on their own or with the total CACs population. This highly suggests that the Shh pathway is involved in some of the functional benefits seen above. It has been shown that the injection of Shh plasmid into $CD34^+$ cells greatly improves their therapeutic effect compared to normal $CD34^+$ cells when used to treat MI, therefore the induction of Shh by the matrix serves as a promising therapeutic option (Mackie, Klyachko et al. 2012). Notably, $CD34^+$ cells isolated from CACs at the end of the culture period exhibited greater expression of both Gli1 and Hes1, compared to $CD34^+$ cells that were cultured on their own. This suggests that the activation of the Shh pathway is increased when $CD34^+$ cells are in proximity to other CACs, and that the optimal therapeutic potential of $CD34^+$ cells may depend on the presence of surrounding supportive cells. A study by Bautz, Rafii et al. supports this possibility. In their work, it was shown that $CD34^+$ cells secrete more VEGF when incubated with other cytokines (Bautz, Rafii et al. 2000), suggesting that the proximity of other cells and their cytokine signaling would promote the secretion of cytokines from $CD34^+$ cells, thus favoring a total CAC population culture. However, in the present

study, it is important to note that CACs and $CD34^+$ cells were cultured at different seeding densities, in 10cm tissue culture dishes for CACs, and 12-well plates for $CD34^+$ cells. Ideally, experiments would need to be conducted with normalized cell culture densities in order to come to conclusive results. If the results were to favor $CD34^+$ cells from a total CAC population (in terms of function and secretome), then culturing the whole CAC population and isolating the $CD34^+$ cells just prior to the injection or injecting CACs with a greater concentration of $CD34^+$ cells may be therapeutically superior.

Since the results suggest that the functional superiority of matrix-cultured cells may be partly due to paracrine effects, we wanted to further investigate this concept. Mackie, Klyachko et al. demonstrated that $CD34^+$ cells can secrete Shh via exosomes and thus it was of interest to see if a similar beneficial response could be activated by culturing $CD34^+$ cells on matrix (Mackie, Klyachko et al. 2012). $CD34^+$ cells were cultured on matrix or fibronectin for 3 days in normoxic conditions and were transferred to hypoxic conditions for the last 24 hours. The media was then taken and placed on HUVECs in an angiogenesis assay. The next day a trend was noticed towards increased branching using media from the cells cultured on matrix. Another member of the lab (Brian McNeill) has shown that HUVECs in an angiogenesis assay with exosomes from matrix-cultured $CD34^+$ cells exhibited branching, while the exosomes from fibronectin-cultured $CD34^+$ cells were unable to elicit a similar response. Further analysis and follow-up studies are necessary to determine the mechanism and significance of these results. For example, angiogenesis assays using exosomes from $CD34^+$ cells cultured under different conditions (e.g. ±CACs, normoxia, hypoxia, matrix versus fibronectin)

need to be performed to determine under what conditions the secreted exosomes are more beneficial to the angiogenesis process. Since both Shh (Mackie, Klyachko et al. 2012) and VEGF(Bautz, Rafii et al. 2000) are known to be secreted by CD34$^+$ cells, it remains to be determined whether the matrix is able to enhance their secretion via exosomes. The secretion of VEGF remains to be thoroughly investigated, but is a promising candidate as it is involved in angiogenesis and vasculogenesis (Dvorak, Brown et al. 1995, Ferrara 1995), is known to induce EC proliferation (Ferrara and Henzel 1989), as well as enhance EC survival (Gerber, McMurtrey et al. 1998), migration and adhesion (Byzova, Goldman et al. 2000). The culture of CD34$^+$ cells on matrix was able to affect the CD34$^+$ cells in a fashion similar to the effects reportedly seen with VEGF, hence VEGF is a promising candidate for secretion by the CD34$^+$ cells.

Since it was shown that the matrix could successfully enhance the function of CACs (Kuraitis, Hou et al. 2011)and their pro-angiogenic subpopulation CD34$^+$ cells, the second aim of this study was to investigate if the matrix could enhance the function of CAD patient CACs. The inclusion criteria for the CAD population consisted of males 50 or older going for CABG or EP procedures; this was established in order to reduce the variability within the disease population.

