Cardiac Tissue Engineering

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ABSTRACT

The limited treatment options available for heart disease patients has lead to increased interest in the development of embryonic stem cell (ESC) therapies to replace heart muscle. The challenges of developing usable ESC therapeutic strategies are associated with the limited ability to obtain a pure, defined population of differentiated cardiomyocytes, and the design of *in vivo* cell delivery platforms to minimize cardiomyocyte loss. These challenges were addressed in Chapter 2 by designing a cardiomyocyte selectable progenitor cell line that permitted evaluation of a collagen-based scaffold for its ability to sustain stem cell-derived cardiomyocyte function ("A P19 Cardiac Cell Line as a Model for Evaluating Cardiac Tissue Engineering Biomaterials"). P19 cells enriched for cardiomyocytes were viable on a transglutaminase cross-linked collagen scaffold, and maintained their cardiomyocyte contractile phenotype *in vitro* while growing on the scaffold. The potential for a novel cell-surface marker to purify cardiomyocytes within ESC cultures was evaluated in Chapter 3, "Dihydropyridine Receptor (DHP-R) Surface Marker Enrichment of ES-derived Cardiomyocytes". DHP-R is demonstrated to be upregulated at the protein and RNA transcript level during cardiomyogenesis. DHP-R positive mouse ES cells were fluorescent activated cell sorted, and the DHP-R positive cultured cells were enriched for cardiomyocytes compared to the DHP-R negative population. Finally, in Chapter 4, mouse ESCs were characterized while growing on a clinically approved collagen I/III-based scaffold modified with the RGD integrin-binding motif, ("Collagen (+RGD and –RGD) scaffolds support cardiomyogenesis after aggregation of mouse embryonic stem cells"). The collagen I/III RGD+ and RGD- scaffolds sustained ESC-derived cardiomyocyte growth

and function. Notably, no significant differences in cell survival, cardiac phenotype, and cardiomyocyte function were detected with the addition of the RGD domain to the collagen scaffold. Thus, in summary, these three studies have resulted in the identification of a potential cell surface marker for ESC-derived cardiomyocyte purification, and prove that collagen-based scaffolds can sustain ES-cardiomyocyte growth and function. This has set the framework for further studies that will move the field closer to obtaining a safe and effective delivery strategy for transplanting ESCs onto human hearts.

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List of Abbreviations

ALCAM	Activated leukocyte cell adhesion molecule or CD166
α-mem	Alpha-minimal essential media
ANP	Atrial natriuretic peptide
BMP	Bone morphogenetic protein
CDB	Cell dissociation buffer
CIHR	Canadian Institute of Health Research
CMs	Cardiomyocytes
DHP-R	Dihydropyridine receptor
DMEM	Dulbecco's modified eagle media
DMSO	Dimethyl sulfoxide
DTT	DL-dithiothreitol
EBs	Embryoid Bodies
EC	Embryonal carcinoma
ECM	Extracellular matrix
ED	Embryonic day
EGFP	Enhanced green fluorescent protein
EPC	Endothelial Progenitor Cells
ES	Embryonic stem
ESC	Embryonic stem cell
FACS	Fluorescent activated cell sorting

FBS	Fetal bovine serum
GFP	Green fluorescent protein
H&E	Hematoxylin and eosin
HCA	Human cardiac α-actin
hES	Human embryonic stem
hESC	Human embryonic stem cell
HSC	Hematopoietic stem cells
iPS	Induced pluripotent stem
LIF	Leukemia inhibitor factor
MACS	Magnetic activated cell sorting
MEA	Microelectrode array
mES	Mouse embryonic stem
mESC	Mouse embryonic stem cell
MHC	Myosin heavy chain
MSC	Mesenchymal stem cells
MI	Myocardial infarction
OGS	Ontario Graduate Scholarship
PBS	Phosphate-buffered saline
PEG	Polyethylene glycol
Percoll	Polyvinylpyrrolidone (PVP)-coated silica
PFA	Paraformaldehyde
PMSF	Phenylmethylsulfonyl fluoride
QPCR	Quantitative real-time polymerase chain reaction

RGD	Arginine-Glycine-Aspartic Acid
RGDS	Arginine–glycine–aspartic acid–serine
ROCK	p-160-Rho-associated coiled kinase
SC	Stem cell
SD	Standard deviation
SDS	Sodium dodecyl sulfate
SE	Standard error
SEM	Standard error of measurement
SP	Side population
sPBS	Stockholm's phosphate buffered saline
TEM	Transmission Electron Microscopy
TMRM	Tetramethylrhodamine methyl ester perchlorate
VEGF	Vascular endothelial growth factor
VEGF-R2	Vascular endothelial growth factor receptor-two
VPCs	Vascular progenitor cells

CONTRIBUTIONS OF CO-AUTHORS

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CHAPTER 3:

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CHAPTER 1: Introduction

1.1 - Heart Disease and Cell Therapy

Heart disease is the leading cause of death in Canada (Public-Health-Agency-of-Canada, 2011). Myocardial infarction (MI), the major cause of death, is associated with loss in functional cardiac muscle, or cardiomyocytes (Thygesen et al., 2007), which if not replaced by functional muscle could lead to heart failure. The average MI that induces heart failure has been estimated to cause a loss of approximately 1 billion cardiomyocytes (Gepstein, 2002). Cardiomyocytes are terminally differentiated, and myocardium has very limited abilities to undergo functionally significant regeneration to compensate for the muscle loss (Jiang and Liao, 2010). Following myocardial damage, scar tissue is formed, followed by tissue hypertrophy. Hypertrophy can lead to further damage and heart failure. Heart transplantation is one of the only options for patients with severe heart failure (Stevenson, 1993). The morbidity and mortality related to cardiomyocyte loss and the shortage of donor hearts for transplantation has lead to significant interest in the development of cell therapy treatment options (Zhang M, 2010).

Apart from MI, congenital malformations exist where ventricular or atrial muscle is lacking (Zimmermann and Cesnjevar, 2009). The application of donor cardiomyocytes and cardiac tissue engineering for improving congenital heart defects or malformations has been recently reviewed (Choi et al., 2011; Zimmermann and Cesnjevar, 2009). Children with malformations, such as severe ventricular malformations, have limited treatment options, with heart transplantation being one of the only possibilities (Conway and Dipchand, 2010). Heart transplantation in infants or children is limited by a lack of suitable donors (Mathur et al., 2011), and complications involved include life-long immunosuppression (Milanesi et al.,

2007). Cardiomyocyte transplantation therapy that provides new muscle may improve this prognosis (Zimmermann and Cesnjevar, 2009).

The theory of cell replacement therapy suggests that if the defect can be re-muscularized it may be able to support the failing heart, inhibit hypertrophy, and decrease further deterioration (Mummery et al., 2010; Zimmermann, 2009). Many cell types have been proposed for transplantation, however pre-clinical animal studies using embryonic stem cell (ESC)-derived cardiomyocytes have indicated that ESCs may be an ideal cell source for cardiomyocyte transplantation.

1.2 - Cell Types for Delivery to the Heart

As summarized in Figure 1, many different cell types are being investigated as candidates or models for regenerating damaged muscle in the heart. These include: skeletal myoblasts, bone marrow-derived multi-potent progenitor cells, embryonic or neonatal cardiomyocytes, resident adult cardiac progenitor cell populations, induced pluripotent stem cells (iPS cells), reprogrammed somatic cells, and ESCs (Vunjak-Novakovic et al., 2010).

1.2.1 - Skeletal myoblasts

Adult skeletal muscle myoblasts or progenitor cells have been explored extensively for their application to repair damaged myocardium. Skeletal myoblasts can be obtained autologously, and expanded *in vitro*. Although there is limited evidence that skeletal myoblasts can trans-differentiate into cardiomyocytes (Iijima et al., 2003; Reinecke et al., 2002; Tamaki et al., 2008), myoblast transplantation in animal infarct models can enhance cardiac function (Siltanen et al., 2011; Taylor et al., 1998). Research to date indicates that skeletal myoblasts do not have the ability to form functional electromechanical connections

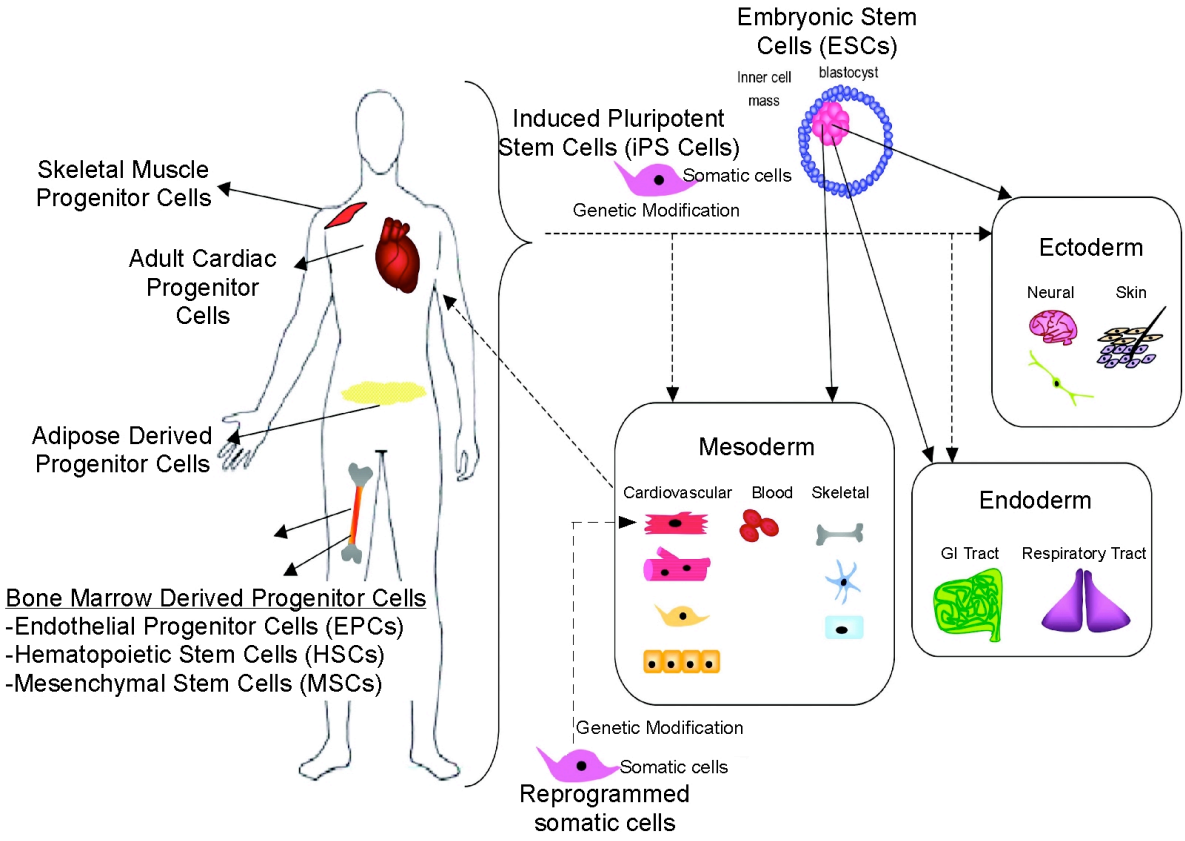

Figure 1.1 - Proposed cell sources for cardiac tissue engineering.

Embryonic stem cells, induced pluripotent stem cells (iPS cells), direct cardiac re-programmed somatic cells, resident cardiac progenitor cells, skeletal muscle progenitor cells, adipose-derived progenitor cells, and bone marrow derived cells, are under investigation as potential sources for repairing damaged myocardium. Figure adapted from (Vunjak-Novakovic et al., 2010).

with the host myocardium, and although they show contractile activity, they cannot integrate and beat synchronously with the host, leading to a higher potential for arrhythmias (Eisen, 2008; Gepstein et al., 2010; Menasche et al., 2003). There is evidence that increasing myoblast connexin 43 expression can improve functional integration (Lee et al., 2009; Tolmachov et al., 2006). There are also multiple ongoing clinical trials with different attempts to use skeletal myoblasts to repair damaged heart muscle, with some positive effects reported (Dib et al., 2009; Haider et al., 2008; Siminiak et al., 2004).

1.2.2 - Adult Bone Marrow Stem Cells

Human bone marrow contains a progenitor cell pool, including endothelial progenitor cells (EPCs), hematopoietic stem cells (HSCs), and mesenchymal stromal cells (also known as mesenchymal stem cells or MSCs). These progenitor/stem cells although not totipotent like ECSs, are multipotent and can still differentiate into more restricted lineages. EPCs can differentiate into endothelial cells that make up the lining of the blood vessels, with a few limited reports of their ability to trans-differentiate into cardiomyocytes (Badorff et al., 2003; Condorelli et al., 2001). It has been shown that compared to HSCs and MSCs, EPCs resulted in better neovascularization and contractility when implanted in a rat cardiac infarct model (Suuronen et al., 2007). Clinical testing is underway to assess the ability of EPCs to grow new blood vessels and repair the human myocardium, as reviewed in (Mosna et al., 2011; van Laake et al., 2006).

HSCs give rise to all blood cell types, including myeloid and lymphoid lineages, as reviewed in (Muller-Sieburg et al., 2002). Despite some reports that HSCs can regenerate

the myocardium (Orlic et al., 2001), *in vivo* and *in vitro* testing demonstrates that HSCs have a very limited ability to acquire a cardiomyocyte phenotype (Murry et al., 2004).

MSCs have been isolated from bone marrow, adipose and organ adventitia. In the adult bone marrow, MSCs are a rare population of cells. When isolated and cultured, they have been shown to acquire the phenotype of cardiomyocytes, osteoblasts, chondrocytes, adipocytes, neurons, smooth muscle, and endothelial cells, as reviewed in (Paul et al., 2009). Many groups have shown that MSCs can improve cardiac function after injection into preclinical rat and pig infarct models, as reviewed in (Nesselmann et al., 2008). Advantages of MSCs include the potential for autologous transplantation, ease of isolation, and expandability in culture.

Adipose tissue is composed of adipocytes but, also contains a stromal-vascular fraction containing endothelial cells, smooth muscle cells, and stem cells (Zuk et al., 2001). MSC-like cells isolated from adipose tissue have been shown to differentiate into cardiomyocytes, reviewed in (Palpant and Metzger, 2010). Adipose-derived cardiomyocytes transplanted onto an infarcted mouse heart contributed to cardiac repair (Bai et al., 2010). The advantage to using cardiomyocytes derived from human adipose tissue for cell transplantation is the accessibility of a patients own adipose by liposuction for autologous transplantation (Palpant and Metzger, 2010).

Disadvantages of using MSCs and adipose-derived stem cells include the potential for a patient's disease state to affect their stem cell pool (Heeschen et al., 2004), and conflicting reports as to whether MSCs actually differentiate into cardiomyocytes (van der Bogt et al., 2009), or if any observed functional improvements are due to cell fusion and/or paracrine effects (Burdon et al., 2011).

Clinical trials using MSCs for cardiac repair report mixed results, as reviewed in (Paul et al., 2009), and there is at least one clinical trial presently on-going, testing human adipose-derived progenitor cells on patients with acute MI and chronic ischemia (Meliga et al., 2007).

1.2.3 - Fetal or Neonatal Cardiomyocytes

Primary cardiomyocytes acquired from neonatal or fetal rodent hearts have been used as models to test cardiomyocyte delivery strategies and integration into the myocardium, as reviewed in (Dowell et al., 2003). Cultured neonatal cardiomyocytes show limited proliferative potential for *in vitro* expansion, and due to limitations of obtaining neonatal human cardiomyocytes, this model is not an appropriate cell source for delivery to human hearts. Regardless, neonatal and fetal cardiomyocytes are an excellent positive control for both *in vivo* and *in vitro* cardiomyogenesis and cardiac tissue engineering studies, and have been well utilized in the literature as such. A recent study, using one-day old neonatal mice, reported significant cardiomyocyte regeneration potential after partial surgical resection of the heart (Porrello et al., 2011). Interestingly, the neonates lost this heart repair capacity by the time they were seven days old. This observation is the first report of cardiac regeneration in mammals after birth, and will have profound implications on the field of cardiac development and regeneration if the signals directing this regeneration can be elucidated.

1.2.4 - Adult Cardiac Progenitor Cells

There is mounting evidence that a small proportion of cells in the adult heart re-enter the cell cycle after injury (Anversa and Kajstura, 1998; Beltrami et al., 2001; Quaini et al., 1994). Adult cardiomyocyte progenitors have been identified based on their pattern of

surface protein expression, such as Sca-1, c-kit or Isl-1 positive cells, or their side population (SP) properties, as reviewed in (Barile et al., 2007). Adult cardiomyocyte progenitor cells have been shown to reconstitute myocardium following an infarct in the rodent model (Beltrami et al., 2003; Dawn et al., 2005). A GATA4 positive population of cells that may contribute to cardiac repair has also been shown to be present in the adult heart epicardium (Vieira and Riley, 2010). Disadvantages include potential limitations in obtaining clinically significant cell numbers for treatment, and finding appropriate donor hearts.

1.2.5 - Embryonic Stem Cells

ES cells are derived from the inner cell mass of the blastocyst, and were first derived from mouse embryos in 1981 (Evans and Kaufman, 1981; Martin, 1981), and from human embryos in 1998 (Thomson et al., 1998). Numerous human ESC (hESC) and mouse ESC (mESC) lines have since been developed and are now available for research purposes. ESCs are characterized by their ability to self-renew in culture for extended time periods in their undifferentiated state, and by their pluripotency, the ability to differentiate into cell types from all three germ layers of the embryo, as reviewed in (Liu et al., 2007). ES differentiation into cardiomyocytes is reviewed in section 1.3.2. HES-derived cardiomyocytes display ultrastructural and functional properties similar to embryonic cardiomyocytes (Kehat et al., 2001), and numerous *in vivo* animal studies report that ES-derived cardiomyocytes integrate functionally into damaged cardiac tissue. HESCs differentiated into cardiomyocytes improve function in rodent hearts following a MI (Laflamme et al., 2007; Stevens et al., 2009a; van Laake et al., 2009), and integrate into the host and contribute to pacing in a swine atrioventricular block model (Kehat et al., 2004). Monkey ES cell-derived cardiomyocyte precursors differentiated into cardiomyocytes, and replaced 1/5 of a cardiac scar in primates

(Blin et al., 2010). MESC derived cardiomyocytes transplanted in rodent hearts (Adler et al., 2010; Christoforou et al., 2010; Lu et al., 2010b), and sheep hearts (Menard et al., 2005), also contributed to cardiac repair.

The challenges associated with using ES cells to repair a human heart include:

- Obtaining a pure population of ES-derived cardiomyocytes for delivery to the heart and the related risk of tumour formation from the accidental transplantation of an undifferentiated ES cell (Blum and Benvenisty, 2008; Fong et al., 2011) (Reviewed below in section 1.4).
- Cell survival issues related to the dissociation and processing of hES-derived cardiomyocytes in preparation for cell delivery (discussed below in section 1.5 and in Chapter 3).
- Dissipation of the cells away from the site of interest (Discussed in sections 1.5 and 1.6).
- Potential immune rejection issues.
- Genetic stability of the cells

There is conflicting evidence regarding the potential for ES cells to evoke an immune response. Studies have confirmed that hESCs express low levels of class 1 major histocompatibility complex molecules, and that the levels increased with differentiation (Drukker et al., 2002). Many hES cardiomyocyte delivery studies used either immunodeficient animal models (Kofidis et al., 2006; Laflamme et al., 2007; Stevens et al., 2009a; van Laake et al., 2009), or drugs to curb immunorejection were administered (Kehat et al., 2004).

1.2.6 – Embryonal Carcinoma Cells

Pluripotent embryonic carcinoma (EC) cells are derived from teratocarcinoma tumours that arise in the testes of humans and mice. Teratocarcinomas are malignant tumours that contain many different tissue types, including undifferentiated stem cells. By isolating the teratocarcinoma-derived stem cells in culture, EC cell lines have been generated (van der Heyden and Defize, 2003). EC cells can be expanded and grown *in vitro* without the use of feeder cells or leukemia inhibitory factor, can be genetically manipulated, and are an excellent tool to investigate the molecular mechanisms underlying embryonic development and ES cell differentiation (Skerjanc, 1999). The P19 cell line was derived from a teratocarcinoma induced in C3H/HC mice (McBurney and Rogers, 1982). P19 cells have been used extensively as a model for studying cardiac and skeletal muscle development, as reviewed in (Skerjanc, 1999; van der Heyden and Defize, 2003). Due to their tumorigenic origin, EC cells are not suited for clinical applications.

1.2.7 - Induced Pluripotent Stem Cells

Pluripotent stem cells can be obtained from somatic cells by nuclear re-programming. Termed iPS cells, human fibroblast cells were first induced in 2007 to form pluripotent stem cells by the transduction of c-myc, Oct3/4, SOX2, and Klf4 (Takahashi et al., 2007), or Nanog, SOX2, Oct3/4, and Lin28 (Yu et al., 2007). IPS cells maintain an ES cell-like characteristic both *in vitro* and *in vivo*, and are capable of differentiating into cells originating from the three germ layers of the embryo. Human iPS cells have been generated from many somatic cell sources, such as, skin fibroblasts (Park et al., 2008; Takahashi et al., 2007; Yu et al., 2007), keratinocytes (Aasen et al., 2008), and blood (Giorgetti et al., 2009;

Kunisato et al., 2010; Loh et al., 2009). In addition, the reprogramming of somatic cells has been refined, removing the requirement for transduction with oncogenes (Martinez-Fernandez et al., 2010; Nakagawa et al., 2008), and finding potentially safer alternatives to retro or lentivirus–based transduction methods (Kaji et al., 2009; Okita et al., 2008; Stadtfeld et al., 2008; Woltjen et al., 2009).

IPS cells can be induced to differentiate into cardiomyocytes in a similar fashion as hESCs, including embryoid body (EB)-based differentiation with serum (Zhang et al., 2009), activin A and bone morphogenic protein (BMP) 4 treatment (Kattman et al., 2011), and co-culture with a stromal layer (Gupta et al., 2010). Human iPS derived cardiomyocytes have been shown to have a similar contractile phenotype as ES-derived cardiomyocytes (Yokoo et al., 2009). The advantages of using iPS cells for cardiac therapy are similar to ES cells, but they also provide the potential for autologous transplantation. Similar to ES cells, iPS cells also carry a risk of teratoma formation from undifferentiated cells (Kooreman and Wu, 2010), and thus must be predifferentiated into a cardiac-restricting lineage and purified. The risk of implanting cells containing transgenes into humans (Hacein-Bey-Abina et al., 2003), and the length of time required to create pluripotent cells and differentiate them into cardiomyocytes (Zhang et al., 2009), decrease the clinical feasibility of this approach.

Recent advances in somatic cell reprogramming report that mouse skin fibroblasts can be reprogrammed directly to cardiomyocytes, without the need to first be in a pluripotent state (Efe et al., 2011). Efe *et al.* demonstrated that cardiomyocytes could be generated from fibroblasts by using the same gene transduction approach used for iPS cell generation, but for a shorter time period. Beating cardiomyocytes were generated by exposing the mouse cells to cardiac-promoting signals such as BMP4, serum, and a small molecule inhibitor. Tests to determine if the cardiomyocytes retain their phenotype over time, and the tendency to form

teratomas are presently being investigated (Efe et al., 2011). Srivastava's group demonstrated that mouse cardiac and dermal fibroblasts can be reprogrammed into cardiomyocyte-like cells using MEF2C, GATA4, and Tbx5 gene transduction (Ieda et al., 2010).

1.3 - Cardiomyogenesis: From the embryo to stem cells

1.3.1 - Cardiomyogenesis in The Embryo

The heart is the first organ to form in the embryo. At the early blastocyst stage, cells of the inner cell mass are totipotent, having the ability to differentiate into all the cell types of the organism. With the onset of gastrulation, however, mesoderm is formed in the primitive streak and the cells rapidly become committed to distinct lineages. Cardiomyogenesis begins when cells from the posterior portion of the primitive streak migrate to form the cardiac crescent in the mesoderm (Garcia-Martinez and Schoenwolf, 1993; Tam et al., 1997), as depicted in Figure 1.2 (Buckingham et al., 2005). The cardiac crescent fuses at the midline to form the cardiac tube, and the tube subsequently undergoes rightward looping to form the structural basis of the heart chambers, as summarized in (Harvey, 1998). Two separate progenitor populations arise in the mesoderm, and are commonly known as the primary heart field and the secondary heart field. Although these fields originate from a common progenitor population during gastrulation, they segregate to form two distinct populations of cardiac precursors in the mesoderm (Meilhac et al., 2004). Cells in the primary heart field originate from the anterior splanchnic mesoderm and contribute to the early heart tube, left ventricle, and atria. The secondary (anterior) heart field is located in the pharyngeal mesoderm, and gives rise to the atrial end of the heart tube, the right ventricle and the outflow tract (Cai et al., 2003; Kelly et al., 2001; Zaffran et al., 2004).