In terms of angiogenic potential, it was demonstrated that the matrix increased the incorporation of patient CACs into capillary-like structures in an *in vitro* angiogenesis assay to an extent comparable to that of healthy donor CACs. MI leads to irreversible myocardial damage and results in necrosis of the affected areas. The ability of the matrix to enhance the angiogenic potential of patient CACs is essential to the

ability of the patient CACs to contribute to the restoration of the blood flow, thus reducing damage to the myocardium. The matrix was also able to promote similar viability between CACs from patients and healthy donors. Transplanted cells post-MI are quickly lost with only a small number retained and surviving within the myocardium (Lu, Li et al. 2012). While a number of reasons exist to explain this phenomenon, the main cause is apoptosis (Baldi, Abbate et al. 2002, Lu, Li et al. 2012). Therefore the ability of the matrix to enhance the survival capabilities of patient CACs to similar rates of healthy CACs in hypoxic conditions, presents a promising therapeutic option. Additionally, it was found in animal studies that endothelial cell proliferation tapers off 1 week post-MI and is essentially non-existent at the 2-week time point (Virag and Murry 2003). Due to high levels of necrosis post-MI, cell proliferation of existing cells and transplanted cells is desired in order to aid the revascularization process; the culture of patient CACs on matrix led to an increase in proliferation comparable to that of healthy CACs. The ability of the matrix to enhance the function of patient CACs was better than anticipated. Initially, it was expected that the dysfunctional patient cells would not be able to respond to the matrix as well as healthy CACs, however the culture of patient CACs on matrix appears to be a promising method for cell preconditioning.

Since it was demonstrated that the matrix has a similar effect on both patient and healthy CACs in terms of cell function, cell-ECM interactions and intracellular signaling were further examined to better understand the mechanisms involved. Specifically, gene expression of a number of integrins (cell surface proteins that interact with the ECM and activate important survival pathways) and TAL1 (important transcription factor in angiogenesis) were assessed.

The expression of these genes was first examined in the PBMNCs from patient and healthy donors, prior to any cell culture. There were no significant differences in gene expression between the cells isolated from these 2 groups. However, when looking at CACs from 4-day cultures, there was a significant down-regulation of integrin α1 in patient CACs compared to healthy CACs. Integrin α1 plays a role in adhesion to collagen I (Leitinger 2011) and angiogenesis (Pozzi, Moberg et al. 2000) and TAL1 plays a role in angiogenesis, adhesion and migration (Palii, Vulesevic et al. 2014). Therefore, these genes may be playing a role in the functional loss observed for CACs from CAD patients; and it was of interest to see if culture on the matrix could restore integrin α1 and TAL1 expression in patient CACs to the same level as those of the healthy donors. After culture on matrix, the expression of TAL1 in patient CACs was corrected to that of healthy CACs, but the integrin α1 gene expression was significantly lower in patient CACs compared to its up-regulation in healthy donors CACs. As mentioned, integrin α1 is involved in collagen I adhesion (Leitinger 2011) and angiogenesis (Pozzi, Moberg et al. 2000); typically, integrin α1 is up-regulated by angiogenic signals (Senger, Perruzzi et al. 2002). Despite the lower expression of integrin α1 in patient CACs, the patient CACs performed similarly in the adhesion and angiogenesis assays compared to healthy CACs. This suggests that there may be a compensatory mechanism activated within the patient CACs when cultured on matrix or that the molecule is redundant. Additionally, a recent study showed that the blocking of integrin α1 in healthy CACs did not impact the ability of CACs to adhere to matrix, thus indicating again that compensatory or alternate mechanisms may be activated by matrix culture (Ahmadi, McNeill et al. 2014). The study concluded that integrin α2 played the

main role in CACs-matrix interaction. However, the study did not evaluate the effects that integrin α1 blocking had on other important CAC functions such as angiogenesis. Another study found that α1-null mice were viable and fertile and had no overt vascular phenotype, thus once again suggesting a compensatory mechanism or that it is a redundant molecule (Gardner, Kreidberg et al. 1996). However, one study showed that Obtustatin, a selective inhibitor of α1β1 was able to inhibit angiogenesis *in vivo* (Marcinkiewicz, Weinreb et al. 2003) and another set of studies showed that α1 was implicated in VEGF-driven angiogenesis (Senger, Claffey et al. 1997, Senger, Perruzzi et al. 2002). Even though the blocking of α1 does not have an effect on adhesion and has mixed results on angiogenic properties, the up-regulation of integrin α1 in patient CACs may still result in increased adhesion and angiogenic properties, and thus overall an increased therapeutic effect; however, this remains to be determined. The effect of culturing patient and healthy CACs on matrix was only investigated on 5 out of 26 possible integrin subunits; there is a possibility that some of the unexamined integrins may be playing important roles in the cells' function.