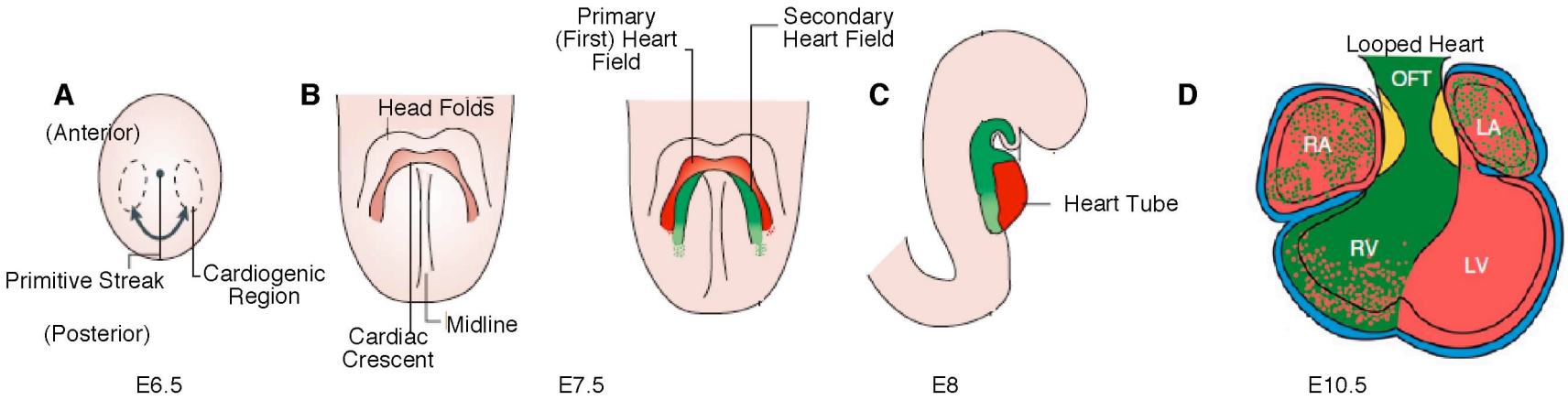

Figure 1.2 - Cardiomyogenesis in The Mouse Embryo.

Cardiomyocyte progenitor cells migrate from the posterior portion of the primitive streak on embryonic day (ED) 6.5 to the anterior portion of the embryo (A), and form cardiac crescent mesoderm located underneath the head folds on ED 7.5 (B). Differentiated cardiomyocytes are found in the cardiac crescent, and the crescent contains two distinct heart fields, the primary and secondary heart fields. The cardiac crescent fuses at the midline to form the heart tube (C), which subsequently undergoes rightward looping to form the structural basis of the heart (D). LV, left ventricle; OFT, outflow tract RA, right atria; LA, left atria; RV, right ventricle. Red = contributions of the primary heart field, Green = contributions of the secondary heart field. Figure adapted from (Buckingham et al., 2005; Vincent and Buckingham, 2010).

At the molecular level, cells of the inner cell mass can be identified by their expression of pluripotency genes such as Oct4 (Pou5fl), Nanog, and Sox2, as discussed in (Fernandez-Tresguerres et al., 2010). During gastrulation, an early marker of mesoderm is the transient expression of the T-box transcription factor Brachyury (Kispert and Herrmann, 1994). Migration to cardiac crescent mesoderm is thought to be driven by the transient expression of Mesp1 (Bondue et al., 2008; Saga et al., 2000). Myogenesis is regulated by extracellular signals from the ectoderm, endoderm and neural tube, such as signals from the Activin/Nodal/TGF-β, Wnt, and BMP pathways, as reviewed in (Brand, 2003; Gessert and Kuhl, 2010). Several cardiac-related transcription factors become activated, including Nkx2.5 (Komuro and Izumo, 1993; Lints et al., 1993) and GATA4 (Grepin et al., 1994). Cardiac targets of these transcription factors such as MEF2C, cardiac *a*-actin, atrial natriuretic peptide (ANP), and cardiac isoforms of myosin heavy chain (MHC) encode transcriptional regulators and structural proteins that are important for cardiomyocyte development and promote cardiomyocyte formation (Charron et al., 1999; Chen et al., 1996; Durocher et al., 1996; Molkentin et al., 1994; Morin et al., 2000).

1.3.2 – Cardiomyogenesis in Stem Cells

Cardiomyogenesis in P19 cells can be induced by aggregating the cells in the presence of dimethyl sulfoxide (DMSO) with serum (McBurney et al., 1982). Cardiomyogenesis in hES and mESCs can be induced using an EB-based differentiation regime also with serum (Kehat et al., 2001; Puceat, 2008). Recent advances in directing cardiomyogenesis in hESCs have lead to an efficient monolayer induction method involving temporal activin A and BMP4 treatment in hESCs (Kattman et al., 2011; Laflamme et al., 2007). Activin A increases induction of non-specific mesoderm (Smith et al., 1990), and

BMP4 further specifies the mesoderm towards a cardiac lineage (Ladd et al., 1998). The sequential treatment of activin A and BMP4 in hESCs was reported to increase the percentage of hES cardiomyocytes in culture from 1% to 30% (Laflamme et al., 2007). A similar BMP2/Activin A cardiomyogenesis-induction protocol using an EB based approach in hESCs and iPS cells, has been shown to generate highly pure populations of cardiomyocytes ranging from 60-80% purities (Kattman et al., 2011; Yang et al., 2008). In addition, a combination of BMP2 treatment and fibroblast growth factor inhibitor treatment induced hES and human iPS cells to form SSEA-1 positive cardiomyocyte progenitors (Blin et al., 2010). HESCs can also be induced to form cardiomyocytes in monolayer by co-culturing them with a visceral endodermal-like cell line called END-2 (Mummery et al., 2003), or with combined END-2 co-culture and BMP2 treatment (Bin et al., 2006).

Embryonic stem cell cardiomyogenesis parallels P19 cell cardiomyogenesis to a great extent, and begins when pluripotent, or "undifferentiated", ES cells are induced to form mesoderm, as reviewed in (Wobus and Boheler, 2005). Similar to the inner cell mass, undifferentiated ES cells express a network of transcription factors such as Nanog, Oct3/4 and Sox2, that maintain the undifferentiated state and repress differentiation, as reviewed in (Boyer et al., 2005). Upon induction, multipotent mesoderm, capable of differentiating into cardiac, vascular, and endothelial lineages is often identified by the transient upregulation of Flk-1 (KDR in humans), a receptor for VEGF (VEGF-R2) (Ema et al., 2006; Kattman et al., 2006). Mesodermal cells destined to become cardiomyocytes begin to express cardiac-related transcription factors Nkx2.5, GATA4, and MEF2C. Further maturation of the cardiomyocytes is monitored by the expression of myocyte structural genes such as MHC, sarcomeric α-actinin, cardiac α-actin, and members of the cardiac troponin family, as reviewed in (Sachinidis et al., 2003). ES-derived cardiomyocytes beat spontaneously in

culture, and show structural and functional characteristics similar to embryonic cardiomyocytes (Kehat et al., 2001). MESCs grown as EBs show spontaneously beating clusters of cardiomyocytes starting around day 7-10 of differentiation, and L-type calcium channel function starting on day 7 of differentiation (Kolossov et al., 1998), mediating the calcium-induced-calcium-release increase in free cystolic calcium levels and myocyte contractions.

The efficiency of cardiomyogenesis in ES cells varies widely between cell lines, and can range from 5-15% in P19 cells (Skerjanc, 1999) and unpublished data, and 1-80% in mES and hES and iPS cells (Chen et al., 2010; Kattman et al., 2011; Laflamme et al., 2007; Puceat, 2008). In addition to BMP and activin A treatment, many chemicals and small molecule inhibitors have been demonstrated to increase cardiomyogenesis in ES models (Graichen et al., 2008; Wang et al., 2010; Xu et al., 2008a).

1.4 - Purifying ES-derived Cardiomyocytes

Directing ESC differentiation towards cardiac muscle lineages is becoming well characterized, and differentiation procedures have improved greatly creating a relatively high percentage of cardiomyocytes, however we do still not have the ability to culture a pure population of cardiomyocytes. The pluripotent capacity of ES cells that allows them to differentiate into cardiomyocytes also means that the potential for other cells derived from any of the three germ layers may be high. In addition, there is the possibility that cells at various stages of differentiation, including undifferentiated cells, may be present in the mixed cultures.

As summarized in Table 1.1, various approaches aimed at purifying ES-derived cardiomyocytes have been taken. Dissection of beating clusters (Asp et al., 2010; Caspi et

al., 2009; Passier et al., 2005; Segev et al., 2005), procedures taking advantage of differences in cardiomyocyte density (Laflamme et al., 2007; Xu et al., 2002), fluorescent or antibiotic selection of transgenic cultures (Anderson et al., 2007; Huber et al., 2007; Kita-Matsuo et al., 2009; Xu et al., 2008b), cell sorting based on mitochondrial activity (Hattori et al., 2009), cell sorting using an antibody-based approach with lineage restricting cell surface proteins (Adler et al., 2010; Nelson et al., 2008; Yang et al., 2008), and cell sorting using an antibody-based approach with cardiomyocyte lineage specific cell surface proteins (Blin et al., 2010; Honda et al., 2006; Rust et al., 2009; Van Hoof et al., 2010). Finally, as summarized in section 1.3.2, the directed differentiation approach has led to high cardiomyocyte culture purities, and can be combined with all of the aforementioned purification strategies.

Table 1.1 - Previously published strategies to enrich cardiomyocyte cultures.
Abbreviations within the table: CMs (cardiomyocytes), EPCs (Endothelial Progenitor Cells), VPCs (Vascular progenitor cells), antibiotic (antibiotic-based selection).

Cell Type	**Purification Technique**	**Purity**	***In Vivo* Results**	**References**
Density-based enrichment				
hESC	Percoll Gradient	70%	N/A	(Xu et al., 2002)
mESC	Percoll Gradient	Not reported	Loss of teratoma forming ability	(Lin et al., 2010)
hESC	Percoll Gradient + directed differentiation	83%	Loss of teratoma forming ability, contributed to repair in rat infarct model	(Laflamme et al., 2007)
Genetic-based selection				
mESC	MLC-2v-eGFP FACS + Percoll Gradient	97%	N/A	(Muller et al., 2000)
hESC	MLC-2V FACS	93%	N/A	(Huber et al., 2007)
mESC	Connexin40 eGFP FACS	⇑ CM transcript	N/A	(David et al., 2008)
mESC	Nkx2.5-GFP day 6 FACS	55-65%	N/A	(Christoforou et al., 2008)
mESC	cardiac α-actin GFP FACS	95%	N/A	(Kolossov et al., 1998)
mESC	αMHC antibiotic, bioreactor stirring	60%	N/A	(Zandstra et al., 2003)
mESC	α-SmM actin antibiotic	75%	N/A	(Potta et al., 2010)

mESC	αMHC antibiotic	99%	Form stable intracardiac grafts on rat hearts	(Klug et al., 1996)
mESC	αMHC antibiotic selection, bioreactor	100%	N/A	(Schroeder et al., 2005)
mESC	αMHC antibiotic	99%	N/A	(Zandstra et al., 2003)
hESC	αMHC antibiotic	96%	N/A	(Kita-Matsuo et al., 2009)
hESC	αMHC antibiotic	99%	Loss of teratoma ability	(Xu et al., 2008b)
hESC	αMHC -GFP FACS and manual dissection		Loss teratoma ability, GFP+ cells restricted to striated muscle	(Ritner et al., 2011)
hESC	αMHC antibiotic	90%	N/A	(Anderson et al., 2007)
Surface Marker Purification				
hES	Elastin microfibril interfacer 2 FACS	Low % CMs	N/A	(Van Hoof et al., 2010)
hESC mESC Marmot ESC	TMRM Mitochondrial Dye FACS	99% CMs	Loss of teratoma forming ability	(Hattori et al., 2009)
hESC	ALCAM (CD166) MACS	85% CMs	N/A	(Rust et al., 2009)
hESC	KDR(low)/c-kit(-)	57% CMs, EPCs, VPCs	N/A	(Yang et al., 2008)
hESC	KDR(FLK-1)+ /PDGFRa FACS	80%	N/A	(Kattman et al., 2011)
mESC	Flk-1+/PDGFRa FACS	5X⇑ CMs	N/A	(Hirata et al., 2006)
Monkey ES hESC iPSC	SSEA-1, MACS	CMs, SmMC, EPC	Monkey ESC repaired 20% scar in non-human primate	(Blin et al., 2010; Leschik et al., 2008)
mESC	Flk-1+-bryT GFP d4.25 FACS	CM<50%, EPCs, VPCs	N/A	(Kattman et al., 2006)
Tet-notch mESC	Flk-1+ with notch4 induction (tet Notch4)	CMs, EPCs, VPCs	CMs, re-populated heart to greater extent then EnPCs, VPCs.	(Adler et al., 2010)
mESC	Flk-1 (d4) FACS	14X⇑ CM	Re-populated rodent heart, improved cardiac function	(Baba et al., 2007b)
mESC	CXCR4/ Flk1 FACS	40% beating clusters	N/A	(Nelson et al., 2008)
mESC	Ncadherin FACS	⇑ CM markers	N/A	(Honda et al., 2006)
mESC	Sca-1 FACS	100% EBs contracting	N/A	(Lam et al., 2010)
Signalling Based Enrichment Strategies (Directed Differentiation)				
mESC, hESC, iPSC	Activin A / BMP4 + bFGF	60-80% CMs	N/A	(Kattman et al., 2011)
hESC	Activin A / BMP4, DKK, VEGF + Percoll	83% CMs	Loss of teratoma forming ability, contributed to repair in rat infarct model	(Laflamme et al., 2007)
hESC	Activin A / BMP4	31% CMs	Grafts were viable 4 weeks after	(Xu et al., 2011)

			transplantation onto rat hearts.	

Manual cardiomyocyte culture enrichment techniques, such as dissecting out clusters of beating cardiomyocytes from an ES culture using a micropipette or scalpel, can be useful for studying ES-derived cardiomyocytes (Asp et al., 2010; Passier et al., 2005; Segev et al., 2005), and in drug screening assays (Caspi et al., 2009). Limitations include the inability to scale up and prepare large quantities of cardiomyocytes, and contamination from other cell types growing in close proximity to the beating clusters.

Protocols taking advantage of differences in cell density have been well utilized for cardiomyocyte enrichment strategies in ES cell cultures. The use of discontinuous percoll gradient centrifugation, (polyvinylpyrrolidone (PVP)-coated silica), allow cardiomyocytes in a heterogeneously growing ES cell culture dish to be separated at a purity of 70-83% (Laflamme et al., 2007; Xu et al., 2002). hES-derived cardiomyocyte cultures that were enriched using percoll gradient centrifugation to a purity of 83%, showed no evidence of teratoma formation and partly remuscularized myocardial infarcts when they were injected into rat hearts (Laflamme et al., 2007). Disadvantages of percoll gradient enrichment include a risk of contaminating cells due to limitations in purity, and a lack of clinical approval due to potential toxicity of its polyvinylpyrrolidone component (Fong et al., 2009). Percoll enrichment is also less effective in progenitor cardiomyocyte purifications (Xu et al., 2006).

1.4.1 - Fluorescent Activated Cell Sorting

Cell sorting of cardiomyocytes based on their unique gene and protein expression patterns and mitochondrial activities rely heavily on technological advances in fluorescent activated cell sorting (FACS) and magnetic sorting. FACS sorted cells are highly homogenous (Orfao and Ruiz-Arguelles, 1996), and due to its specificity and accuracy, FACS is the most efficient technique for separating low frequency cell-populations from

heterogeneous samples (Johnson et al., 2007). High levels of cell death following FACS are a disadvantage, and are especially pertinent when sorting for a rare or unstable population of cells. Losses ranging from 81-98% of viable undifferentiated hESCs were noted after FACS for markers of pluripotency (Emre et al., 2010). Measures to increase cell recovery by inhibiting p-160-Rho-associated coiled kinase (ROCK) improved the cell survival, but still lead to cell losses ranging from 65-92% following FACS (Emre et al., 2010).

FACS sorting can be applied to various cell enrichment approaches, such as genetic selection using stable cell lines expressing fluorescent markers, sorting live cells based on differences in the retention or uptake of specific dyes, and sorting cells that have been stained with fluorescently-linked antibodies residing on the cell surface.

1.4.2 – Magnetic Activated Cell Sorting

Magnetic activated cell sorting (MACS) is a second method for separating populations of cells based on surface antigens. To perform MACS, magnetic beads or particles are covalently coated with a selected antibody against a particular surface antigen of interest. Cells are incubated with the magnetic particles and the cells expressing the surface antigen can be separated (Orfao and Ruiz-Arguelles, 1996). Advantages of MACS include the ability to sort large numbers of cells, and fewer viability issues compared to FACS. Disadvantages include fluctuations in the level of purity, and the need to perform flow cytometry on a fraction following MACS to evaluate the purity (Dainiak et al., 2007).

1.4.3 - Genetic Selection of Cardiomyocytes

Strategies using genetic selection to purify ES-derived cardiomyocytes can result in nearly pure populations of cardiomyocytes. Many groups have established hES, mES, and P19 cell lines stably expressing cardiac lineage selectable markers (reviewed in Table 1). In the hES model, the human myosin light chain-2V promoter, expressed in the ventricle of the heart in both humans and rodents, coupled with an enhanced green fluorescent protein (GFP), permitted the identification and FACS sorting of hES-derived cardiomyocytes with up to 93% purity (Huber et al., 2007). A similar method was employed to identify mES-derived cardiomyocytes using GFP expression under the control of the cardiac α-actin promoter (Kolossov et al., 1998). The MHC-promoter coupled with a gene for antibiotic resistance permitted three different groups to obtain 90-99% purity in populations of hES-derived cardiomyocytes (Anderson et al., 2007; Kita-Matsuo et al., 2009; Xu et al., 2008b). An advantage of the genetic selection method for purifying cardiomyocytes, especially antibiotic-based selection, is the ease of the ability to "scale-up", and produce large quantities of highly pure cardiomyocytes (Zandstra et al., 2003). ES-derived cardiomyocytes purified using genetic selection have many effective applications, including their potential use in drug screening, using purified populations for molecular analysis (Christoforou et al., 2008), biomaterial testing (Dawson et al., 2009), and *in vivo* animal model cell delivery studies (Xu et al., 2008b). Disadvantages include potential risks involved with introducing cells containing viral particles into humans. There are also concerns over the random nature of the transgene integration that can lead to activation or repression of endogenous genes, such as oncogenes, at the site of insertion (Hacein-Bey-Abina et al., 2003). There are new hES genetic selection strategies reported, including transposon-based gene delivery of selectable cardiac markers (Orban et al., 2009), and the use of multiple stage-specific

promoter plasmids inserted into hESCs for identification or selection of cardiomyocytes (Guddati and Kessler, 2010).

1.4.4 – Mitochondrial-based Cardiomyocyte Purification

Another successful report of purifying ES-derived cardiomyocytes is using a mitochondrial dye to separate cells based on their high mitochondrial content. Differentiated ESC cultures that were FACS sorted based on their staining by tetramethylrhodamine methyl ester perchlorate (TMRM), gave highly enriched populations of cardiomyocytes, and *in vivo* studies are ongoing by this group (Hattori et al., 2009).

1.4.5 - Surface Marker Purification of ES Cells

"Surface markers" or "cell surface antigens" are receptors, channels, or other proteins located on the exterior of a cells membrane (Deb et al., 2008; Laursen et al., 2007). Once identified, lineage-specific surface markers can be used as tags for identification of living cells and are an efficient means for characterizing and purifying many different cell types. Although proteomic advances have been made in identifying cell surface markers specific to cardiomyocytes and progenitors (Van Hoof et al., 2010), at the present time a cardiac muscle-specific cell surface protein that can be used to efficiently obtain pure populations of differentiating cardiomyocytes has not been reported. Other common approaches to identifying novel cell surface markers on ES-derived cardiomyocytes include analyzing neonatal cardiomyocytes and embryos (Hirata et al., 2006; Murakami et al., 2007), and molecular studies aimed at identifying novel cell surface markers through understanding the molecular basis that underlies muscle development (Honda et al., 2006).

Activated leukocyte cell adhesion molecule (ALCAM), or CD166, is a transmembrane protein whose expression is restricted to the developing heart in early embryogenesis, and is expressed in a wide range of adult cell types (Hirata et al., 2006; Murakami et al., 2007). MACS was used to separate ALCAM labeled hESCs, and the ALCAM positive fraction was approximately 66% positive for cardiomyocyte markers (Rust et al., 2009). Difficulties with *in vitro* growth and survival of the enriched cells were reported.

N-Cadherin was also tested as a candidate surface marker for the purification of cardiomyocytes from ES cells. N-Cadherin plays a critical role in early heart development (Radice et al., 1997), and FACS sorted N-Cadherin positive mESCs showed between a 2-8 fold increase into their differentiation into cardiomyocytes compared to the unsorted cells (Honda et al., 2006).

The lateral mesodermal marker VEGF receptor 2, commonly referred to as "Flk-1", or kinase insert domain protein receptor (Kdr) in humans, has been the most heavily investigated candidate surface marker for isolating developing cardiomyocytes, lead by Dr. Keller at the University of Toronto (Adler et al., 2010; Yang et al., 2008). Embryonic mouse cell tracking and ESC differentiation studies have lead many groups to believe that myocardial and endothelial lineages develop from a common Flk-1-positive progenitor (Kattman *et al.*, 2006). The Flk-1 positive cell population isolated from mESCs differentiates into a higher portion of cardiac muscle than the Flk-1- negative cell population *in vivo*, however this marker was not specific enough on its own to generate a pure population of precursors cells that exclusively differentiated into cardiomyocytes (Baba et al., 2007a; Baba et al., 2007b; Iida et al., 2005; Nelson et al., 2009). When Flk1+ mESCs were transplanted *in vivo* into the hearts of a cardiomyopathic mouse, significant

improvement in cardiac function was recorded (Adler et al., 2010; Baba et al., 2007b). Kdr has also been used to isolate populations of differentiating hESCs. A c-kit negative population of cells expressing Kdr at "low" levels was identified, and when FACS sorted, Kdr^{low}/c-kit(-) cells were characterized as a mesoderm-like population of cells that had cardiac, vascular, and endothelial potential (Yang et al., 2008). Since Flk-1/Kdr receptors are expressed on a wide range of progenitor cells, including neurons (Yang and Cepko, 1996), smooth muscle, and endothelial precursors (Kattman *et al.*, 2006), several groups are attempting to purify myogenic precursors based on their co-expression with other surface proteins, including CXCR4 (Nelson *et al.*, 2008) and platelet-derived growth factor receptor alpha (PDGFR-α) (Hirata et al., 2007; Kattman et al., 2011).

In summary, although there have been considerable advances in purifying ES-derived cardiomyocytes, the need for cardiac-specific cell surface marker that can purify different stages of cardiomyogenesis is clear. A mixed population of donor cells, including cells with vascular potential and other supporting cell types, may be ideal for delivery to the heart (Stevens et al., 2009a; Yang et al., 2008). However it is the donor cardiomyocytes that are important for integrating into the myocardium and eliciting an improvement in cardiac function after an infarct (Adler et al., 2010; Song et al., 2010). Regardless of the donor cell population, (cardiomyocytes and vascular cells, or cardiomyocytes alone), purifying the cells prior to delivery is essential in order to reduce the risk of tumour formation or other adverse effects.

1.5 - Strategies to improve cardiomyocyte survival

Due to the large numbers of cardiomyocytes lost during a MI, cardiomyocyte repair strategies must at least replace millions of cardiomyocytes in order to be clinically relevant.

Improving the survival of cardiomyocyte grafts is a challenge that must be overcome. Reports from the literature indicate that cell survival following injection into the heart is extremely variable, and ranges from 0-90% depending on the species, as reviewed in (Robey et al., 2008). In addition, cell survival is also dependent on the diseased state of the myocardium and the number of cells grafted (Zhang et al., 2001).

Cardiomyocytes are dependent on their cell-cell and ECM interactions for survival, and are sensitive to disruption. In order to prepare cardiomyocytes and purify them for delivery, most protocols require that cells be disrupted from their ECM and cell-cell interactions and be in single cell suspensions. Cells being sorted can be left in suspension for hours, increasing the likelihood of anoikis, or apoptosis due to cell detachment. Anoikis is proposed to be a significant cause of cell death during the cardiomyocyte dissociation step, as reviewed in (Robey et al., 2008). Although the pathways of cardiomyocyte anoikis are not well elucidated, apoptosis is hypothesized to be at least partially induced by a loss of integrin-receptor binding of the cells, which triggers apoptosis by interrupting cell survival signals (Robey et al., 2008; Zhan et al., 2004). Various supplementation-based approaches have been taken to increase cardiomyocyte survival, as reviewed in Chapter 3, and they include measures to decrease anoikis or apoptosis (Braam et al., 2010; Laflamme et al., 2007). Providing dissociated cardiomyocytes with an *in vitro* native heart-like extracellular matrix (ECM)-based environment, such as tissue engineered scaffolds or ECM-based injectable gels, are another approach to decrease cell loss and encourage survival of grafted cardiomyocytes.

1.6 - Cardiac Tissue Engineering

Tissue engineering refers to a broad area of research, with the goal to develop biocompatible substitutes to aid in the formation or regeneration of living tissue. Cardiac tissue engineering involves *in vitro* assembly of the cardiomyocytes on a biomaterials "patch" or scaffold prior to delivery to a recipient, as recently reviewed in (Codina et al., 2010; Martinez and Kofidis, 2009; Sui et al., 2010; Vunjak-Novakovic et al., 2010; Zimmermann, 2009). Advantages of a tissue engineering approach to treating damaged myocardium compared to cell injections alone include, decreasing transplanted cell loss within the host, and the ability to organize the cardiomyocytes into functional muscle patches prior to delivery. Several proof of principal studies using ES-derived cardiomyocytes or vascular cells, substantiate these notions and will be reviewed below. Presently one cardiac tissue engineering clinical trial has been completed using autologous bone marrow derived cells incorporated into a collagen sponge scaffold (Chachques et al., 2008). The clinical study recipients showed improvements at the one-year follow-up; however further studies are necessary to rule out confounding factors such as concomitant coronary artery bypass graft surgery (Chachques et al., 2007).

Numerous promising tissue engineered substrates have been developed, and some of these grafts have shown preliminary success when implanted into mammalian hearts. Many challenges remain, including optimizing the scaffold design, vascularization, and functional integration of the patch.

1.6.1 - Scaffold Design for Cardiac Tissue Engineering:

The ideal cardiac graft scaffold structure must be strong enough to resist damage from the contracting heart, yet porous enough to allow for cell incorporation, diffusion of growth factors and nutrients, and penetration of blood vessel, as reviewed in (Vunjak-

Novakovic et al., 2010). The mechanical strength needs to be coupled with sufficient elasticity to allow for contractility. It would also be biocompatible and degradable at a rate that coincides with the repair process. The scaffold may also contain specific biochemical information that controls cellular growth and development.

Scaffolds can be made of natural or synthetic materials. Synthetic scaffolds, using materials such as polyethylene glycol (PEG), have the advantage of allowing greater control over scaffold properties such as strength, elasticity and biodegradability (Marsano et al., 2010). Synthetic scaffolds are often limited with respect to their functional interactions with cells and need modification to mimic endogenous conditions, as reviewed in (Zhu, 2010). Biological based scaffolds, using naturally occurring polymers and ECM proteins, have the ability to provide a native-like environment for cardiomyocytes. Biological scaffolds can be difficult to process without losing their functional structure, lack control over mechanical and chemical properties compared to synthetic scaffolds, and have the potential for pathogen transmission when using xenoproteins, as reviewed in (Schussler et al., 2010).

1.6.2 – Extracellular Matrix-based Cardiac Biomaterials

In the heart, cardiomyocytes are surrounded by a basement membrane containing collagen type IV, laminin, fibronectin, and proteoglycans, and the extracellular space consisting mainly of interstitial collagen types I and III, along with many other macromolecules (VanWinkle et al., 1996). These ECM components are essential in heart development and in mechano-electrical function, as reviewed in (Parker and Ingber, 2007). Collagen-based cardiac scaffolds have been under development by numerous groups, and mES EB-derived cardiomyocytes have been shown to adhere and form beating clumps of cardiomyocytes on collagen scaffolds (Guo et al., 2006; Wang et al., 2006). Neonatal rodent

cardiomyocyte collagen I-Matrigel scaffolds improve contractility compared to non-contractile grafts and controls (Zimmermann et al., 2006). Matrigel is a commercially available basement membrane matrix extracted from cultured tumour fibroblasts, and made up of collagen IV, laminin and various other growth factors (BD Biosciences). Matrigel has also been successfully used in many cardiomyocyte growth applications, including as a means for delivering hES cardiomyocytes along with a prosurvival cocktail to improve ventricular function in infarcted rodent hearts (Laflamme et al., 2007), as reviewed in Chapter 3. However, Matrigel is an undefined, extracted protein cocktail from animal sources, and as such has the potential for disease transmission, so Matrigel-free versions of the above collagen-I scaffold have also been developed (Naito et al., 2006). In a separate approach, decellularized heart tissue was used as a biomaterial to deliver cardiomyocytes, and shows preliminary success (Ott et al., 2008).

1.6.3 - The use of Bioactive Molecules and Growth Factors in Scaffolds

Natural and synthetic-based cardiac scaffolds can be designed to contain growth factors and cell adhesion peptides to gain improvements in cell attachment and differentiation, or for the controlled release of growth factors and cytokines following transplantation. Rat neonatal cardiomyocytes grown within RGD-collagen scaffolds were shown to have improved contractile function and viability compared to control collagen cardiomyocyte scaffolds (Schussler et al., 2009). The use of the RGD motif in scaffolds is reviewed in detail in Chapter 4. Recently, a novel application for functionalized cardiac scaffolds in tissue engineering was reported, and involved designing a collagen scaffold that could enrich for endogenous progenitor cell populations by presenting antibodies recognizing specific cell surface markers (Shi et al., 2011).

1.6.4 - Functional Integration and Mechanical Coupling of Tissue Engineered Scaffolds

Despite preliminary reports of successes with delivering cardiomyocytes within a biomaterial to heart infarcts, functional integration of the grafts and mechanical coupling leading to clinically detectable improvements remain poor, as discussed in (Hattori and Fukuda, 2010; Mummery et al., 2010; Robey et al., 2008). It has recently been shown that cardiomyocytes are more effective than vascular progenitors at improving function after MIs in the rat model (Adler et al., 2010). With the aim of improving survival and functional integration, many groups are beginning to utilize mixed cell populations, including supporting cells along with cardiomyocytes in their tissue engineered constructs. A mixture of hES-derived cardiomyocytes, hES-derived endothelial cells, and embryonic fibroblasts were *in vitro* delivered into a porous synthetic cardiac scaffold with Matrigel (Caspi et al., 2007). The resulting tissue was shown to recapitulate ultrastructural, molecular and functional properties specific to cardiomyocytes, and when transplanted onto rat hearts, stable grafts were formed with evidence of functional integration into the host vasculature (Lesman et al., 2010). The Murry group used hES derived cardiomyocytes and vascular progenitors grown in suspension within an orbital shaker to form clumps or patches of pre-vascularized cardiac tissue (Stevens et al., 2009b). These scaffold free patches integrated into rat skeletal muscle and formed functional microvessels that connected with the host circulation to deliver blood to the grafts, and were found to contain significantly more viable cells than patches implanted with cardiomyocytes alone (Stevens et al., 2009a).

In order to mimic *in vivo*-like heart conditions and obtain greater control over the *in vitro* differentiation environment, tissue engineered constructs containing cardiomyocytes are often grown within bioreactors. Bioreactors are vessels designed so that cells can receive a

constant flow of nutrients and oxygen, and be subjected to electromechanical conditions that are similar to what native cardiomyocytes growing in the heart would experience, as reviewed in (Vunjak-Novakovic et al., 2010).

1.7 – Rationale and Hypothesis

Many hurdles remain before ESC cardiomyocyte therapy can move forward to clinical testing. These include: directing ESCs to the cardiac lineage, purifying cells for delivery, reducing ESC-derived cardiomyocyte cell loss, and determining a suitable delivery method for the cells, such as, within a cardiac-specific tissue engineered substrate. The overall goal of this research is to contribute to the field of cardiac-repair and cardiac tissue engineering by enriching populations of ESC-derived cardiomyocytes and performing *in vitro* testing to determine if the use of collagen-based tissue engineered substrates is a viable delivery method for transplantation. **I hypothesize that ESC cultures can be enriched for cardiomyocytes, and ES-derived cardiomyocytes can grow on biomaterials that support the cardiomyocyte phenotype.**

1.7.1 - Research Objectives for Manuscripts and Specific Hypotheses

The following summary and specific sub-objectives is given to direct the progression through chapters 2, 3, and 4 (collection of manuscripts).

Chapter 2: "A P19 Cardiac Cell Line as a Model for Evaluating Cardiac Tissue Engineering Biomaterials", published in 2009 in *The Open Tissue Engineering and Regenerative Medicine Journal*. **My hypothesis was that a P19 cell line that is genetically modified to select cardiomyocytes is an effective model to perform *in vitro* testing on novel cardiac biomaterials.** In these experiments, a P19 model cell line utilizing genetic selection to

increase the purity of cardiomyocytes was developed. The utility of this model is demonstrated for preliminary *in vitro* testing of a novel cross-linked collagen-based scaffold, which was determined to be suitable to sustain cardiomyocyte function and growth.

Chapter 3: "Dihydropyridine Receptor Surface Marker Enrichment of ES-derived Cardiomyocytes". **My hypothesis was that dihydropyridine receptor (DHP-R) surface marker staining and cell sorting may be used to enrich ES-derived cardiomyocyte cultures.** As an alternative to using a genetic approach to purifying cardiomyocytes, the novel use of DHP-R surface staining to enrich mESC-derived cardiomyocytes was tested. By performing FACS using antibodies against DHP-R, a fraction of cells enriched for cardiomyocytes were obtained. Thus, DHP-R surface marker selection of ES-derived cardiomyocytes is a suitable method to enrich ES cultures for cardiomyocytes. When combined with previously published lineage-restricting sorting techniques, DHP-R may prove to be an efficient marker to purify cardiomyocytes or cardiomyocyte progenitor cells.

Chapter 4: "Collagen (+RGD and –RGD) scaffolds support cardiomyogenesis after aggregation of mouse embryonic stem cells", submitted. **My hypothesis was that RGD motif modified and non modified collagen I/III scaffolds support ES-derived cardiomyocyte differentiation and function.** MES cardiomyocytes differentiating within EBs were used to test the ability of RGD-modified collagen I/III scaffolds to support ES-derived cardiomyocytes. Similar to the collagen I scaffold tested in chapter 2, the collagen I/III scaffold was able to sustain cardiomyocyte growth and contractile function.

1.8 – Summary

The heart is an extremely complex organ, and thus far our ability to repair damaged myocardium with ES or autologous derived cardiomyocytes remains poor. Despite this,

there has been significant progress made towards designing a biocompatible tissue engineered cardiac patch. The studies presented in chapters 2-4, which combine a defined population of donor cells with biomaterials recapitulating the natural heart environment, contribute to this progress by setting the foundation for clinically relevant studies transplanting ES-derived cardiomyocytes onto human hearts.

CHAPTER 2:

A P19 Cardiac Cell Line as a Model for Evaluating Cardiac Tissue Engineering Biomaterials

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2.1 - Abstract:

Our objective was to develop a suitable cardiomyocyte progenitor cell line for use in testing biomaterials as potential scaffolds in cardiac tissue engineering. We transfected P19 cells with the human cardiac α-actin promoter driving the gene for puromycin resistance, to create a stable cardiomyocyte-selectable P19 cell line, termed P19(CA-Puro). Puromycin selection resulted in a 4-10 fold enrichment of cardiac muscle gene expression and a 3-fold enrichment in cardiomyocytes. Morphological, biochemical, and functional analyses were used to evaluate the properties of P19(CA-Puro) cardiomyocytes in the presence and absence of a novel cross-linked collagen-based biomaterial. The collagen-based biomaterial was able to support appropriate viability, gene expression, and cardiomyocyte function.

Therefore, P19(CA-Puro) cells are suitable for examining biomaterials as potential scaffolds and this approach could be used for rapidly screening biomaterials for designing future human embryonic stem cell therapies.

2.2 - Introduction:

Heart disease such as myocardial infarction is the leading cause of death in developed countries. It is often directly correlated with a loss in functional cardiac muscle and the formation of scar tissue as mature cardiomyocytes have limited abilities to regenerate. Stem cell based cardiomyocyte replacement strategies may offer promise for regenerating the myocardium, demonstrated by recent efforts with human embryonic stem cell (ESC) delivery in the rodent model [1-3]. In addition, the development of tissue engineered cardiac patches, comprised of stem cell derived muscle cells delivered in a biological-based scaffold, is being investigated by numerous groups [4, 5]. These cardiac muscle patches are designed to integrate into the myocardium and improve contractile function at the site, offering improved cell delivery and integration when compared to injected cells alone. It is generally agreed that the ideal scaffold should mimic the native cardiac extracellular matrix (ECM) microenvironment to allow for incorporated stem or progenitor cells to proliferate and differentiate into cardiac-like muscle in a controlled manner. Many different ECM combinations have been reported in the literature, and some of these scaffolds have shown promising albeit limited success when implanted into mammalian hearts, as reviewed in [6-9]. Common challenges include designing a strong yet biodegradable scaffold, controlling cardiomyocyte differentiation, and functional host-graft integration of the patch [5, 7, 10, 11]. A limiting factor to solving these tissue engineering problems is the lack of a homogeneous, readily available source of cardiomyocyte stem cells for use in testing new materials as potential scaffolds. In this study, we report the derivation of a model cardiomyocyte progenitor line for use in evaluating different biomaterials as potential scaffolds for cardiac tissue engineering.

P19 embryonic carcinoma (EC) cells have been widely used to model cellular differentiation during embryonic development. Originally derived from an induced murine embryonic teratocarcinoma [12], P19 EC cells are pluripotent and can differentiate into all three germ layers after cellular aggregation (a process similar to embryoid body formation) [13]. In response to cardiomyogenic inducers in culture, rhythmically contracting areas begin to appear by 8-14 days in 8-25% of the P19 EC embryoid bodies [14], and these express cardiomyogenic-related transcription factors and proteins [15-17]. P19 cell differentiation requires similar signalling pathways and transcription factors as those observed during early embryonic development and in mouse ES cell differentiation [18]. Further, P19 cells can contribute to murine heart formation in chimeric mice *in vivo* [19]. Functional ion channels have been demonstrated in P19 derived cardiomyocytes [20], and were found to be similar to *in vivo* derived neonatal, embryonic and ESC cardiomyocyte cultures [20-23]. Therefore, P19 EC cells can be readily used to model ESC differentiation into cardiac muscle, forming functional, beating cardiomyocytes [24-27].

While human embryonic stem cells are ideal for future cell therapies to regenerate the myocardium, the growth and differentiation of these cells is much more difficult, labour intensive, and time consuming than P19 cell cardiomyogenesis [18, 28]. Thus P19 cells could be used as a rapid screen to optimize methods to use for future human ES cell cardiac muscle therapies. Despite these attractive properties, the actual utility of P19 EC and human ES cells in studying cardiac muscle formation has been limited due to the relatively low efficiency of cardiac muscle induction. In order to optimize utility for P19 cells, we have developed stable P19 cell lines with the ability to select for cardiomyocytes. Cardiac α-actin, often used as a marker of muscle differentiation, is expressed throughout cardiac muscle development and also in embryonic skeletal muscle [29]. In the present study, we

developed a cardiac α-actin selectable cell line, in which the cardiac α-actin promoter drives puromycin resistance, termed P19(CA-Puro), to obtain cultures enriched in cardiomyocytes. We report that our P19(CA-puro) line efficiently differentiates into functional cardiac muscle, and provides a useful tool in characterization of biomaterials for cardiac tissue engineering.

2.3 - Materials and Methods

2.3.1 - P19 Cell Culture:

P19 cells (American Type Culture Collection #CRL-1825, Virginia, USA) were cultured in α-minimum essential media (αMEM) (Invitrogen, Ontario, Canada) supplemented with 10% fetal bovine serum (FBS) (CanSera, Rexdale, Canada) and 50μg/ml of gentamycin (Invitrogen). They were plated at $1x10^5$ cells/ml media and passaged every two days.

2.3.2 - Plasmid Constructs*:*

In order to create our cardiac α-actin selectable cell line, we designed an expression construct, termed Human cardiac actin-puromycin (HCA-puro) in which the puromycin resistance gene is under control of an HCA promoter. The expression construct HCA-puro was cloned into the BSK+ expression vector, and contains a Sal/HindIII 450 base pair HCA promoter (25), driving a HindIII/Bs1II 1 Kb puromycin resistance fragment from PGK-puro. The empty PGK vector was used as a control. The constructs PGK-Neo, B17, and PGK LacZ were previously described [30].

2.3.3 - Stable Cell Line Generation:

For transfections 2.04 μg of CA-Puro, 0.09 μg of PGK-Neo, 0.17 μg of PGK-LacZ, and 0.77 μg of B17, were mixed, added to the FuGENE^{TM} 6 reagent (Roche Applied Science, Quebec, Canada), and incubated with 2.5 x 10^5 cells in 35-mm tissue culture dishes. Controls comprised of P19 cells transfected with CA-LacZ, PGK-Neo, PGK-LacZ, and B17. The B17 construct is a 17 Kb portion of the pgk-1 gene and has been shown to enhance the formation of stable cell lines in P19 cells, by an unknown mechanism [31]. β-galactosidase assays were used to determine the transfection efficiency for each experiment as described [32]. Cells were selected for G418 resistance (500 μg/ml) (Sigma Aldrich, Ontario, Canada) for 7 days. The high ratio of CA-Puro to PGK-neo plasmids increases the chance of G418-resistant clones containing the CA-puro cDNA. 48 stable P19(CA-puro) clonal populations were isolated and examined for their low expression of puromycin transcripts (RNA) and their high content of HCA-puro DNA by slot-blot analysis as previously described [33]. Stable cells lines that met this criteria were termed P19(CA-puro), and further tested for their ability to differentiate into puromycin resistant cardiomyocytes.

2.3.4 - P19(Control) and P19 (CA-puro) Differentiation into Cardiomyocytes:

P19(CA-puro) and control cells were allowed to reach confluence, passaged, and then placed under differentiation conditions. Cells were aggregated in the presence of 0.8% dimethyl sulfoxide (DMSO) (Sigma Aldrich) in 10% FBS (PAA, Austria), αMEM (Invitrogen) for 4 days and then plated onto tissue culture dishes [12]. On days 0, 4, 6, 8 of differentiation, selection was initiated by treating cells with 2μg/ml puromycin in the growth medium. Cultures were maintained until day 12. On day 12, cultures were assessed for cell viability (by trypan blue exclusion), and fixed for immunocytochemistry with myosin heavy

chain (MHC) antibody. P19(Control) cells served as selection controls. For cardiomyocyte selection experiments, cells were treated with 2μg/ml puromycin (or 0μg/ml for controls) starting on day 6 of differentiation. αMEM was changed daily.

2.3.5 - Quantitative Polymerase Chain Reaction (QPCR):

To determine the extent of enrichment of cardiomyocytes with puromycin selection, changes in gene expression of cardiac muscle genes, (Table 1) were examined by QPCR. Briefly, RNA was isolated using Trizol (Invitrogen) on Day 9 of differentiation from P19(CA-puro) cells with and without puromycin treatment starting on Day 6. QPCR was performed, and the fold increase in expression changes associated with the puromycin selection was calculated relative to the P19(CA-puro) Day 9 non-puro treated control RNA. cDNA synthesis was performed using the SuperScript™ III Reverse Transcriptase kit as suggested by the manufacturer (Invitrogen). QPCR was performed using SYBR Green PCR Master Mix (Applied Biosystems, California, USA), following manufacturer's suggestions in a 25ul final reaction volume. PCR amplification was performed using a BioRad iCycler thermocycler with the following conditions: an initial denaturing at 95ºC for 10 minutes, 40 cycles of 95ºC (15 seconds)/60ºC (30 seconds)/72ºC (30 seconds), and a final extension at 72ºC for 10 minutes. A melt curve analysis was performed as follows: 95ºC for 1 minute, 55ºC for 1 minute, and 55ºC for 10 seconds with an increase of 0.5ºC at each successive cycle for 80 cycles. The oligonucleotide primers for α-actin, atrial natriuretic peptide (ANP), MHC, GATA4, Pax-3, MyoD, and GAPDH are given in Table 1. Relative fold differences in expression of these genes between the P19(CA-puro) (+) puro and P19(CA-puro) (-) puro control cells were calculated using the comparative C_T method [34]. Briefly, the difference in cycle time ΔC_T was normalized relative to the housekeeping gene, GAPDH. $\Delta\Delta C_T$ was

calculated as the difference between ΔC_T P19(CA-puro) (+) puro and the ΔC_T control (-) puro cells. The effect of the collagen substrate on gene expression in P19(CA-puro) was investigated by determining the fold change in gene expression of P19(CA-puro) (+) puro cells growing on the substrate, and using P19(CA-puro) (+) puro cells growing on tissue culture plates as the baseline. The relative fold change in gene expression level was determined by calculating $2^{-\Delta\Delta CT}$[35].

Table 2.1 - Primers and Their Respective References Used in QPCR Reactions.

Gene of Interest	Forward Primer	Reverse Primer	Reference
MHC	ACAACCCCTACGATTATGCGT	ACGTTCAAAGGCACTATCCGTG	[61]
GATA4-4	AAACGGAAGCCCAAGAACCT	TGCTAGTGGCATTGCTGGAGT	[62]
ANP	ACTAGGCTGCAACAGCTTCC	TGACACACCACAAGGGCTTA	[63]
Cardiac α-actin	CTGGTATTGCCGATCGTATG	CTTGCTGATCCACATTTGCT	[61]
MyoD	CCCCGGCGGCAGAATGGCTACG	GGTCTGGGTTCCCTGTTCTGTGT	[64]
Pax3	TTTCACCTCAGGTAATGGGACT	GAACGTCCAAGGCTTACTTTGT	[61]
GAPDH	AGGTCGGTGTGAACGGATTTG	TGTAGACCATGTAGTTGAGGTCA	[61]

2.3.6 - Immunocytochemistry:

Differentiated aggregates grown on gelatin coated coverslips were fixed on day 9 or 12 in –20 °C methanol (for MHC), and with 1.6% paraformaldehyde in 0.9% picric acid) for cardiac α-actin, actinin, troponin-T, and GATA4. For MHC immunocytochemistry, cells were rehydrated in 10mM phosphate buffered saline (sPBS), and MHC expression was detected utilizing the monoclonal MF20 antibody supernatant (Developmental Studies Hybridoma Bank, Iowa City, IA USA) as described previously [33]. For cardiac α-actin, actinin, troponin-T, and GATA4 immunocytochemistry, fixed coverslips were blocked with 4% goat serum, 0.3% Triton-X, and incubated with the respective antibodies, as summarized in Table 1.2. In all staining, nuclei were detected with 1:10000 DAPI and washed well with

sPBS. Coverslips were mounted on glass slides and visualized with a Zeiss Axioskop microscope. Isotype matched controls were used for all antibodies and background florescence was taken into account at all magnifications analyzed. Cardiomyocyte and total cell counts were performed to quantify the percentages of MHC positive cardiomyocytes in our cultures. This was performed by counting five images from two coverslips for each data point in two independent experiments.

Table 2.2 - Antibodies Used for Immunocytochemistry Experiments.