The differences in expression of several genes of interest were also investigated in $CD34^+$ cells from patients and healthy donors. Since $CD34^+$ cells only account for a small proportion of CACs, and only 50 ml amount of blood was collected for the patient studies, we only investigated gene expression in $CD34^+$ cells from fresh PBMNCs and from CACs generated after 4 days of culture. There were a number of significant differences in gene expression in $CD34^+$ cells isolated from PBMNCs. Nearly all the genes were down-regulated, with the greatest reductions observed for VEGF and the genes involved in the Shh pathway. These 2 pathways are extremely important for the

angiogenic process and hence the down-regulation within the $CD34^+$ cell population in the CAD patients may account for their reduced therapeutic potential. However, when the $CD34^+$ cells were isolated from the CAC population, there were no significant differences between the expressions for any of the genes. This signifies the importance of using more specific sub-populations for therapeutics, and/or understanding their functional status. The results suggest that the gene expression profile of a patient's circulating $CD34^+$ cells can be restored by culturing the PBMNCs on a collagen matrix for 4 days. Therefore using these cells, increasing their concentration and culturing them on matrix may be more beneficial than a cell treatment using dysfunctional $CD34^+$ cells directly from the isolated PBMNCs. While this needs to be further investigated, it may be a promising option for improving the potency of these cells for therapy, along with the use of apheresis in order to obtain greater quantities of the $CD34^+$ cells. This is an attractive approach since clinical trials using BMMNCs have had inconsistent outcomes, while the use of $CD34^+$ cells for cell therapy appear to have a more beneficial impact on LVEF (Jiang, He et al. 2010). Additionally the culture of $CD34^+$ cells on matrix yielded greater amounts of clusters of cells than CACs as a whole population. This is promising because cell aggregates in stem cell preconditioning have shown to have an increased secretome promoting cell proliferation, integrin signaling and survival (Sart, Ma et al. 2014), and thus suggest that these beneficial survival pathways would be activated to a greater extent in $CD34^+$ cells cultured on matrix than CACs cultured on matrix.

The culture of cells on collagen I matrix has been shown to induce functional benefits in healthy $CD34^+$ cells, as well as healthy and CAD patient CACs. In addition to its in vitro culture use, the matrix is thermosensitive and can be used as a delivery

vehicle for the transplantation of cells. The biomaterial can be inserted in liquid form via catheter and solidify upon reaching physiological temperature. This would allow for the treatment to fill complex shapes (Kuraitis, Hou et al. 2011), and to increase contact with tissues (Hilborn 2011). Such a treatment could serve to be very beneficial because the cells would be preconditioned on the matrix, and then would be inserted with the matrix into the ischemic myocardium; the ongoing interaction between cells and matrix could constitutively activate beneficial pathways, such as those associated with angiogenesis and survival. Additionally, injecting the cells with a biomaterial could overcome common concerns associated with cell transplantation, such as poor engraftment and lack of persistence of transplanted cells in the target tissue (Suuronen, Zhang et al. 2009).

5.0 Conclusion

CAD is the leading cause of death worldwide and regenerative therapies are in demand to put an end to it. In this study we have shown that a collagen I matrix is able to enhance the function of the pro-angiogenic $CD34^+$ cell sub-population from CACs. The matrix is able to enhance the proliferation, migration, and angiogenic potential of these cells. These benefits are likely elucidated via paracrine effects that may involve the Shh pathway. Additionally, when investigating the effect that the matrix has on CACs from CAD patients, the matrix was able to promote their proliferation, viability and angiogenesis. Despite having similar functional capabilities compared to healthy CACs, integrin α1 was down-regulated in patient CACs after matrix culture, suggesting a compensatory or alternative mechanism is involved in regulating CAC-matrix interactions. Additionally, it was demonstrated that $CD34^+$ cells isolated from the CACs of healthy and CAD patient donors had similar gene profiles, indicating that $CD34^+$ cells from patients may also be able to respond favourably to the matrix. Overall, culturing cells on a collagen I matrix is a promising option for expanding and enhancing cell populations that are needed for cell therapy.

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