Antibody	Antibody Dilution	Company
Myosin Heavy Chain (MF20)	1:2 in sPBS	Developmental Studies Hybridoma Bank, Iowa, USA
Cardiac α-actin	1:500 in 4% goat serum, 0.3% triton X-100	Fitzgerald, MA, USA
TroponinT	1:500 in 4% goat serum, 0.3% triton X-100	LabVision,QC,Canada
Gata4	1:100 in 4% goat serum, 0.3% triton X-100	Santa Cruz Biotech Inc. CA, USA
Sarcomeric Actinin	1:1000 in 4% goat serum, 0.3% triton X-100	Sigma-Aldrich
Goat Anti-mouse Cy3	1:400 in block for secondary staining	The Jackson Laboratory, Maine, USA
Goat Anti-mouse FITC	1:1000 in block	The Jackson Laboratory, Maine, USA
Anti-Goat FITC	1:500 in block	The Jackson Laboratory, Maine, USA
Anti-Goat IgG	1:100 (30 μg/ml) in 4% goat serum, 0.3% tritonX-100	Invitrogen, Ontario, Canada
Anti-Mouse IgG1	2 μg/ml (in 4% goat serum, 0.3% triton X-100)	Invitrogen, Ontario, Canada
Anti-Mouse IgG2b	2μg/ml in SPBS	Zymed Labortories, California, USA

2.3.7 - Collagen Hydrogels:

Rat tail collagen I (4.75mg/ml) (BD Biosciences, Ontario, Canada) was mixed with collagen buffer (1M HEPES, 10x Dulbecco's-MEM (FBS-free), and 7.5% sodium bicarbonate) at a ratio of 7:1 v/v on ice. The pH of the collagen mixture was then adjusted to 7.4 with 1N NaOH, and 5mM calcium chloride and 1mM DL-dithiothreitol (DTT) were added. Cross-linking was performed with 0.22U/ml guinea pig transglutaminase (Sigma Aldrich), and the viscous solution was dispensed into the desired moulds as follows: 200μl

for 12mm transwell tissue inserts (Corning, Massachusetts, USA), and 250 µl for the EcoMEA dishes (Microelectode Array Systems, Germany), and then thermogelled at 37°C for 45 minutes. 10% FBS αMEM media and P19(CA-puro) aggregates were added on top of the gels. Media was replaced every day, and the cells were treated with 2µg/ml puromycin for enrichment on Day 6, 7, 8, and 9 of differentiation as described above.

2.3.8 - Viability Assay:

Cells were grown on top of the collagen gels and control tissue culture dishes (as described above) for one to seven days, and analyzed with the Live/Dead Viability and Cytotoxicity Kit (Invitrogen) according to the manufacturer's instructions. Briefly, gels containing cells were incubated with Calcein AM (esterase activity of viable cells visualized as green fluorescence) and Ethidium homodimer-1 (fluorescent red nuclear stain specific for non-viable cells). Hoechst 33342 staining was used for total cell nuclear detection. The gels were then washed well with PBS and immediately visualized with a Zeiss inverted florescent microscope. Three representative images were acquired for each sample for analysis. (n=3 independent samples).

2.3.9 - Microelectrode Array Analysis:

Collagen hydrogel mix was dispensed as 250 µl aliquots into 22mm microelectrode array (MEA) dishes (Multichannel Systems), and P19(CA-puro) cells were differentiated on top as described above. The MEA dishes contained 60 electrodes recording extracellular potentials. Between Day 9 and 12 of differentiation, the MEA grown cardiac hydrogels were washed with Tyrodes buffer containing Ca^{2+} (pH 7.4) for 5 minutes and the baseline extracellular potential was examined, as described previously [36, 37]. All readings were taken at 37°C. A final concentration of 100µM isoproterenol (Sigma Aldrich) diluted in

warmed tyrodes buffer was then added to the cardiac gel, and readings were taken from 1-5 minutes after the drug was added. After a drug response was observed, the gel was washed with buffer for 5 minutes, and a "drug recovery" extracellular potential recording was taken.

2.3.10 - Statistical Significance:

Values are reported as mean +/- SD (standard deviation). For QPCR, each gene and sample group was tested in duplicate reactions in three biologically independent experiments. Statistical significance was evaluated using unpaired student's t-tests to compare between two treatment groups. Significance level was set at a $p < 0.05$.

2.4 - Results

2.4.1 - Enrichment for Cardiac Muscle and Characterization of the Cardiomyocytes:

Stable P19 cells were isolated after transfection with the gene for puromycin resistance driven by the cardiac α-actin promoter, termed P19(CA-puro). P19(CA-puro) cells responded to DMSO induction by differentiating into beating cardiomyocytes after 6 days of differentiation (Fig. 2.1, panel I). P19(CA-puro) and P19(Control) cells were differentiated and treated with puromycin starting on day 4, 6, or 8, until day 9. Puromycin treatment starting on days 6 or 8 resulted in 65% viability of P19(CA-puro) cells (Fig. 2.1, panel II). Puromycin treatment of P19(Control) cells or P19(CA-puro) cultures on days 0-4, showed 100% cell death, reflecting the lack of cardiac α-actin expression. Immunocytochemistry of P19(CA-puro) cells treated with puromycin from Day 6-9 showed

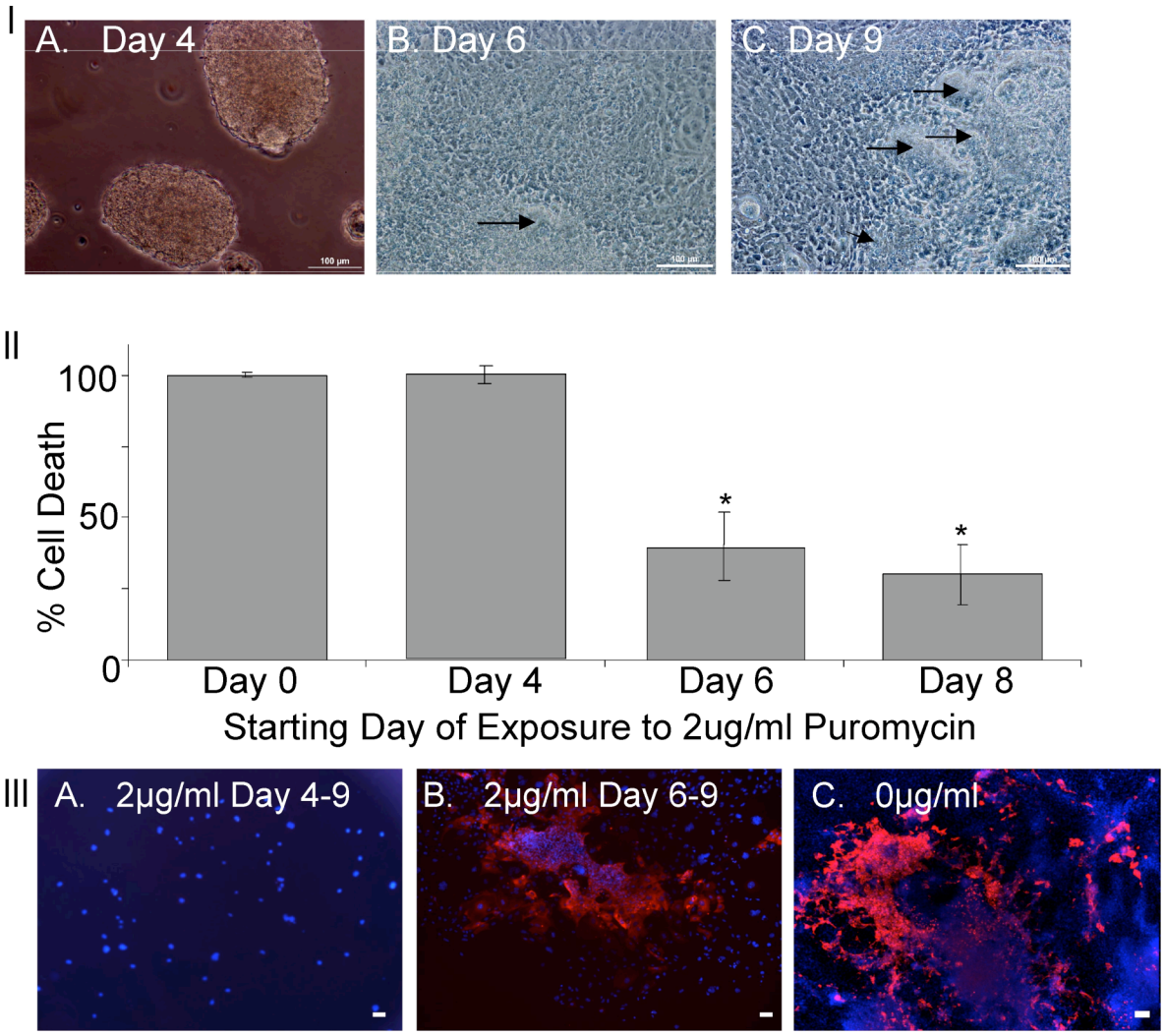


Figure 2.1 - Cardiomyocyte enrichment from P19(CA-puro) cultures during differentiation.

Panel I: P19(CA-puro) cultures differentiated into beating cardiomyocytes by day 6 with DMSO exposure . Arrows depict visibly beating colonies. Panel II: Differentiating P19(CA-puro) cells were exposed to 2µg/ml of puromycin at different timepoints from day 4-9 of differentiation, and percentage of cell death was calculated on Day 12 of differentiation . Data represents the average +/- SD of three experiments, *=$p<0.05$ analyzed by students t-test comparing the P19 control cells to the puromycin enriched cells. Panel III: Muscle formation was visualized by immunocytochemistry with the MF20 antibody against MHC on day 12 (Panel III). Cells were treated with puromycin on days 4-9 (Panel A), days 6-9 (Panel B), or without puromycin (Panel C). For all images (scale bar = 100µm).

a greater loss of total cells, compared to MHC positive cells, resulting in a relative enrichment in MHC positive cells, compared with cells not exposed to puromycin (Fig. 2.1, Panel III). The slight decrease in cardiomyocyte number could be due to selective silencing of the transgene in some cardiomyocytes or the loss of puromycin-resistant cardiomyocytes due to their attachment to the plate via puromycin-sensitive cells. Thus, selection with puromycin from days 6-9 of P19(CA-puro) differentiation appeared to be optimal during DMSO differentiation, reflecting the expected pattern of cardiac α-actin expression [38].

To evaluate the extent of cardiomyocyte enrichment in P19(CA-puro) with puromycin treatment, changes in the expression of cardiomyocyte-specific transcripts were assessed using QPCR. Puromycin selection on Day 6-9 of differentiation in P19(CA-puro) induced a 4-10 fold increase in gene expression of the cardiac muscle related transcripts MHC, ANP, and α actin (Figure 1.2). No significant increase in gene expression was measured for MyoD, which is indicative of skeletal muscle development, or Pax3, which marks both skeletal and neural crest precursors.

To confirm the gene expression results with protein expression analysis, and determine the extent of cardiomyocyte enrichment, immunocytochemistry was performed on fixed P19(CA-puro) cardiomyocytes. MHC expression was evaluated in P19(CA-puro) cultures with or without puromycin treatment, again showing a greater loss of total cells than cardiomyocytes (Fig 2.3A,B). Quantitative analysis indicated that in the absence of puromycin selection 18+/- 9% of the total cells were MHC positive. In contrast, 59+/-19% of the total cells were MHC positive after puromycin selection from Days 6-9 (Fig. 2.3 B and C). P19(CA-puro) derived cardiomyocytes also expressed cardiac α-actin (Fig. 2.3D), troponin T and GATA4 (Fig. 2.3E), and α actinin (Fig. 2.3F) proteins.

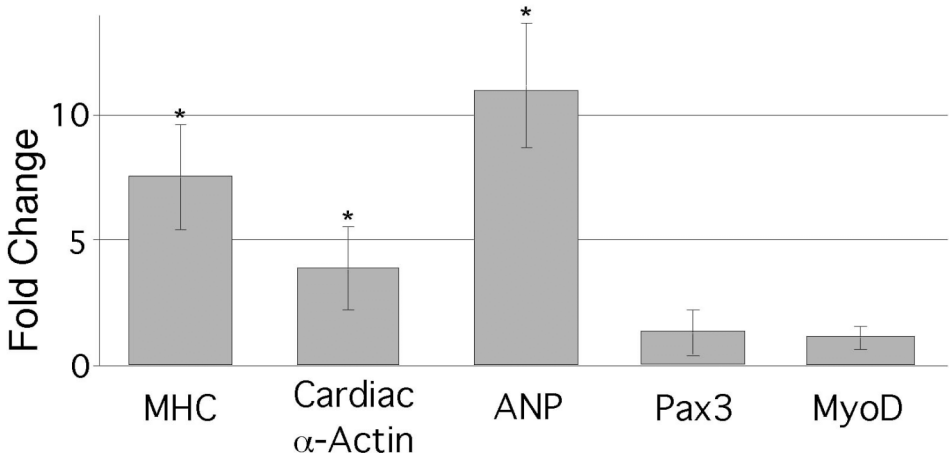


Figure 2.2 - Cardiac muscle gene expression is enriched by puromycin selection of P19(CA-puro) compared to non-enriched P19 cultures.

Differentiated P19(CA-puro) cells were exposed to 0µg/ml or 2µg/ml puromycin from day 6-9 of differentiation. RNA was harvested and analyzed with QPCR for primers specific for cardiomyocytes, skeletal muscle, and neural crest cells. Data represents the average +/- SD of 3 independent experiments done in duplicate, *=$p<0.05$, data analyzed using a students t-test to compare the enrichment of P19(CA-puro) to P19 control differentiated cultures.

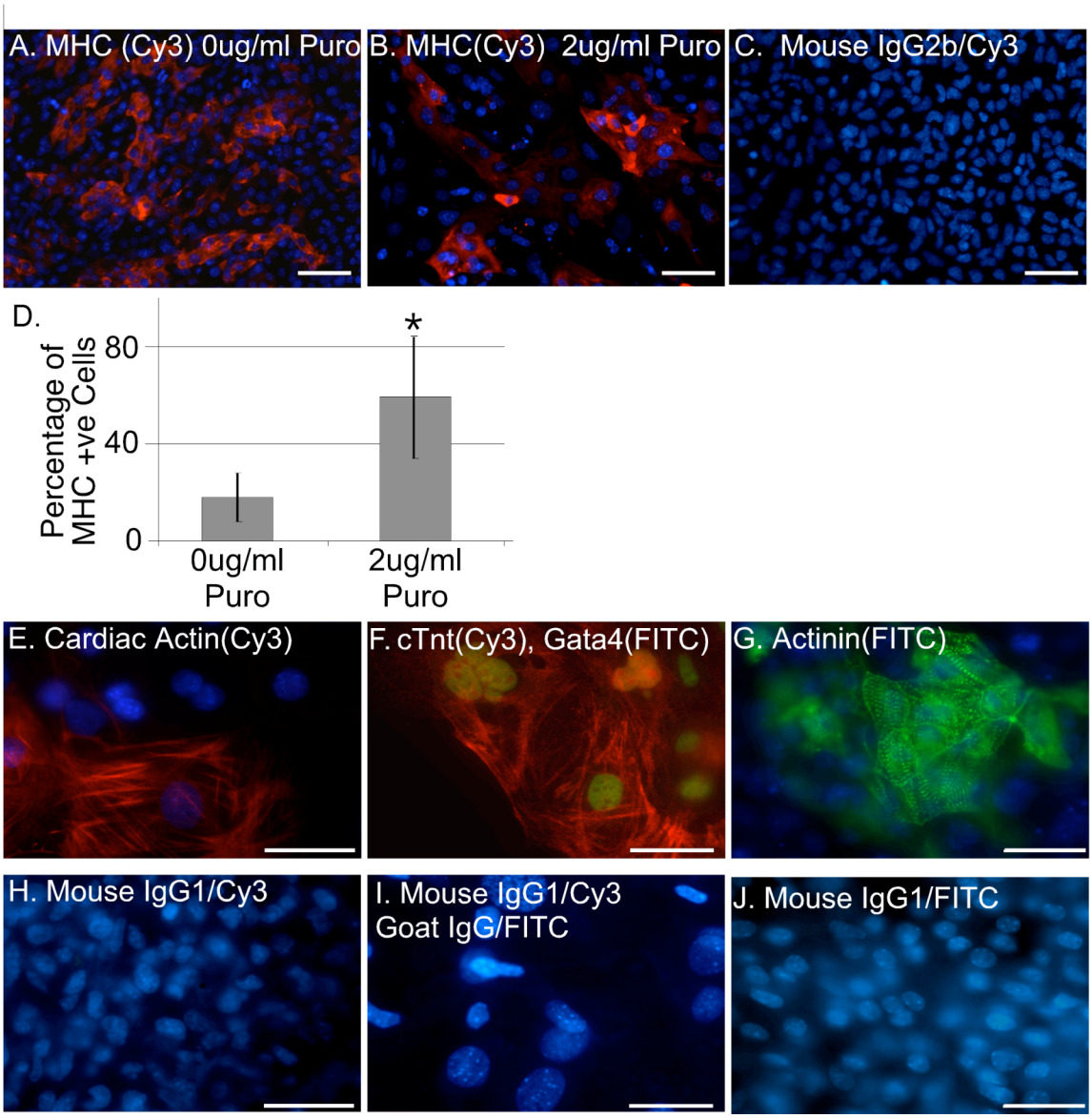


Figure 2.3 - Cardiomyocytes are enriched in cultures treated with puromycin.

Immunocytochemistry was performed for MHC expression in P19(CA-puro) cultures treated with and without puromycin (Panel A, B) and the mouse anti IgG2b isotype control is shown in panel C. The percentage of MHC positive cells was quantified by cell counting, *= $p<0.05$ quantified using a students t-test to compare the P19 control cells to the puromycin enriched P19 cells (Panel D). Cardiomyocytes within the P19(CA-puro) enriched cultures expressed Cardiac α-Actin (E), Troponin T and GATA4 (F), and Sarcomeric Actinin (G). Control coverslips were stained with their respective isotype matched controls and are shown in H-J. Mouse anti-IgG1 (cardiac α-actin, Troponin T, Actinin), Goat anti IgG (GATA4). All coverslips are stained with DAPI for nuclear detection (blue). Scale bars = 100nm.

Therefore, selection of puromycin resulted in a 3-fold enrichment in the number of cardiomyocytes in P19(CA-puro) cell cultures. This cardiomyocyte enrichment was obtained by the selective loss of the non-cardiac muscle cells resulting from the puromycin exposure.

2.4.2 - Evaluation of P19(CA-puro) cardiomyocytes on a collagen hydrogel

To test the compatibility of the transglutaminase cross-linked type I collagen hydrogel as a substrate for cardiomyocytes, the viability of P19(CA-puro) cells was first evaluated. P19(CA-puro) cardiomyocyte viability on collagen hydrogels and on untreated tissue culture dishes was evaluated, and no significant differences in cell death was measured at both the 24 hour or 7 day time point (Fig. 2.4).

To test whether growth on the hydrogel affected the cardiomyocyte phenotype, changes in gene expression were evaluated using QPCR. P19(CA-puro) cardiomyocytes maintained their up-regulation of cardiomyocyte marker transcripts under the selective conditions while growing in the collagen hydrogel (Fig. 2.5). This suggests that the collagen substrate is conducive to maintaining the cardiomyocytes.

To examine the functionality of P19(CA-puro) derived cardiomyocytes, a MEA analysis was performed in the presence and absence of isoproterenol (Fig. 2.6). P19(CA-puro) cardiac muscle cells on collagen gels emitted evenly spaced extracellular electrical events. The number of electrical events per minute were counted and a comparison was made between baseline and isoproterenol treatment groups which was expressed as fold change over baseline. They exhibited an average of 3-fold chronotropic increase between control baseline readings and 100uM isoproterenol readings ($p<0.05$), with subsequent

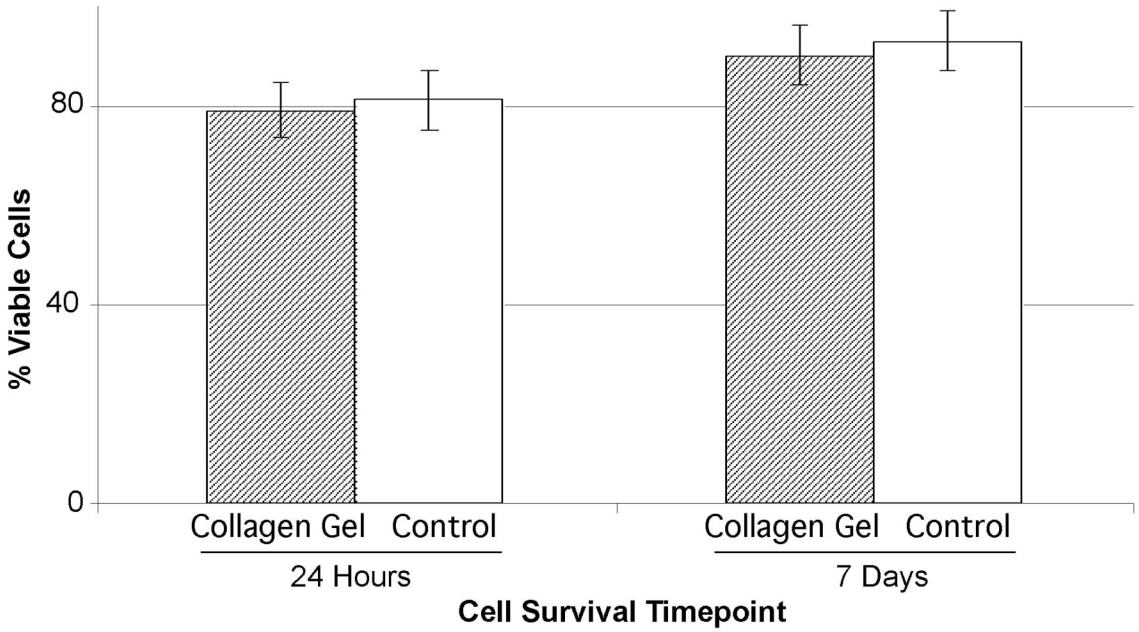


Figure 2.4 - P19(CA-puro) are viable on a collagen I gel.

The cellular viability of P19(CA-puro) was assessed at the 24 hour and 7-day time-points of growth and was used as a preliminary indicator of the suitability of this particular scaffold for the delivery of cardiac cells. No significant differences were observed between the collagen gel and the control surfaces.

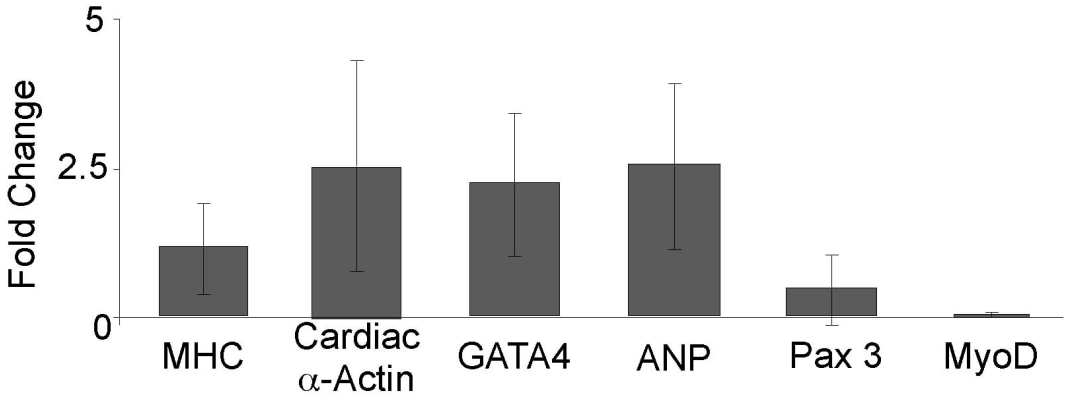
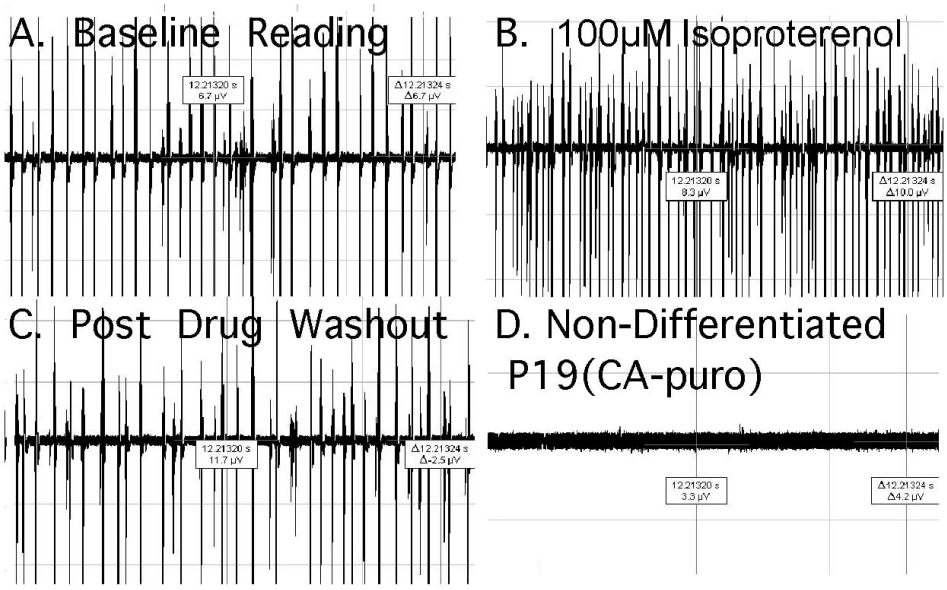


Figure 2.5 - P19(CA-puro) cardiomyocytes maintain expression of cardiac muscle genes while growing on the collagen gel compared to gelatin control coatings.

QPCR analysis of P19(CA-puro) enriched cells growing on the collagen gel and on control tissue culture dishes was performed on Day 9 of differentiation. There was no loss in expression detected in the cardiac transcripts MHC, cardiac α-actin, GATA4, or ANP compared to controls. In addition, no gain in skeletal muscle related transcripts was detected (Pax 3 or MyoD). Data represents the average +/- SD of 3 independent experiments done in duplicate, $p<0.05$.



E

	Trial 1	Trial 2	Trial 3	Trial 4	Ave. Fold Change	SD
Baseline	29	5	4	7		
Isoproterenol	63	12	14	28		
Fold Change	2.2	2.4	3.5	4	3.0	0.9

Figure 2.6 - P19(CA-puro) responds to the adrenotropic agonist isoproterenol.

P19(CA-puro) shows a chronotropic response to isoproterenol while growing on our collagen I crosslinked matrix. A-D are an example of an electrode reading from one trial, the complete data set (Fold change of # events recorded per minute) is shown in E.

recovery back to baseline after 5 minutes of drug removal. Undifferentiated P19(CA-puro) cells did not exhibit any detectible electrical events, nor did they respond to the drug stimulation. Therefore, collagen substrates are compatible with cardiomyocyte differentiation, as determined by the maintenance of synchronous extracellular electrical events, and the appropriate response to isoproterenol.

2.5 - Discussion

Cell based therapies to repair and regenerate cardiomyocytes have shown modest success but are promising. There is evidence that such therapies can be enhanced by incorporation of biomaterial scaffolds to prevent death of implanted cells, as well as to provide appropriate bioactive factors for cell survival and differentiation. A current limitation in cardiac biomaterials research is the availability of large homogeneous populations of cardiac muscle progenitor cells. For the present study we show the creation of a stable cell line that can be enriched for cardiac muscle cells with puromycin selection. Characterization of these derived cardiomyocytes indicated that these cells exhibited many properties of embryonic cardiomyocytes. In addition, we found that the cardiac muscle phenotype of the P19-derived cardiomyocytes was maintained when growing on collagen, a commonly used natural material in tissue engineering

A similar cardiac muscle cell enrichment strategy has been employed using human and mouse ESCs, in which a MHC promoter drove antibiotic resistance or GFP expression [39-47]. A previous publication reported an 99% purity for human ESC-derived cardiomyocytes used the α-MHC promoter driving neomycin resistance approach [44]. The higher enrichment in these ES studies may be due to differences in the promoter specificity

due to an integration site in the cells such as near strong lineage-dependent enhancers, the nature of the construct, or simply differences in the method of assessing enrichment.

Our genetically modified P19 cell line, P19(CA-puro), can be selected for cardiac α-actin expressing cells with puromycin during differentiation, resulting in about 59% of cells consistently differentiating into cardiomyocytes compared to 18% control P19 differentiations. This shows that transfection of P19 cells with the cardiac α-actin promoter driving puromycin resistance is an effective methodology for creating a cardiomyocyte selectable model progenitor line.

In response to DMSO induction, P19 cells will differentiate into cardiac and skeletal myogenic lineages. Cardiac α actin expression begins on approximately Day 6 after DMSO induced differentiation of cardiomyocytes, and on approximately day 8-9 in differentiating skeletal muscle [17]. The promoter for human cardiac α-actin was first tested in P19 cells in 1988, and it was shown that the transfected promoter functions in the appropriate developmental manner during differentiation of P19 EC cells to cardiomyocytes [17, 38, 48, 49]. In our experiments, this α actin promoter was used to enrich for cardiomyocytes in P19(CA-puro) as differentiating cells were exposed to puromycin on Day 6-9 and cells expressing α actin survived the puromycin selection. RNA and protein analysis, morphological analysis, contractile ability, and the appropriate response of P19(CA-puro) cardiac muscle to the cardioactive drug isoproterenol, demonstrate that P19(CA-puro) cells differentiate into functional cardiac muscle cells. In addition, by selecting for α actin expressing cells prior to skeletal expression, we did not observe enrichment for skeletal muscle, as confirmed by the lack of change in expression of the myogenic transcription factors MyoD and Pax3.

Collagen I is the major constituent of the cardiac extracellular matrix, and has been used quite extensively in previous reports as a base for tissue engineered patches, as reviewed in [50, 51]. In this study, we examined the effect of collagen substrate for P19 cardiomyocyte differentiation. We selected a natural crosslinking system for collagen hydrogel preparation to facilitate a natural biodegradable substrate. Transglutaminase is a calcium dependent enzyme that mediates covalent bond formation between glutamine and lysine residues, providing amide bonds which reinforce the scaffold [52, 53], and it has been previously shown that transglutaminase increases the strength of different types of hydrogels, including collagen [54-58]. To date a transglutaminase cross-linked collagen gel has not been published as a substrate for cardiac muscle cells. P19(CA-puro) cells grown on the collagen hydrogel substrate maintained similar levels of cardiomyocyte differentiation, as determined by GATA4, MHC, ANP, and cardiac α-actin and transcript levels, compared to the control substrate (untreated tissue culture dishes). Microelectrode arrays allow for the measurement of extracellular field potentials of cultured cells, similar to electrocardiograms [59]. Analysis of the beating-nature of the cardiomyocytes growing on collagen substrates using microelectrode array analysis to detect electrical events showed cardiomyocyte chronotropic stimulation. The P19(CA-puro) derived cardiomyocytes displayed regular pulsing events that were similar to that described for human ESC derived cardiomyocytes [39, 59, 60], and neonatal isolated cardiac muscle cells [37]. The ability of our substrate to sustain and support functional cardiomyocytes will allow for cardiac muscle cell sheets to be grown in culture for transplantation in the future.

2.6 - Conclusion

In summary, we created a stable selectable P19 embryonic carcinoma cell line, P19(CA-puro), that can be induced to differentiate into functional cardiac muscle cells. This

P19-EC cell based model appears to be a reliable source for evaluating biomaterials, which can next be validated using human ESCs or other muscle cells for therapeutic use such as in tissue engineered scaffolds and/or cell delivery systems for cardiac muscle repair.

2.7 - Acknowledgements

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CHAPTER 3:

Dihydropyridine Receptor Surface Marker Enrichment of ES-derived Cardiomyocytes

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3.1 - Abstract

Cardiomyocyte loss related to myocardial infarcts causes heart failure and in severe cases death. Stem cell (SC) therapy to replace muscle loss is an attractive treatment option and will progress once techniques are developed to prepare pure or defined populations of ES cell-derived cardiomyocytes for delivery to the heart. Various cardiomyocyte purification techniques using cell surface markers have been suggested, however our ability to purify cardiomyocytes is limited by the lack of cardiac muscle-specific surface markers. In the present study we investigate the utility of the dihydropyridine receptor (DHP-R) as a cell surface marker to purify ES-derived cardiomyocytes. DHP-R positive mouse ES cells were enriched for a cardiac isoform of myosin heavy chain and GATA4 transcript levels. Although the DHP-R fraction did not contain a pure population of cardiomyocytes, we conclude that DHP-R is a useful surface marker to increase the purity of ES cardiomyocyte cultures, and its utility may be especially appropriate when used in conjunction with other lineage restricting cell surface purification strategies.

3.2 - Introduction

The high level of mortality and morbidity related to cardiovascular disease reinforces the need to investigate alternate treatment options. Myocardial infarctions, or heart attacks, are characterized by a loss of blood to the heart muscle, and are associated with cardiac muscle (cardiomyocyte) loss due to ischemia which can lead to heart failure (Thygesen et al., 2007). Therapeutic options for severe cases of MI are limited (Stevenson, 1993), and the application of embryonic stem (ES) cell therapies to treat myocardial infarction in humans is being investigated by many different groups (Zhang et al., 2010). ES cells are characterized by their ability to self renew in their undifferentiated state, and their pluripotency, the capacity to differentiate into cell types from the three primary germ layers of the embryo, as reviewed in (Liu et al., 2007). Since the derivation of the first human ES (hES) cell line (Reubinoff et al., 2000; Thomson et al., 1998), there are still no approved hES cell treatments, and only a couple approved clinical trials, including a trial using hES derived oligodendrocyte progenitor cells for the treatment of spinal cord injury (Mayor, 2010). One of the factors that has contributed to the slow progression from treating animal models of disease to treating humans is the high risk of teratoma generation when undifferentiated or uncommitted stem cells are transplanted into mammals, as reviewed in (Blum and Benvenisty, 2008; Fong et al., 2010). In order to decrease this risk and progress to the clinic, a genetically stable and homogenous population of donor cells must be acquired from ES cell cultures to ensure the elimination of unwanted cell types or tumorigenic cells.

Directed *in vitro* differentiation of both hES and mouse ES (mES) cells into cardiomyocytes is becoming more efficient and defined (Puceat, 2008; Xu et al., 2011), and research into signaling mechanisms that can be applied to restrict lineage type is starting to progress, as reviewed in (Perino et al., 2008). Nonetheless, purifying cardiomyocytes to

obtain homogenous populations from the ES cultures is a difficult endeavor, as cell surface and structural proteins highly specific for developing cardiomyocytes have not yet been identified. In addition, inherent difficulties with cardiomyocyte cell survival have impeded progress in this area, as reviewed in (Hattori and Fukuda, 2010; Robey et al., 2008).

Many different approaches have been taken to enrich ES-derived cardiomyocytes, including manual dissection of beating areas (Passier et al., 2005; Segev et al., 2005), density-based enrichment procedures (Xu et al., 2002), transgenic selection of cardiomyocytes or progenitors based on fluorescent or antibiotic selection (Anderson et al., 2007; Huber et al., 2007; Xu et al., 2008), mitochondrial-based enrichment (Hattori et al., 2009), antibody-based cell sorting techniques using potential cardiomyocyte surface proteins (Blin et al., 2010; Honda et al., 2006; Rust et al., 2009; Van Hoof et al., 2010), and antibody-based cell sorting using combinations of lineage restricting cell surface proteins (Adler et al., 2010; Nelson et al., 2008; Yang et al., 2008).

We have identified the L-Type calcium channel α-1 subunit, also know as the dihydryopyridine receptor (DHP-R), as a candidate cell surface protein for sorting cardiomyocytes from ES cell cultures. DHP-R was identified as a potential cardiac and skeletal muscle surface marker based on its down-regulation in a microarray analysis of P19 embryonic carcinoma (EC) cell lines that are defective in myogenic differentiation (unpublished work Al-Madhoun and Skerjanc).

The L-Type calcium channel is an important pathway for calcium to enter cardiomyocytes, and plays a role in excitation-contraction coupling of heart muscle (Bers, 2002). This channel is also present in neurons (Calin-Jageman and Lee, 2008), skeletal myocytes (Flucher et al., 1990), small cell carcinoma cell lines (Morton et al., 1994), and other excitable and secretory cell types, reviewed in (Moosmang et al., 2005). The purpose

of this study is to determine if a commercially available antibody that recognizes DHP-R on the cell surface can be used as a surface marker to sort cardiomyocytes from ES cell cultures.

DHP-R is expressed during P19 EC and mES cardiomyogenesis, and follows a similar temporal pattern of expression as other cardiac-related transcripts. In this study, previously published ES-derived cardiomyocyte cell dissociation and survival techniques were systematically tested in an attempt to improve cell viability following dissociation. FACS sorting with DHP-R on 8-day mES cardiomyocyte cultures led to the enhancement of MHC and GATA-4 transcript levels and an enrichment of MHC positive myocytes compared to negatively sorted control cells. Therefore, we conclude that the DHP-R is a useful surface marker to increase the purity of ES cardiomyocyte cultures, and may be a suitable marker for purifying cardiomyocytes when used in conjunction with other lineage restricting surface markers or strategies.

3.3 – Methods

3.3.1 - P19 Cell Culture and induction of myogenesis:

P19 EC cells (American Type Culture Collection, ATCC) were grown in α-minimum essential media (αMEM) (Invitrogen), 10% fetal bovine serum (CanSera) and 100 μg/ml penicillin (Invitrogen), and passaged every second day. Myogenic differentiation was induced by culturing the cells in suspension culture conditions (in a Petri dish), in the presence of 0.8% DMSO growth media (McBurney et al., 1982). The cells were grown in suspension culture conditions for 4 days to form embryoid bodies (EBs), and then plating onto tissue culture dishes or gelatin coated coverslips.

3.3.2 - D3 mES Cell Culture and induction of myogenesis:

D3 mES cells were obtained from ATCC, and undifferentiated cells were grown in complete media consisting of high glucose Dubecco's modified eagle media (DMEM, Gibco) containing 15% fetal bovine serum (Wisent), 0.1 mM of non-essential amino acids (Gibco), 0.1mM β-mercaptoethanol (Sigma), 100 μg/ml penicillin (Invitrogen), and 1000U/mL leukemia inhibitory factor (LIF) (Chemicon).

Cardiomyocyte differentiation was induced by growing the cells in suspension culture conditions in the absence of LIF as described (Puceat, 2008). Cells growing in monolayer were brought into single cell suspension by treating for 5 minutes with 1X trypsin-EDTA (Invitrogen), and re-suspending in complete media (without LIF). Cells were grown for 2 days in hanging drops (800 cells/ 20μl drop), and then transferred to a petri-dish to grow in suspension for an additional 3 days prior to plating onto tissue culture treated dishes or gelatin coated coverslips.

3.3.3 - Western Blotting:

P19 EC cell and mES cells were grown in monolayer (Day 0) or differentiated (days 1-9 for P19 EC cells and day 6 or day 10 for mES cells) following the differentiation protocol described herein. Total protein was harvested in modified RIPA buffer (50mM Tris-HCl (pH 7.4), 1% NP-40, 0.25% Na-deoxycholate, 150mM NaCl, 1mM EDTA, 1mM phenylmethylsulfonyl fluoride and 1X protein inhibitor cocktail, (Roche Applied Sciences). 50μg of total protein for P19 cells and 80μg for mES cells was separated on a 10% polyacrylamide gel with sodium dodecyl sulfate (SDS) in SDS buffer, and transferred onto a polyvinylidene fluoride membrane (Bio-Rad, Hercules, CA). DHP-R protein was detected

using anti-DHP-R antibodies (1:100 dilution; Chemicon) and visualized with horseradish peroxidase-conjugated secondary antibodies (Chemicon). β-actin proteins were detected with anti- β-actin antibody (1:10000 dilution; Sigma).

3.3.4 - RT-PCR:

Total RNA from day 0-9 differentiating P19 EC cell cultures was harvested, and 1μg was used to synthesize the first strand cDNA as described (Gianakopoulos and Skerjanc, 2005). The oligonucleotides and PCR conditions for GATA4, GAPDH, and Mef2c are described in (Gianakopoulos and Skerjanc, 2005; Kennedy et al., 2009), and the oligonucleotides used for DHP-R are listed in Table 3.1. PCR conditions for the DHP-R were: 1 minute 94°C (denaturation), 2 minutes 72°C (annealing), and 2 minutes 72°C (extension), for 26 cycles. Negative controls included a water (no cDNA) control in the PCR reactions, and for the cDNA synthesis, water and no-RT enzyme controls. The PCR product was hybridized to DNA probes and visualized by autoradiography.

3.3.5 - Cell Dissociation and Viability:

6-day old mES cardiomyocyte cultures were dissociated using either 0.5mM EDTA, Liberase TM (Roche Applied Sciences), collagenase B (Roche Applied Sciences), trypsin-EDTA (Invitrogen), or Cell Dissociation Buffer (CDB) (Gibco). Table 3.2 outlines the detailed protocol followed for each of the dissociation reagents, and the corresponding references. Briefly, at day 6 of differentiation, mES cultures were washed with HBSS or PBS, incubated with the cell dissociation reagent at 37°C, washed with 4% FBS-HBSS or 4% FBS-PBS, and passed through a 40μm sterile filter. Cells were monitored under the

Table 3.1 - Primers and their respective references used in PCR reactions.

Gene of Interest	Forward Primer	Reverse Primer	Reference
βMHC	ACAACCCCTACGATTATGCGT	ACGTTCAAAGGCACTATCCGTG	(Wang and Seed, 2003)
GATA4	AAACGGAAGCCCAAGAACCT	TGCTAGTGGCATTGCTGGAGT	(Zhang et al., 2007)
Pax3	TTTCACCTCAGGTAATGGGACT	GAACGTCCAAGGCTTACTTTGT	(Wang and Seed, 2003)
Cardiac α-actin	CTGGTATTGCCGATCGTATG	CTTGCTGATCCACATTTGCT	(Wang and Seed, 2003)
Nkx 2.5	AAGCAACAGCGGTACCTGTC	GCTGTCGCTTGCACTTGTAG	Primer3 (NIH)
GAPDH	AGGTCGGTGTGAACGGATTTG	TGTAGACCATGTAGTTGAGGTCA	(Wang and Seed, 2003)
MyoD	CCCCGGCGGCAGAATGGCTACG	GGTCTGGGTTCCCTGTTCTGTGT	(Oustanina et al., 2004)
Nestin	CCCTGAAGTCGAGGAGCTG	CTGCTGCACCTCTAAGCGA	(Wang and Seed, 2003)
Mash1	TCT CCT GGG AAT GGA CTT TG	GGT TGG CTG TCT GGT TTG TT	(Wang and Seed, 2003)
MHC6 (αMHC)	GCCCAGTACCTCCGAAAGTC	GCCTTAACATACTCCTCCTTGTC	(Wang and Seed, 2003)
DHP-R	TCCCGAGCACATCCCTACTC	ACTGACGGTAGAGATGGTTGC	(Wang and Seed, 2003)

Table 3.2 Comparison of the protocols used for testing the various published cell dissociation reagents and protocols.

Dissociation Reagent	Concentration and dilutent	Incubation Time at 37°C (minutes)	Cell Filtering (40um sterile filter)	# of Day 6 Trials	Reference
EDTA	0.5mM in PBS	15	YES	7	
Liberase TM (Roche), formally called "blendzyme"	0.25 mg/ml in serum-free DMEM	20	YES	3	(Laflamme et al., 2007)
Collagenase B (Roche)	1mg/ml in serum-free DMEM	30	YES	2	(Akasha et al., 2008)
Trypsin-EDTA	0.25% in PBS	10	YES	3	(Yang et al., 2008)
Gibco Cell Dissociation Buffer (CDB),	Not diluted, HBSS-based	20	YES	4	(Baba et al., 2007)

microscope throughout the dissociation incubation period, swirled, and gently pipetted every 5 minutes to disperse clusters. The analysis included differentiating mES cultures at day 5, 8, and 11 using CDB (Table 3.2). Cell viability was determined by the trypan blue dye exclusion method using a hemocytometer.

3.3.6 - Testing the effects of ROCK (Rho-associated Kinase) Inhibitor and Prosurvival Cocktail (PSC) on Cardiomyocyte Survival Following Dissociation:

8-day old differentiated mES cells were dissociated with CDB as described above, and the viability was immediately verified. Dissociated cultures that contained 70% or higher live cells following dissociation were re-plated on Matrigel, PSC-Matrigel, or untreated tissue culture dishes. Dissociated cells were grown in either control growth media, PSC supplemented growth media, or ROCK inhibitor supplemented growth media (10μM Y-27632, Calbiochem), for two days prior to RNA harvesting the cells for QPCR analysis or fixing coverslips for MHC IF (as described above). The PCS cocktail cell culture conditions included coatings of 50% (vol/vol) Matrigel (Invitrogen Life Sciences) and growth media, both supplemented with ZVAD (100 mM, benzyloxycarbonyl-Val-Ala-Asp(O-methyl)-fluoromethyl ketone, Calbiochem), Bcl-XL BH4 TAT peptide, 50 nM, Calbiochem), IGF-1, (100 ng/ml, Peprotech), and pinacidil (50 mM, Sigma), modified from (Laflamme et al., 2007).

3.3.7 - Fluorescent Activated Cell Sorting (FACS):

P19 EC cells were dissociated using the 0.5% EDTA-PBS method and mES cells were dissociated using CDB, as described above. The viability of the single cell suspension

was verified prior to proceeding with FACS using the trypan blue dye exclusion method. Cell suspensions with 70% or higher total live cells were processed for FACS. Cells were maintained on ice during antibody staining and sorting. Single cell suspensions were washed two times in FACS buffer (cold 4% FBS-PBS), spinning at 500g for 5 minutes at 4°C. DHP-R antibody titration was performed with dilutions of 1:1000-1:100. For FACS, mES and P19 EC cells were incubated for 30 minutes with anti-DHP antibody (Chemicon 1:200 diluted in FACS buffer). Cells were washed twice with FACS buffer and re-suspended in 50μl goat anti-mouse Alexa488 secondary antibody (Invitrogen, 1:200 diluted in FACS buffer) and incubated for 30 minutes in the dark. Cells were washed twice with FACS buffer. "Alexa 488- alone" controls and "auto" (no primary or secondary antibody) were prepared in parallel and subjected to identical washes/spins as the cells prepared for sorting. Cells were sorted into FBS using a BeckmanCoulter MoFlo FACS machine. Sorted and control unsorted P19 EC cells were re-aggregated for 2 days in hanging drops (1500 cells/20μl), and cultured for 2 additional days on tissue culture dishes or gelatin coated coverslips prior to analysis. Sorted and control mES cells were either analyzed immediately after sorting, or plated on Matrigel coated dishes and coverslips (BD Biosciences) for two days prior to analysis.

3.3.8 - Real Time Quantitative Polymerase Chain Reaction (QPCR):

QPCR was performed to quantify changes in gene expression of DHP-R, Nkx2.5, GATA4, β and α-myosin heavy chain (MHC), cardiac α-actin, MyoD, Pax3, Mash1 and Nestin. Primer sequences are given in Table 3.1. Total RNA was isolated using an RNeasy Kit (Qiagen). The QuantiTect Reverse Transcription Kit with DNase I treatment was used to synthesize the cDNA using 0.8μg of purified RNA as a template (Qiagen). QPCR was

performed using FastStart SYBR Green with ROX (Roche Applied Sciences), following manufacturer's suggestions in a 25μl final reaction volume. PCR amplification was performed using an ABI 7300 thermocycler (Applied Biosystems) as described (Savage et al., 2009). The primers have been previously validated for their efficiency of amplification under these conditions, including a melt curve analysis and by comparison with results from Northern Blot analysis. Relative fold differences in expression of these genes were calculated using the comparative CT method (Livak and Schmittgen, 2001). The results were normalized to GAPDH and the fold change was calculated relative to either unsorted cells or undifferentiated cells ("Day 0"). Controls included a no cDNA (water only) control for each primer combination, and also a cDNA reaction for each RNA sample that did not contain the reverse transcriptase enzyme to ensure that there was no DNA contamination prior to cDNA synthesis. All reactions were performed in duplicate.

3.3.9 - Immunohistochemistry and Cell counting:

MHC immunofluorescence was performed using the hybridoma antibody anti-MHC, MF20 (Developmental Studies Hybridoma Bank, University of Iowa) as described (Ridgeway and Skerjanc, 2001). A 1:200 dilution of goat anti-mouse Cy3-linked antibody in PBS (Jackson ImmunoResearch Laboratories, West Grove, PA) was used as a secondary antibody. For sarcomeric actinin, cardiac troponin T, and DHP-R immunofluorescent staining, coverslips were fixed using freshly made ice cold 4% paraformaldehyde (PFA) in PBS for 20 minutes at room temperature. Samples were washed 3 times using 1xPBS, and blocked for 1 hour at room temperature using 0.3% triton X-100, and 4% goat serum PBS. Coverslips were incubated overnight at 4 ºC with their respective antibodies, 1:500 Sarcomeric actinin (Sigma-Aldridge), 1:500 cardiac troponin T (Neolabs/Labvision), and

1:200 DHP-R (Chemicon), diluted in blocking solution. Coverslips were washed three times with block, and incubated for 1 hour at room temperature with 1:200 dilution of goat anti-mouse Cy3-linked secondary antibody in PBS (Jackson ImmunoResearch Laboratories, West Grove, PA), diluted in block. The coverslips for MHC, sarcomeric actinin, cardiac troponin T, and DHP-R staining were washed, mounted with 1:1000 Hoechst nuclear stain to detect nuclei, and the fluorescence was detected using a Nikon microscope. The percentage of MHC-positive cardiac and skeletal myocytes were quantified by cell counting. Cardiomyocytes were identified based on their rounded clusters, and skeletal myocytes based on their elongated morphology.

3.3.10 - Intracellular Calcium Measurements:

Spontaneously beating mES coverslips were observed under a Nikon light microscope to identify contracting areas Beating cells grown on coverslips were washed in serum-free DMEM and then treated with 2μM fura-2 AM in DMSO (Sigma Aldridge) for 20 minutes at 37 ºC in the dark. Fura-2 AM solution was removed, and cells were left in serum-free DMEM to perform the analysis. Calcium transients were observed by exciting the fura-2 AM loaded cells with alternating excitation wavelengths of 340 and 380 nm, and recording the fluorescent emission intensity at 510nm (340/380 ratio). Calcium measurements were made using an IonOptix™ Myocyte Calcium and Contractility Recording System with a HyperSwitch dual excitation light source (Ionoptix, Milton, MA, USA). Cell shortening was measured simultaneously on the Ionoptix Contractility System using the cell edge motion detector (Ionoptix, Milton, MA, USA).

3.3.11 - Statistical Analysis:

Statistical analysis was performed between two groups using two-tailed students t-tests, and values are expressed as mean +/- standard error (SE) of the mean. For the mESC cardiomyocyte differentiation transcript time course experiment, statistical analysis was performed using a repeated measures ANOVA with a Tukey post hoc test. Statistical analysis was performed using XLStat software and Microsoft Excel. $p < 0.05$ probability values were considered statistically significant for all analyses.

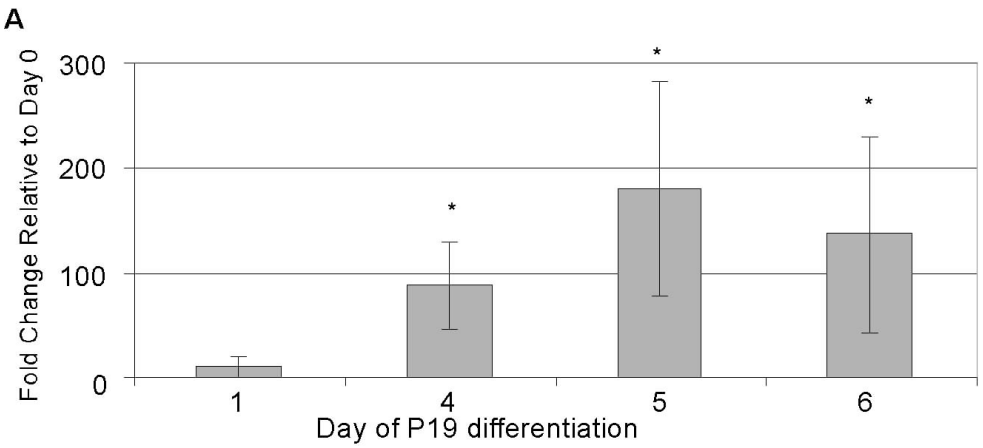
3.4 – Results

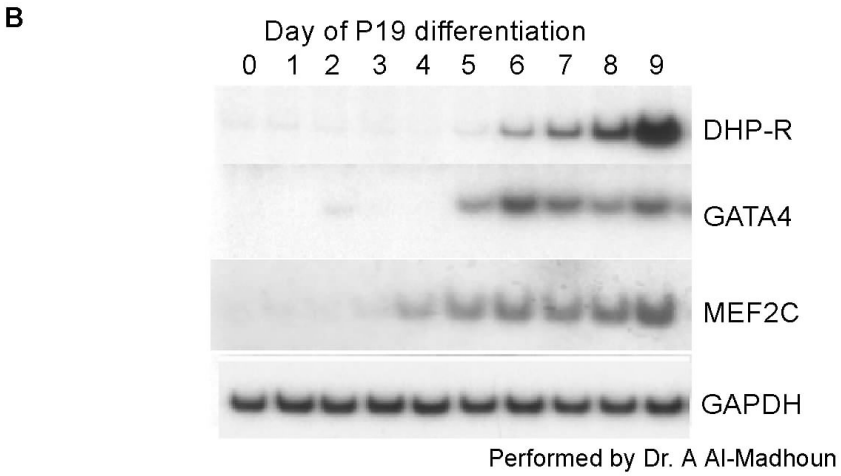
3.4.1 - P19 EC cells upregulate DHP-R at the protein and RNA level during myogenesis.

To characterize the temporal pattern of expression of DHP-R, P19 cells were induced to differentiate into myocytes using the cell suspension and DMSO induction protocol (McBurney et al., 1982). RNA and protein expression were analyzed by QPCR, RT-PCR, and western blotting. DHP-R RNA transcript levels are upregulated starting on day 4 of differentiation during P19 EC cell cardiomyogenesis as detected by QPCR (Fig. 3.1 Panel A), and detected on day 5 by semi-quantitative RT-PCR (Fig. 3.1, Panel B). RT-PCR analysis was also used to demonstrate that DHP-R is upregulated in a similar temporal pattern as the cardiogenic factors GATA4 and MEF2C during cardiomyogenesis. The observation was further confirmed using Western blot analysis, which shows that DHP-R protein levels were increased on days 4-5 (Fig. 3.1, Panel C). Thus, P19 EC cells express DHP-R during myogenesis, starting on days 4-5 of differentiation.

3.4.2 - DHP-R-positive P19 cells are enriched for cardiomyocytes and skeletal myocytes.

An 18% population of day 5 differentiating P19 cells was identified as expressing DHP-R on the cell surface by flow cytometry, which was not present in undifferentiated P19





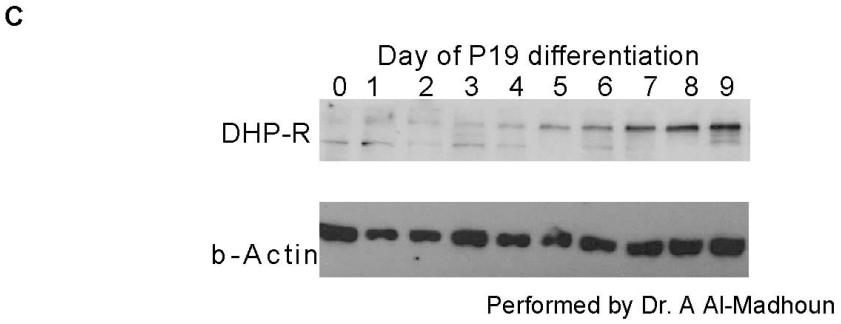


Figure 3.1 - DHP-R is upregulated during P19 EC cardiac and skeletal myogenesis at the RNA and protein level.

(A) DHP-R RNA transcript levels increase on Day 4 of P19 EC differentiation, as detected by QPCR. *=$p<0.05$ compared to D0 undifferentiated P19 cultures assessed using a repeated measures ANOVA and Tukey post hoc test, (n=3). (B) DHP-R is upregulated in a similar pattern to GATA4 transcript during cardiomyogenesis following MEF2C upregulation, as detected by RT-PCR. (C) DHP-R protein levels also increase starting on Day 4 of differentiation, visualized by Western blot analysis.

cells (Fig. 3.2). In order to determine if DHP-R selects for a cardiac muscle-specific population in P19 cells, the DHP-R + and – fractions were isolated from day 5 P19 cultures using FACS. After re-aggregation and culture, DHP-R+ cells have a trend for enrichment of cardiac α-actin, MyoD, and DHP-R transcripts compared to either the unsorted cells or the negatively sorted cells (Fig. 3.3 Panel A). DHP-R + cultures also show a trend to be enriched for MHC-positive cardiomyocytes and skeletal myocytes by immunofluorescent staining (Fig. 3.3 Panel B, C). DHP-R + colonies exhibited spontaneous beating (n=1). Spontaneous beating was not seen in the unsorted or DHP-R - fractions. Therefore, it appears that DHP-R antibodies can be used to enrich cardiac and skeletal myocytes from P19 cells.

3.4.3 - DHP-R is upregulated during mES cardiomyogenesis.

D3 mES cells were differentiated following the hanging drop method, and QPCR analysis of a timecourse revealed an early expression of GATA-4 by day 3 and cardiac muscle-related structural transcripts MHC and cardiac α-actin by day 5. DHP-R was significantly upregulated starting on day 5 of differentiation (Fig. 3.4, Panel A). MES cardiomyocytes showed positive immunofluorescent staining for MHC, cardiac troponin T, sarcomeric α-actinin, and DHP-R (Fig. 3.4 Panel B). DHP-R immunofluorescent staining revealed a distinct punctate staining pattern on the DHP-R positive cells. Previous reports have shown a similar punctate pattern of DHP-R on muscle fibers (Leach et al., 2005; Takagishi et al., 2000). The percentage of cardiomyoctes appeared to peak on day 8 of differentiation at approximately 28% MHC-positive cardiomyocytes (Fig. 3.4 Panel C). Protein expression of DHP-R was also confirmed using western blot analysis, revealing an

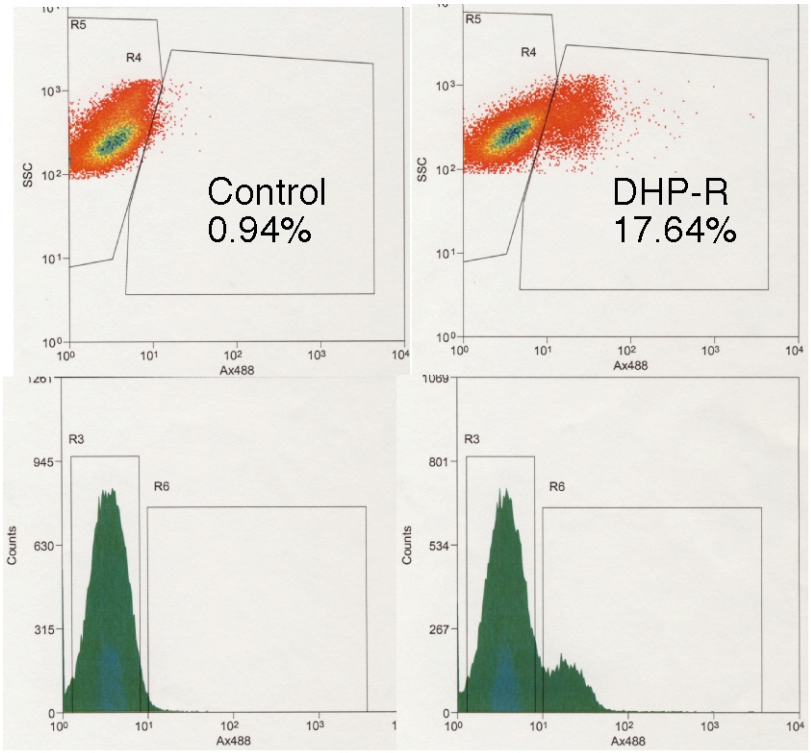


Figure 3.2 - An 18% DHP-R positive cell population was identified on Day 5 of P19 EC differentiation by FACS analysis.

Day 5 differentiating P19 cells were analyzed by using anti-DHP-R primary antibody and FITC secondary. An 18% positive population was measured by gating the stained cells against cells that were stained with the secondary antibody alone (FITC alone control), (representative image of n=2).

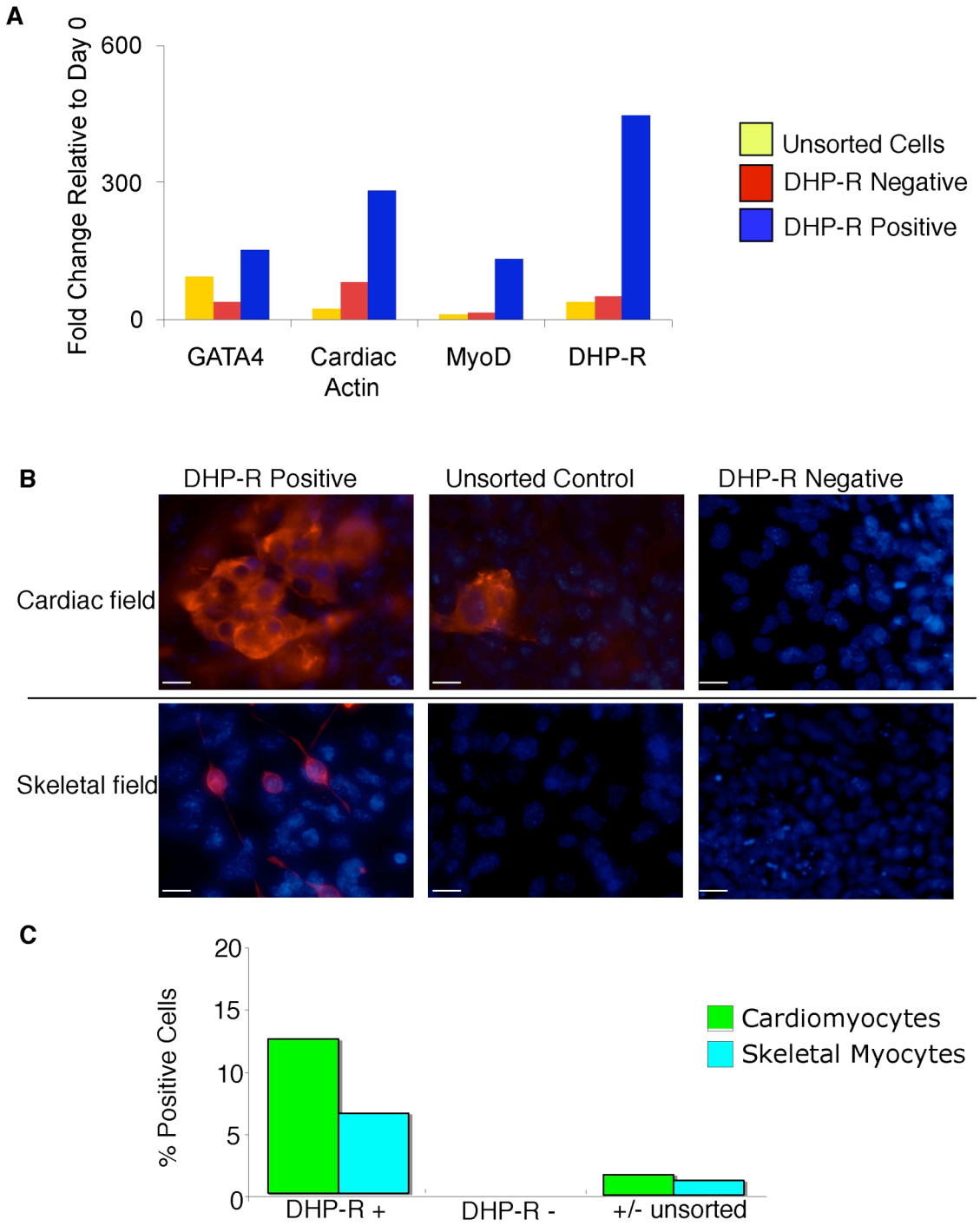


Figure 3.3 - DHP-R positive P19 cells are enriched for cardiac and skeletal myocytes. P19 EC cells were FACS sorted on Day 5.

DHP-R positive cell populations were compared to the DHP-R negative and unsorted populations on Day 9 of differentiation using QPCR analysis (A), and MHC immunofluorescent staining (B) and MHC positive cell counting C). DHP-R + cultures show a trend for enriched RNA transcript levels for GATA4, cardiac α-actin, MyoD, and DHP-R by QPCR analysis. (n=2), Scale bars = 20μm.

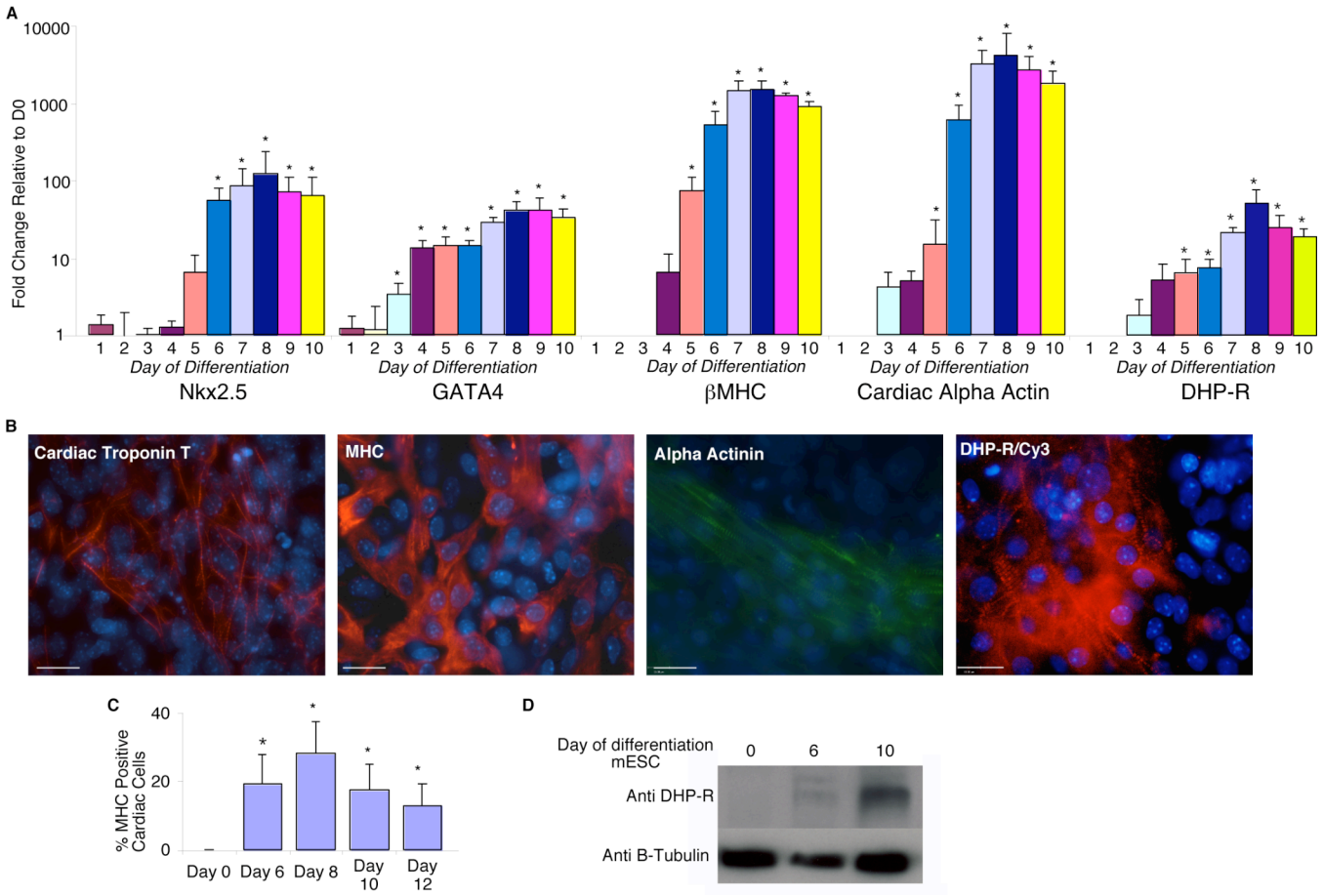


Figure 3.4 - DHP-R expression during mES cardiomyogenesis follows a similar temporal pattern to cardiac-related transcript upregulation.

MES cardiomyocyte differentiation was monitored using QPCR from RNA extracted on Days 0-10 of differentiation (A), and immunofluorescent staining on Day 10 for cardiac troponin T, MHC, and sarcomeric actinin, DHP-R (B). To assess cardiomyogenesis % MHC positive cardiomyocytes were counted on Days 0, 6, 8, 10, and 12 (C). DHP-R protein expression is detectible on Day 6 and 10 by Western blot analysis (D). *=Statistical significant $p<0.05$, n=3, assessed using a repeated measures ANOVA with a tukey post hoc test. Scale bars=20μm.

increase in DHP-R protein levels between days 6 and 11 of cardiomyogenic differentiation (Fig. 3.4, Panel D).

MES cultures began to show spontaneous rhythmical contractions on day 7 of differentiation and beat in culture until days 12-18 of differentiation (data not shown). Intracellular Ca^{2+} handling was examined by simultaneously recording the contraction and intracellular calcium transient from the contracting cardiomyocytes (n=3) (Fig. 3.5). Contraction of the cardiomyocytes is accompanied with an intracellular calcium transient, similar to previous documentation in ES-derived cardiomyocytes (Dolnikov et al., 2006; Kehat et al., 2001).

Therefore, DHP-R upregulation at the RNA and protein level accompanies cardiomyogenesis, forming functional cardiomyocytes in the mES model.

3.4.4 - MES cell dissociation lead to a high amount of variability between dissociation trials:

In order to optimize the cell dissociation step prior to FACS, 6-day differentiated mES cells were dissociated with various cell dissociation buffers that have been published for use in either mES or hES cardiomyocyte cultures. Liberase TH from Roche Applied Sciences (Laflamme et al., 2007), Gibco's CDB (Honda et al., 2006), collagenase B (Akasha et al., 2008), 1x trypsin-EDTA (Yang et al., 2008), and 0.5mM EDTA-PBS were tested. The percentage of live cells was assessed immediately after termination of the cell dissociation step. As shown in Figure 3.6, the survival rate of differentiated mES cultures dissociated with 0.5% EDTA-PBS, Liberase TH, collagenase B, and trypsin-EDTA was highly variable among the different experiment trials. Cells dissociated with the CDB tended to have a higher rate of survival than the other buffers. It should be noted that dissociation

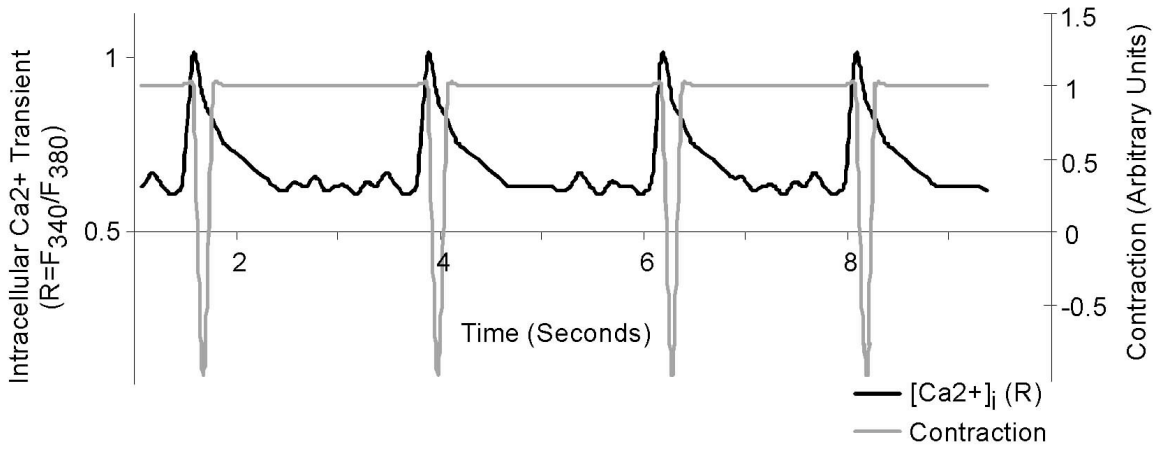


Figure 3.5 - MES-derived cardiomyocytes beat spontaneously and rhythmically under tissue culture conditions, with intracellular calcium levels increasing during the contractions.

The calcium transient ratio (R) was calculated based on the ratio between calcium influx and efflux in Fura-2 AM loaded cardiomyocytes (fluorescence measured at 340nm and 380nm). Corresponding physical contractions were observed under the microscope and measured using a cell motion edge detector. (n=3).

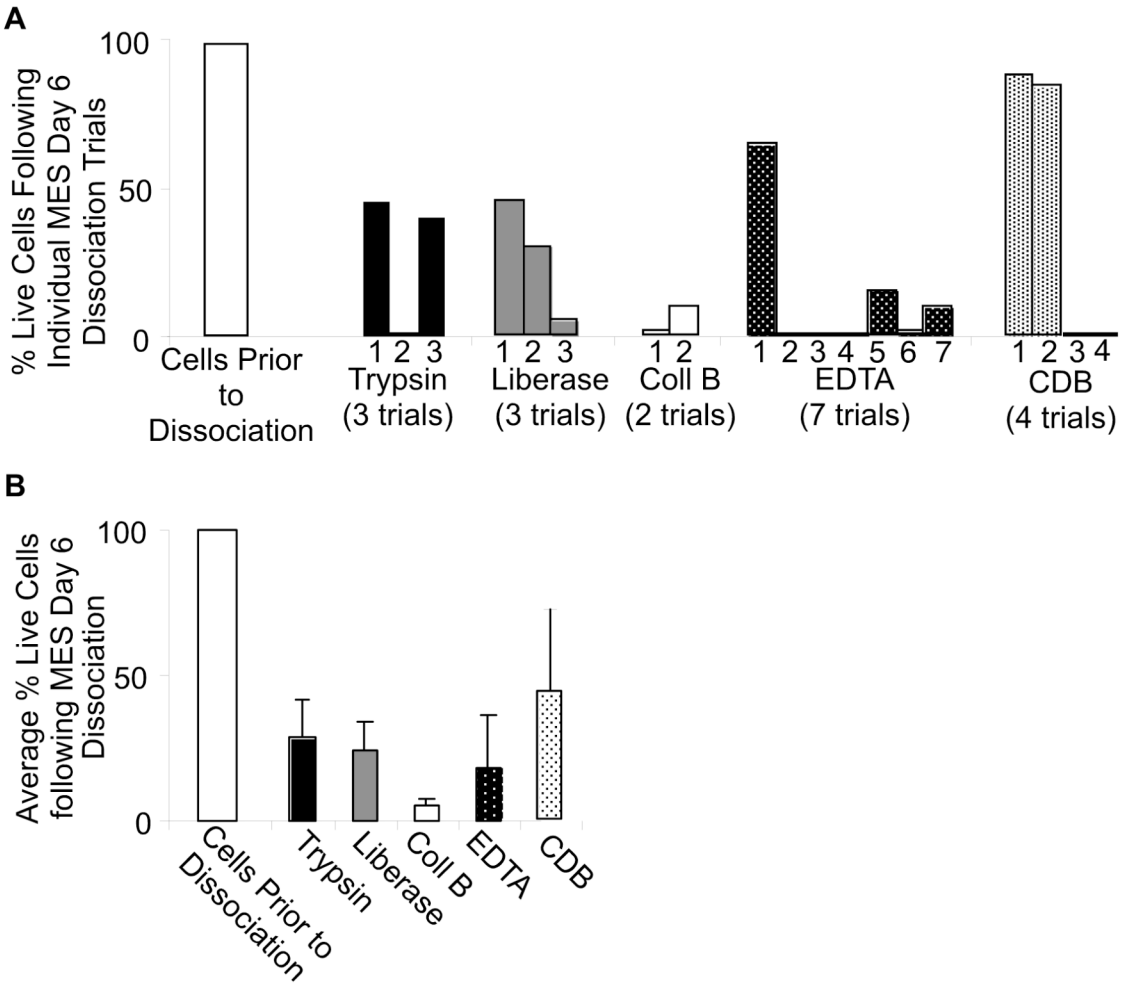


Figure 3.6 - MES cardiomyocyte cultures dissociated with Gibco's Cell Dissociation Buffer (CDB) on Day 6 of differentiation resulted in a range from 0-80% of live cells following dissociation.

Five different cell dissociation reagents were compared for their efficiency of dissociation on Day 6 of mES differentiation. Dissociation with CDB appeared to lead to the least amount of cell death, however results varied from experiment to experiment in the individual dissociation trials. Panel A- individual trials plotted to demonstrate the variability between trials, Panel B- averages of the individual trials (Error bars = SE, number of trials range from n=2 - n=7).

with CDB, although yielding a slightly higher percentage of live cells, still resulted in a great deal of variability between experiments even when all other conditions were maintained constant. No differences in the percentage of total live cells remaining after dissociation were detected between day 5, 6, 8, and 11 mES cardiomyocyte cultures, dissociated with CDB (Figure 3.7).

As a test of the effect of cell dissociation specifically on mES-derived cardiomyocytes, day 8 mES cells were dissociated with the CDB and grown on Matrigel-coated coverslips. Dissociation trials that yielded less than 70% of live total cells immediately following CDB dissociation were not grown on Matrigel. 2-days following dissociation, MHC-positive cardiomyocytes were present in the Matrigel cultures, however a trend of a 50% decrease was observed compared to non-dissociated day 10 differentiating cardiomyocytes (Fig. 3.8). Furthermore, the loss of cardiomyocytes after dissociation varied from experiment to experiment (8%+/-6% cardiomyocytes remained in culture).

In an attempt to improve cardiomyocyte survival following dissociation, two previously published cell-survival-promoting supplement regimes were tested on dissociated mES cells. As shown in Figure 3.9 panel A, mES cells dissociated with CDB on day 8 and grown for 2 days on tissue culture dishes or Matrigel-coated dishes showed a trend for averages of 19, 2, and 3-fold decreases in cardiac markers α–MHC, β–MHC, and cardiac α-actin respectively, compared to undissociated mES cardiomyocyte cultures. Supplementation with PSC or ROCK inhibitor did not improve the decrease observed in gene expression. No significant differences were noted for GATA4 and Nkx2.5 transcript levels. MHC IF did show a small proportion of myocytes that survived the dissociation step, however no major differences in the number MHC-positive myocytes were observed between treatment groups (Fig. 3.9, panel B).

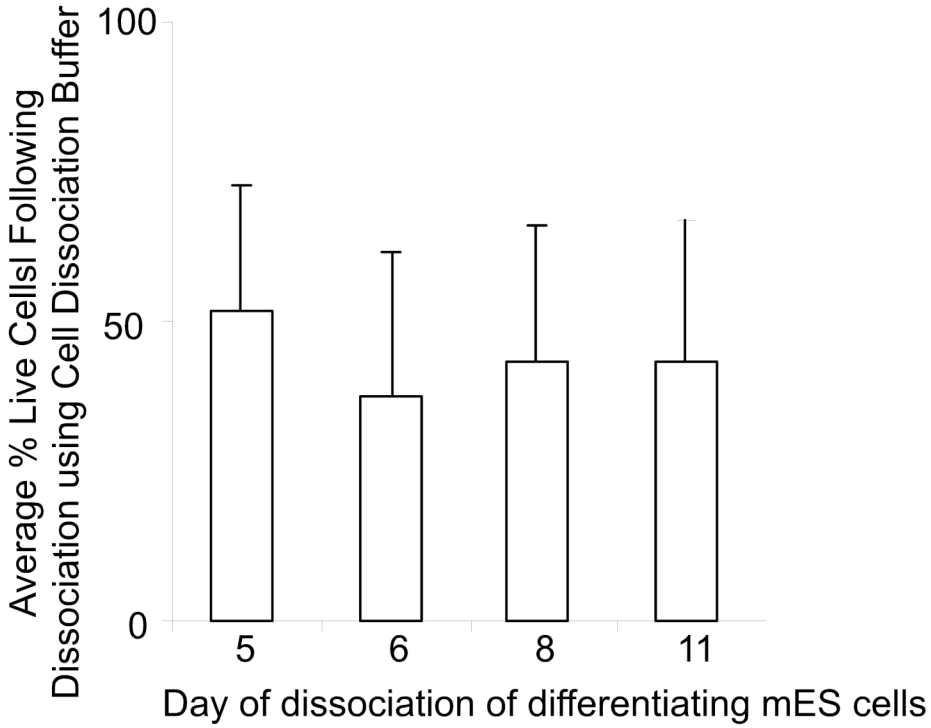


Figure 3.7 - No significant differences were detected between mES cells that were dissociated on Days 5, 6, 8, and 11.

MES cardiomyocyte cultures were dissociated with CDB, and the % live cells was assessed using trypan blue dye exclusion. The percentage of live cells varied from 0-85 %, and the averages are shown in the above graph. Error bars = SE (n=3).

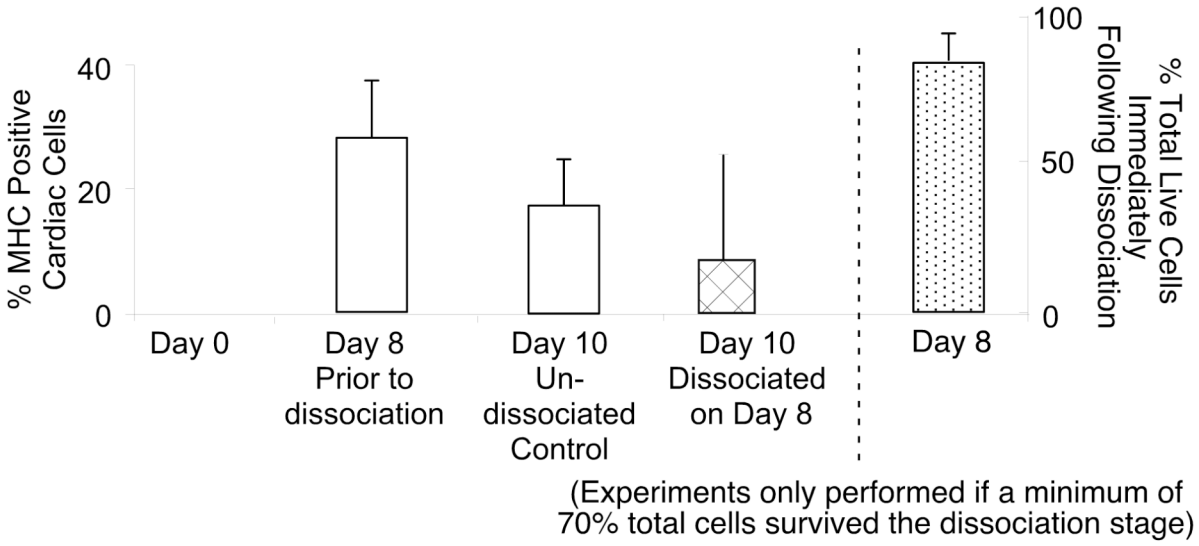


Figure 3.8 - Dissociation of the mES-derived cardiomyocytes lead to a 50% loss in MHC-positive cardiomyocytes compared to undissociated control cultures.

Day 8 mES-derived cardiomyocytes were dissociated, plated on Matrigel coated tissue culture dishes, and assessed for the percentage of MHC-positive cells (Error bars = SE, n=3). Experiments were only performed if a minimum of 70% total cells survived the dissociation stage.

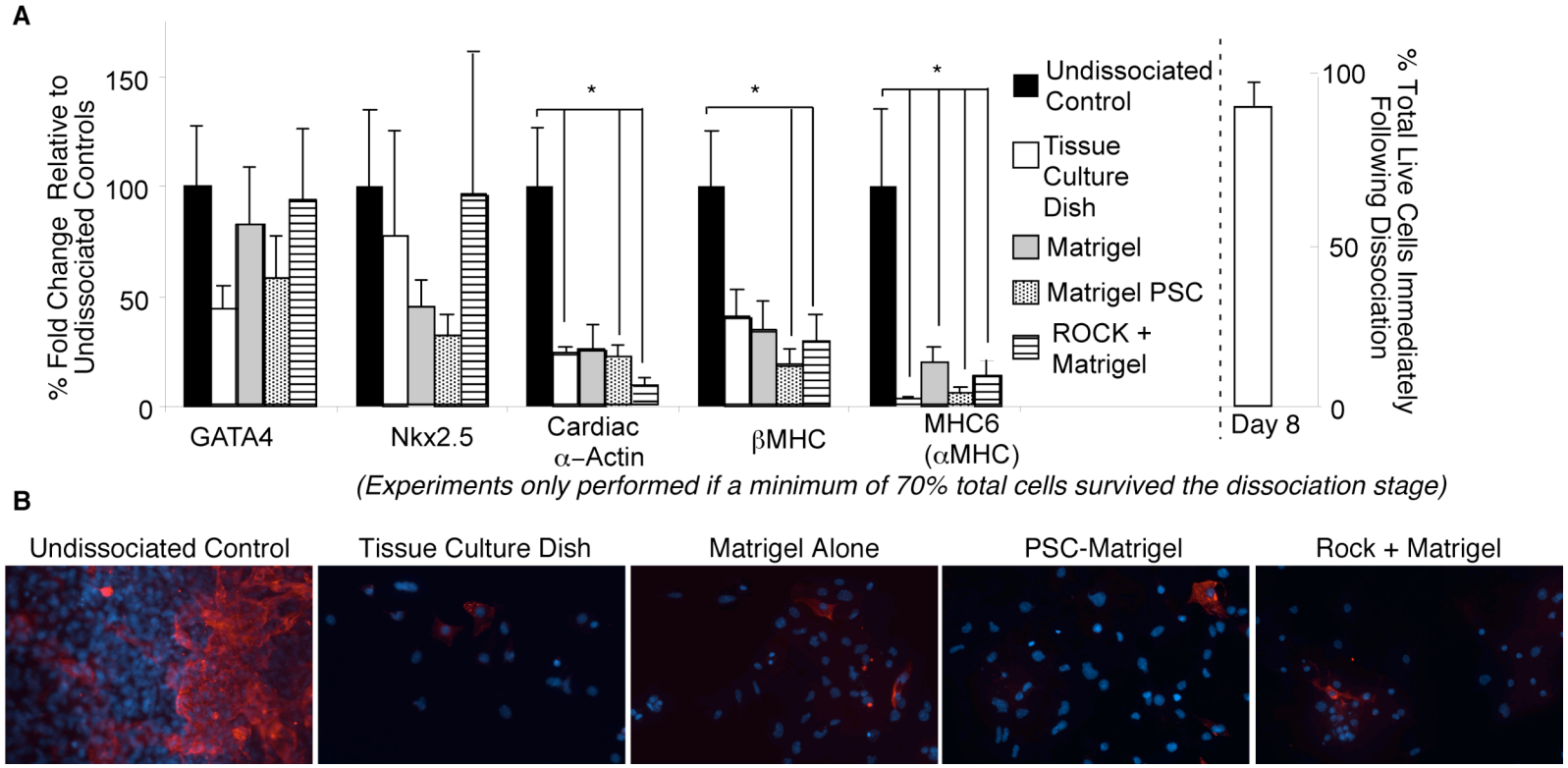


Figure 3.9 - Growing dissociated mES cardiomyocytes with ROCK inhibitor supplementation or PSC-Matrigel survival mix does not improve cardiomyocyte survival.

Day 8 mES cells were dissociated and seeded onto matrigel or PSC-Matrigel coatings, or tissue culture treated control dishes and grown for two additional days in either ROCK-inhibitor or PSC supplemented media, or control growth media (Matrigel and tissue culture controls). (A) QPCR was performed on day 10 of mES differentiation. Compared to undissociated cultures, the transcript levels of αMHC, βMHC, and cardiac α-actin were reduced. No differences were noted between GATA4 and Nkx2.5 transcript levels. Treatment with PSC-Matrigel or ROCK inhibitor did not improve the loss in αMHC, βMHC, and cardiac α-actin transcript levels compared to untreated dissociated cells (tissue culture dish controls). (n=3), *=$p<0.05$ compared to undissociated cultures using a student's t-test. (B) MHC staining was performed on day 10 to demonstrate the loss of cardiomyocytes following dissociation. No MHC positive staining differences were detected between any of the dissociated cultures. Scale bars = 100μm. Note: Experiments only performed if a minimum of 70% of total live cells were obtained following dissociation.

Thus, upon comparison with multiple previously published protocols, the Gibco CDB technique resulted in the highest percentage of viable cells. MES-derived cardiomyocytes are still highly sensitive to the cell dissociation step and PSC or ROCK inhibitor supplementation for two days following dissociation did not improve cardiomyocyte survival.

3.4.5 - DHP-R positive mES cells were enriched for cardiac α-actin and GATA4.

Optimization of flow cytometry analysis revealed that when the appropriate antibody concentration was used to stain the cells, the percentage of DHP-R positive mES cells was detected to increase during cardiomyogenesis (Figures 3.10 and 3.11). On days 6, 8, and 11, a 6%, 12%, and 25% respectively of total cells stained positive for DHP-R (Figure 3.11). FACS was performed on day 6 (n=2), day 8 (n=3), and day 11 (n=2) samples. Day 6 DHP-R sorting trials did not result in any DHP-R positive viable cells (data not shown). Day 8 DHP-R sorting yielded a viable population of DHP-R positive cells that were enriched for GATA4 and cardiac α-actin compared to the negative fraction, after growing on Matrigel for two days, ($p<0.05$, n=3) (Fig. 3.12 Panel A). Day 8-sorted cells were also not enriched for MyoD, Pax3, Nestin or Mash1. No spontaneous beating was observed in the cultures, however cells did stain positive for MHC in the positive and unsorted fractions, (Fig. 3.12, Panel B), with a trend for a higher % MHC in the DHP-R positive fraction.

RNA was isolated from day 11 DHP-R sorted cells immediately after sorting and analyzed by QPCR (n=2) (Fig. 3.13). The DHP-R positive fraction showed a trend to be enriched for cardiac α-actin and GATA4, compared to the negative fraction, and did not

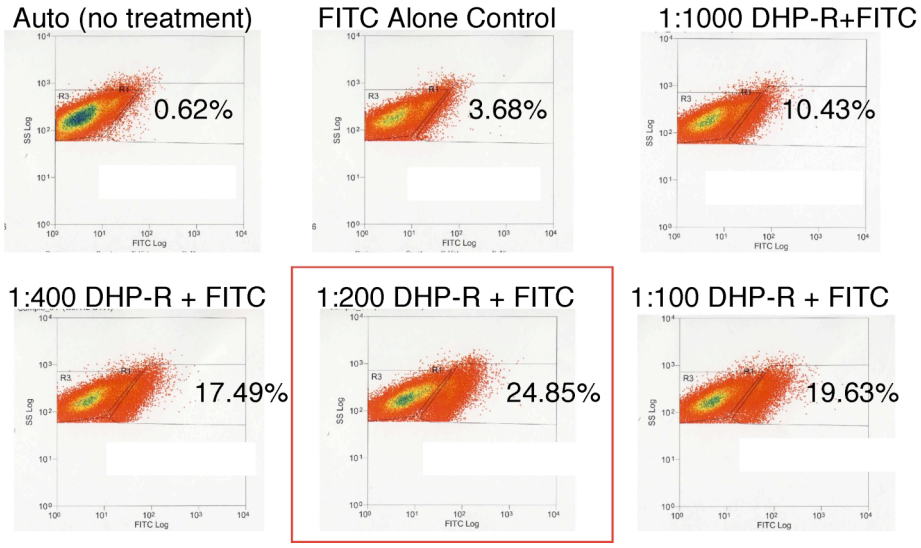


Figure 3.10 - Anti-DHP-R antibody staining revealed a distinct population of DHP-R positive mES cells from differentiated cultures.

Titration of DHP-R antibody for flow analysis and FACS in differentiated mES cultures was performed, and an increase in DHP-R positive cells compared to the FITC-alone control was observed as the concentration of DHP-R increased up to the 1:200 dilution (representative image, n=2).

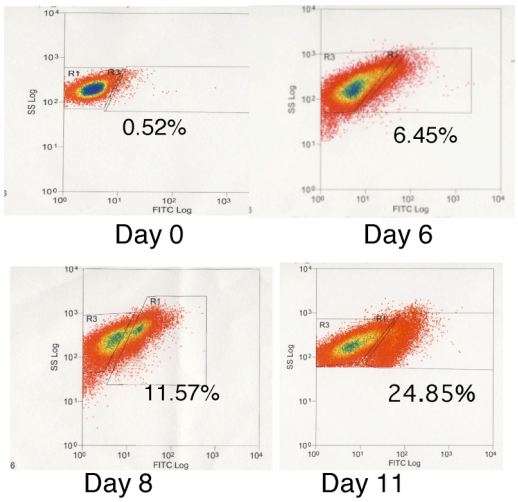


Figure 3.11 - An increase in DHP-R positive mES cells was observed as cardiomyocyte differentiation proceeded.

DHP-R positive cells were analyzed by flow cytometry using anti-DHP-R antibody staining on Day 0, 6, 8, and 11 of mES differentiation (representative image, n=2)

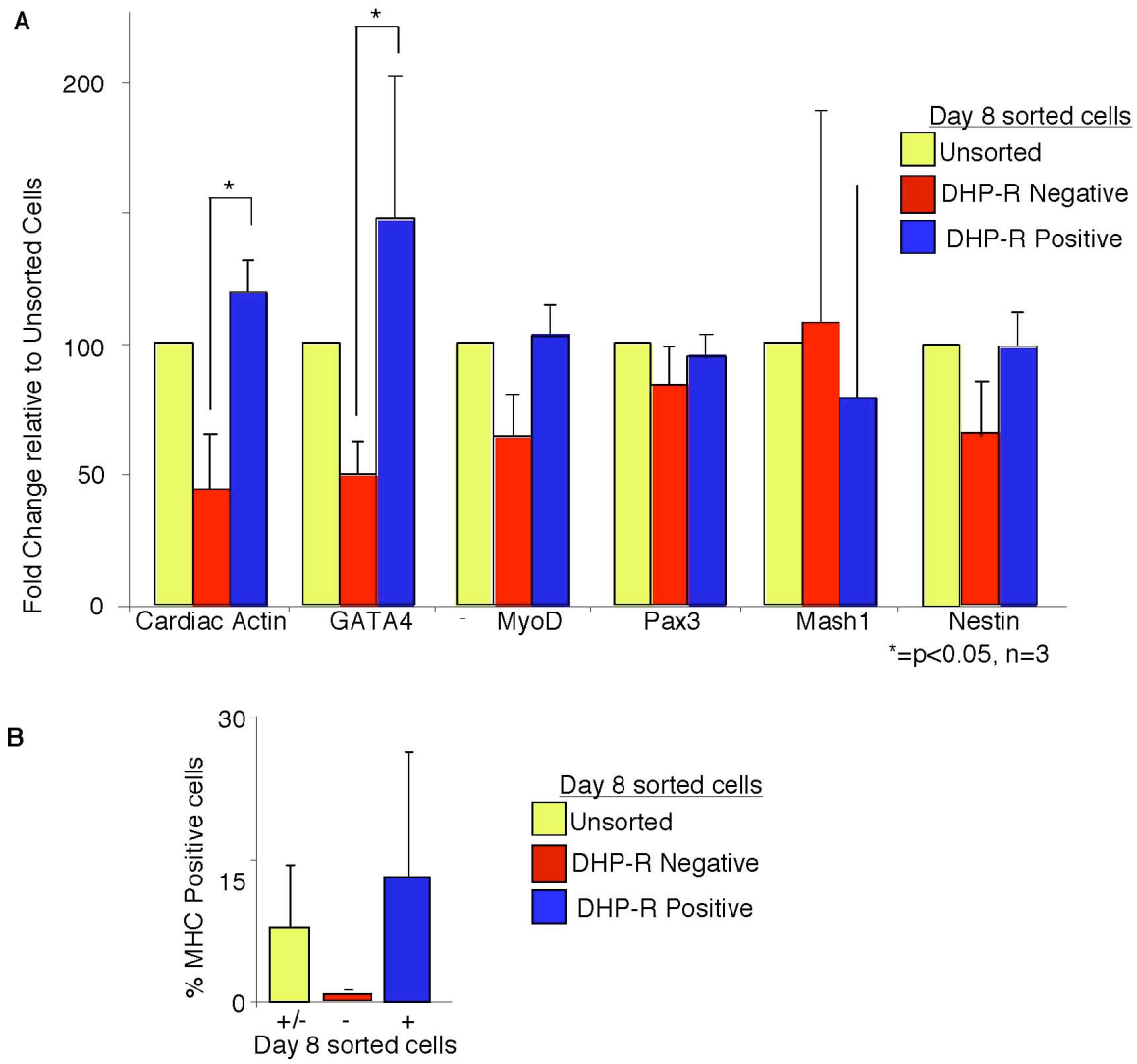


Figure 3.12 - DHP-R positive cells were enriched for GATA4 and cardiac α-actin when FACS was performed on Day 8 of differentiation.

MES cardiomyocyte cultures were FACS sorted on Day 8, plated on Matrigel for 2 days and analyzed for cardiac, skeletal and neuronal markers. RNA was isolated for QPCR analysis (A), * = $p<0.05$ compared to the DHP-R negative culture, but not the unsorted cells using individual student's t-tests. QPCR results are presented as % fold change relative to the unsorted control samples. Sorted and control cells growing on Matrigel-coated coverslips were stained for MHC and the percentage of MHC-positive cells was assessed by cell counting (B). n=3 independent trials.

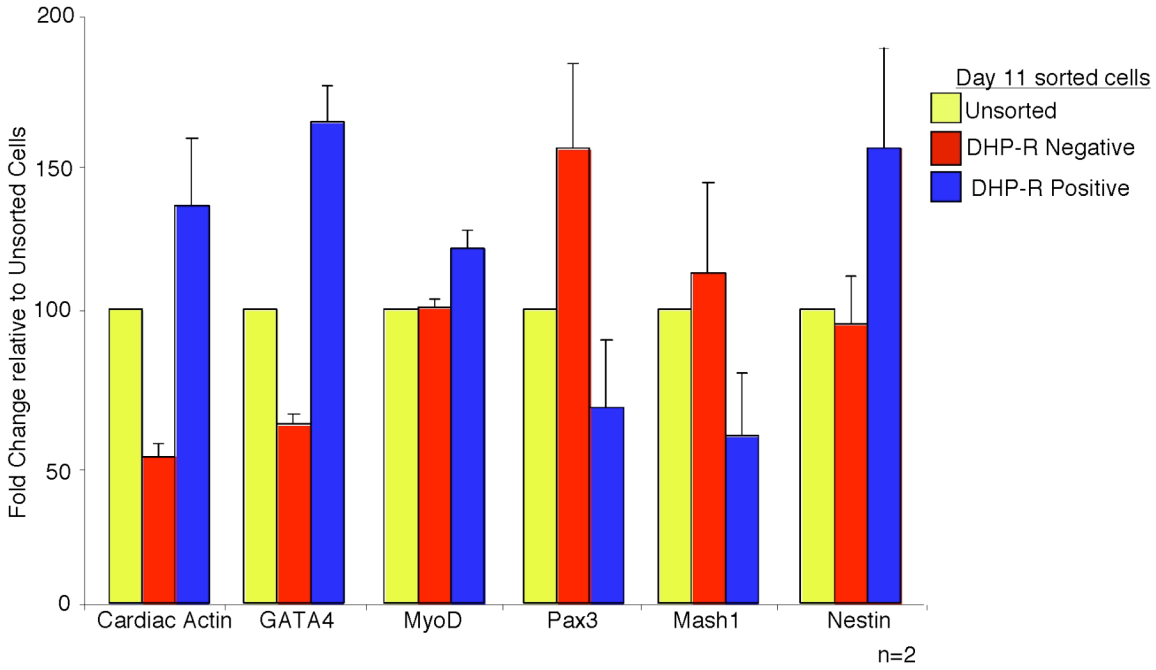


Figure 3.13 – A trend for GATA4 and cardiac α-actin enrichment was observed in the Day 11 DHP-R-positive fraction immediately following cell sorting.

DHP-R positive and negative cells were FACS sorted on Day 11, and RNA was extracted immediately after sorting and analyzed for the expression of cardiac, skeletal, and neuronal markers (n=2). QPCR results are presented as % fold change relative to the unsorted control samples.

appear to be enriched for MyoD, Pax3, Nestin, or Mash1 (n=2). Day 11 sorted cells that were grown for 2 days on Matrigel did not contain any viable cells (data not shown).

Thus, DHP-R sorting enriches differentiating mES cultures for cardiomyocytes. However, the poor viability of the cardiomyocytes hinders the overall success of the protocol.

3.5 - Discussion

Identifying mES-derived cardiomyocytes based on their expression pattern of cell surface markers may allow for a source of pure cardiomyocytes, thus decreasing the risk of teratoma formation or side effects from transplantation of a pluripotent or unwanted cell type. The novel use of DHP-R as a surface marker to enrich cardiomyocyte cultures in ES cells was investigated, and despite the observation that a large proportion of MHC-positive cardiomyocytes are not surviving the dissociation and cell sorting steps, DHP-R is a suitable cell surface protein to pursue for further testing and purification of ES-derived cardiomyocyte cultures.

DHP-R is involved in cardiac muscle development. The absence of DHP-R is embryonic lethal, with knockout mice dying by gestational day 14.5 (Seisenberger et al., 2000), and zebrafish knockout embryo's show lethal defects in the development of ventricular myocytes (Rottbauer et al., 2001). In our mES model, DHP-R transcript levels follow a similar temporal pattern to that of NKX2.5, GATA4, cardiac α-actin and MHC transcript up-regulation. This is in accord with previous studies indicating that DHP-R function is developmentally regulated in mES cell cardiomyogenesis (Kolossov et al., 1998). MES cardiomyogenesis has been well documented in the literature. In order to set a baseline level of cardiomyogenesis by which to compare potential enrichment, we first characterized

the pattern of gene expression, structural-related protein expression, and function of DHP-R in mES-derived cardiomyocytes. It should be noted that the timing and level of cardiomyogenesis is quite variable between mES cell lines and between laboratories, and also depends on the specific lot of serum used to supplement the media and the passage number of the cells.

Although DHP-R mES sorting did not give us a pure population of cardiomyocytes, the up-regulation of cardiac-related transcripts lead us to conclude that DHP-R can be used as a surface marker to enrich an ES cell population for cardiomyocytes. DHP-R expression is not specific to cardiomyocytes and DHP-R protein is present in many excitable cell types, including neurons and skeletal muscle as reviewed in (Moosmang et al., 2005). We found that the day 8-sorted mES cells were not enriched for skeletal or neuronal precursors, as demonstrated by the lack of up-regulation of skeletal or neuronal precursor transcript levels in the DHP-R positive sorted cells. Skeletal myogenesis in D3 mES cell cultures occurs at a later time point in differentiation compared to cardiomyogenesis, as MHC-positive skeletal myocytes are not visible by immunofluorescent staining until approximately days 12-20, ((Kennedy et al., 2009) and unpublished work). We hypothesize that by sorting on day 8 in mES differentiation, we see reduced contamination from skeletal muscle. Notably, DHP-R positive P19 cells, sorted on day 5 of differentiation, showed a trend for enrichment of both cardiac and skeletal markers. Skeletal myogenesis in P19 EC cultures proceeds faster than in mES cultures, with MHC-positive myocytes visible by immunofluorescent staining by day 9 of differentiation, as reviewed in (Skerjanc, 1999). Also, addition of cardiac muscle-specific signaling molecules, such as BMP-4 and activin A, should enhance overall cardiomyogenesis and inhibit skeletal myogenesis in P19 cells and ES cells (Kennedy et al., 2009; Laflamme et al., 2007). We predict that our documented experiment-to-experiment problems with

retrieving viable cells may explain our lack of success at growing the day 6 and 11 sorted mES cells.

Due to the lack of available cardiac-specific cell surface proteins identified to date, the most successful method of purifying ES-derived cardiomyocytes using surface markers is taking a lineage-restricting approach, by sorting cells based on their Flk-1/Kdr (vascular endothelial growth factor receptor 2) expression pattern (Kattman et al., 2011; Yang et al., 2008). Vascular, endothelial and cardiac progenitor cells are proposed to arise from Flk-1 expressing cells within the mesoderm (Kattman et al., 2006). Sorted human ES Flk-1 populations resulted in 57% cardiomyocytes (Yang et al., 2008), and Flk-1 positive mES cells improved heart function in an infarcted mouse model (Baba et al., 2007). Although ES-derived donor grafts containing cardiomyocytes and vascular progenitors improve cardiomyocyte graft survival and vascularization within the host (Stevens et al., 2009), Flk-1 positive cardiomyocytes were shown to be more effective than Flk-1 vascular progenitors at improving myocardiac function following an infarct (Adler et al., 2010). Since Flk-1 and DHP-R selection are lineage restricting and not cardiomyocyte specific approaches, in order to increase the purity of the sorted cells, we propose to use DHP-R sorting in combination with Flk-1 sorting strategies.

The death of dissociated ES-derived cardiomyocytes from stem cells is a common occurrence in the literature, as reviewed in (Robey et al., 2008), and is a major limitation of this approach. We attempted to first circumvent this problem by testing various cell-dissociation agents in order find a protocol that would permit reproducible dissociation of a high yield of viable cardiomyocytes. The non-enzymatic CDB appeared to be the best candidate tested. CDB is an EDTA-based buffer, and functions by chelating calcium and magnesium, releasing the cells from their calcium-dependent cadherin and integrin-based

interactions, as reviewed in (Wary, 2005). Unlike the digestive enzyme trypsin, which is routinely used to dissociate monolayer mES cells for passage or for differentiation, using non-enzymatic buffers reduces the risk of degradation of membrane surface markers or ligands that may be important for antibody binding during the FACS labeling process. Regardless of the protocol we followed to increase the amount of viable cells after dissociation, we found that a minimum of 20-30% of the total cells did not survive the cell dissociation step. When this cell loss figure is compounded with an additional 40-80% of cells lost due to the pressures of cell sorting as reported in the literature (Dainiak et al., 2007; Emre et al., 2010), the importance of developing techniques to preserve cells is imperative.

Dissociating and purifying viable cardiomyocytes from ES cells is not a simple task, yet it may be imperative in order to progress to the clinical stage and deliver hES-derived cardiomoycytes to patients. Cardiomyocytes are dependent on their cell-cell and extracellular matrix interactions for survival, and thus are also sensitive to disruption, as reviewed in (Hattori and Fukuda, 2010; Robey et al., 2008). Strategies improving cardiomyocyte survival have been reported, and include reducing cardiomyocyte loss during transplantation by using a mixture of anti-apoptotic factors and prosurvival factors (Laflamme et al., 2007), treating dissociated cardiomyocyte cultures with a ROCK inhibitor (Braam et al., 2010), protecting cardiomyocytes from oxidative stress using isoflurane anesthetic preconditioning (Sepac et al., 2010), co-culturing dissociated cardiomyocytes with embryonic fibroblasts to improve attachment to substrates and EB formation (Pfannkuche et al., 2010), re-aggregating the cells following dissociation and cell sorting (Hattori et al., 2009; Hattori and Fukuda, 2010), and heat shock treatment conditioning (Zhang et al., 2001). Unlike previously published observations using hES-derived cardiomyocytes (Braam et al., 2010; Laflamme et al., 2007), in our model, ROCK inhibitor treatment and PSC

supplementation did not improve mES cardiomyocyte survival following dissociation. Potential explanations for these discrepancies include differences in the potency or activity of the PSC or of ROCK inhibitor between mES and hES models, as both ROCK inhibitor and the PSC have been published using hES cell models. Re-aggregating dissociated cardiomyocyte cultures to form EBs has also been suggested as a means of reducing cardiomyocyte loss (Hattori et al., 2009; Hattori and Fukuda, 2010), and there is a possibility that a combination of supplementation with PSC and/or ROCK inhibitor along with re-aggregation may improve mES-derived cardiomyocyte survival. The published PSC was shown to improve the survival of hES-derived cardiomyocytes when delivered *in vivo* (Laflamme et al., 2007), and cells were suspended directly into the Matrigel-PSC mix for injection onto damaged rat hearts. In order to test the PSC in our mES *in vitro* model, we modified the PSC procedure by plating the cells onto gelled Matrigel-PSC using PSC supplemented growth media. We cannot rule out the possibility that suspending the ES-derived cardiomyocytes within the Matrigel-PSC may have resulted in reduced cell death following dissociation.

In our cardiomyocyte dissociation studies, despite a decrease in MHC-positive cardiomyocytes following dissociation, the transcript levels of the early cardiac muscle development-related transcription factors Nkx2.5 and GATA4 were not observed to decrease. This may indicate that cardiomyocyte-progenitor cells, not yet differentiated to express MHC and cardiac α-actin, may survive the dissociation stage. We are interested in further investigating the viability of cardiomyocyte progenitor cells following dissociation, and applying these findings to purification strategies targeted at progenitor populations. Functional L-type calcium channel activity has been detected as early as day 7 of mES cardiomyocyte differentiation (Kolossov et al., 1998), and DHP-R protein is also evident in

the mouse embryonic heart tube starting on embryonic day (ED) 9.5, and becoming stronger by ED 10.5 (Acosta et al., 2004). In our mES studies, trends of DHP-R RNA transcript level increases are detectible as early as day 4 of mES cardiomyogenesis. DHP-R therefore can be considered a candidate for cell surface purifying cardiomyocyte progenitor cells.

In summary, we tested the novel use of DHP-R surface staining for the enrichment of mES-derived cardiomyocytes by FACS. We propose to use DHP-R positive cell selection in conjunction with other lineage-restricting cell sorting approaches in order to obtain a pure population of ES-derived cardiomyocytes. DHP-R co-sorting, when combined with tactics to increase cardiomyocyte survival, such as using cardiomyocyte progenitors with an optimized anti-apoptotic supplementation regime, may yield a highly pure population of viable cardiomyocytes for *in vivo* delivery.

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CHAPTER 4:

Collagen (+RGD and –RGD) scaffolds support cardiomyogenesis after aggregation of mouse embryonic stem cells

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4.1 – Abstract

Cell-based cardiac muscle repair is an attractive prospective treatment for patients suffering from heart disease. Tissue engineered scaffolds containing mouse embryonic stem (mES) cell-derived cardiomyocytes could allow for *in vitro* development of the tissue substitute prior to implantation. The objectives of this study were to determine the optimal seeding conditions and to characterize mES cell-derived cardiomyocytes growing on collagen I/III scaffolds, modified with the adhesion peptides arginine–glycine–aspartic acid–serine (RGD). Unaggregated mES cells and cells aggregated to form embryoid bodies (EBs) for 3 or 5 days were seeded onto the collagen (+RGD and –RGD) scaffolds or onto gelatin control substrates. On day 10, unaggregated mES cells or 3-day old EBs grown on any of the three substrates failed to differentiate into beating cardiomyocytes. In contrast, when 5-day old mES-derived EBs were seeded onto the collagen scaffolds or gelatin-coated control plates, the cells differentiated efficiently into beating cardiomyocytes. QPCR analysis and immunofluorescent staining on day 10 showed that cardiomyocytes expressed cardiac muscle-related transcripts and proteins, respectively. Analysis of cardiomyocytes by electron microscopy identified muscle fiber bundles and Z bands, typical of ES-derived cardiomyocytes. The mES-derived cardiomyocytes growing on the collagen scaffolds contained larger beating areas with a slower beating rate as compared to those grown on gelatin. These results indicate that a collagen I/III scaffold supports cardiac muscle development and function after 5 days of EB formation, and that this scaffold appears suitable for future *in vivo* testing.

4.2 - Introduction:

Myocardial infarction leads to loss of functional cardiac muscle (cardiomyocytes). Over the years, the well-described morbidity and mortality of systolic heart failure due to cardiomyocyte loss has led to significant interest in the development of cell therapy treatment options [1].

Evidence thus far suggests that embryonic stem (ES) cell-derived cardiomyocytes or cardiomyocyte progenitor cells can repair damaged myocardium in animals; in this regard, human ES cells differentiated into cardiomyocytes have been shown to improve function in rodent hearts [2-4], and monkey ES cell-derived cardiomyoyctes can replace 20% of a cardiac scar in a non-human primate model [5]. Mouse ES (mES) cells pre-differentiated into cardiomyocytes contributed to cardiac repair in a rat infarct model [6], and sheep model [7]. While injecting cell suspensions alone or with a growth factor cocktail may seem attractive, recent clinical studies using non-embryonic cell sources have reported that the majority of injected cells dissipate away from the site of interest, and have cell survival issues [8, 9].

The use of a tissue engineered scaffold, with implanted ES cell-derived cardiomyocytes, acts as a tissue mimic substitute in which cells can proliferate and differentiate in a controlled manner prior to implantation. Three-dimensional biological based scaffolds to aid in the delivery of cardiomyocytes to the heart have been well reviewed in the literature [10-16]. Cardiac scaffolds may contain specific chemical and structural information that control tissue formation in a manner mimicking cell-cell communication and patterning during embryonic development, as reviewed in [17]. Common difficulties with tissue engineered patches include vascularization, the structural design and strength of the implant, host-immunoreactivity, and functional integration of the donor cells, as reviewed in [12].

Numerous naturally-derived biomaterials have undergone *in vitro* and *in vivo* testing for their use in cardiac tissue engineering. Studies have shown that incorporating integrin binding domains into biomaterials may improve cell adhesion, survival and differentiation of some cell types, as reviewed in [18]. Different attempts have been made to modify biomaterials with the adhesion peptides arginine– glycine– aspartic acid– serine (RGD) for cardiac tissue engineering applications [19-30]. The RGD motif is effective in enhancing contractility and cell adhesion in primary cultures of rodent neonatal cardiomyocytes [24-26], although the context of the RGD motif is important in soliciting a maximal biological response in a variety of cell types [18, 22, 23, 31]. Enhanced cardiomyogenesis in the presence of RGD was observed in P19 embryonal carcinoma (EC) cells after cells formed aggregates in a dextran-based hydrogel [21]. However, the effect of the RGD motif on mES cell cardiomyogenesis has not yet been examined.

Collagen based substrates are commonly used in cardiac tissue engineering applications, as reviewed in [15, 17]. It was recently observed that a clinically approved cross linked collagen I/III sponge, Avitene Ultrafoam (CR Bard), modified to present the RGD domain, supported neonatal cardiomyocyte contractility and improved cardiomyocyte viability [25]. Collagen type I and III are components of native cardiac muscle extracellular matrix (ECM), and play an important role in contractility and structural integrity in the myocardium [32]. Collagen is an effective biomaterial for cardiac tissue engineering, supporting cell attachment and beating of mES-derived cardiomyocytes [33-35]. In the present study we determined appropriate conditions for the formation of functional mES-derived cardiomyocytes on collagen I/III scaffolds. These scaffolds are relatively easy to functionalize [25], easy to manipulate with forceps and handle in culture, have a porous structure suitable for the diffusion of nutrients and penetration of blood vessels [36, 37], and

are approved for use in humans as a hemostat. In particular, we tested if growth on the collagen scaffold could bypass the requirement for embryoid body formation and if the presence of the RGD motif could enhance cardiomyogenesis. This is the first study looking at the ability of collagen scaffolds containing RGD to support cardiomyocyte formation in mES cells.

4.3 - Materials and Methods

4.3.1 Collagen (+RGD and –RGD) scaffolds:

Coupling of the RGD adhesion peptides to the collagen scaffolds and the specificity of the peptide interactions are described in detail in [25]. Briefly, commercially available Avitene Ultrafoam sheets (CR Bard, Murray Hill New Jersey) were re-hydrated in phosphate-buffered saline (PBS) solution. Linear glycine-arginine-glycine-aspartic actin-serine peptides (GRGDS, Sigma Aldridge, Lyon France) were covalently bound to the collagen scaffolds using 20 mM Sulfosuccinimidyl 6- [3-(2-pyridyldithio)-propionamido] hexonate (Sulfo-LC-SPDP, Pierce Biochemical Rockford, IL). Scaffolds were washed with PBS and reduced with 24 mg/ml dithiothreitol to expose the free sulfhydryl groups. To prepare the RGD peptides, Sulfo-LC-SPDP was coupled at the N-terminal glycine residue in GRGDS, and a pyridyldithio group was added. The reduced SPDP- collagen scaffold was finally mixed with pyridyldithio-RGD peptides for 48 hours in PBS. The coupling process was monitored spectrophotometrically by measuring the release of pyridine-2-thione into the solution, as described [25]. Scaffolds containing 1 mg of GRGDS per 10 mg of collagen were used in this study and are denoted as “collagen +RGD”. Non-modified scaffolds (“collagen -RGD”) and tissue culture dishes coated with 0.1% gelatin were used as control substrates.

4.3.2 - Stem Cell Culture:

4.3.2.1 Undifferentiated (unaggregated) mES cells:

D3 mESC cells were obtained from the ATTC Biological Resource Center (Manassas, Virginia), and differentiated following the protocol suggested in [38]. Unaggregated cells were grown in complete media consisting of high glucose Dulbecco's modified eagle media (DMEM) (Gibco) containing 15% fetal bovine serum (Wisent), 0.1 mM each of non-essential amino acids (Gibco), 0.1 mM ß-mercaptoethanol (Sigma), 100 ug/ml penicillin and strepamycin (Invitrogen), and 1000 U/mL leukemia inhibitory factor (LIF) (Chemicon).

4.3.2.2 Cardiomyocyte formation (EB formation):

Cardiomyocyte formation was induced by growing the mES cells in suspension culture conditions, in the absence of LIF, to form embryoid bodies (EBs). Cells growing in monolayer (termed day 0) were brought into single cell suspension by treating for 5 minutes with 1x trypsin-EDTA (Invitrogen), and re-suspending in complete media, without LIF. Cells were grown for 2 days in hanging drops (800 cells / 20 ul drop), and then transferred into a petri dish to grow in suspension for an additional 3 days (termed 5-day old EBs). On day 5 of differentiation, EBs were plated onto gelatin-coated dishes or scaffolds and examined 5 days later, on day 10 of the differentiation time course. An overview of the experimental timeline is shown in Figure 4.1.

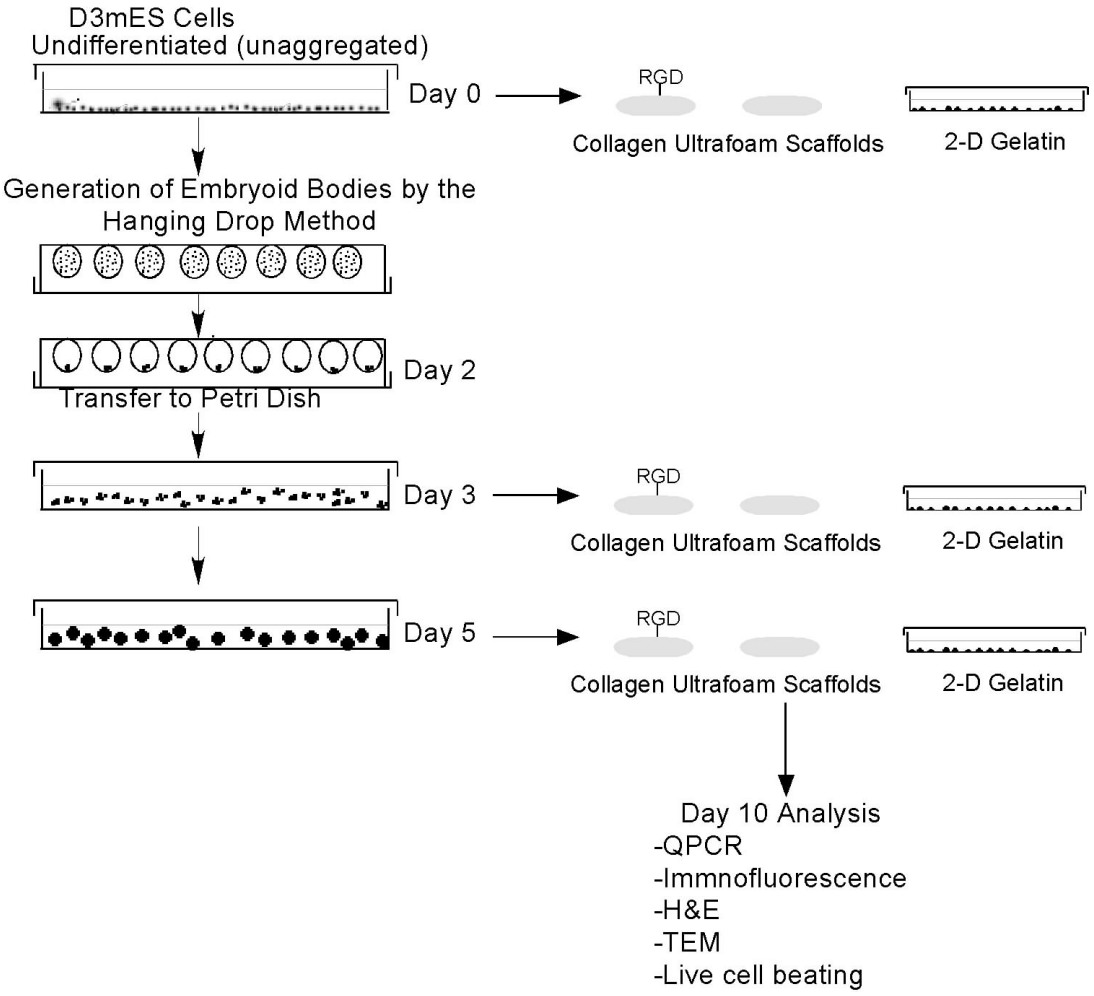

Figure 4.1 - Overview of the timeline of cell seeding on the scaffolds and gelatin control.

4.3.2.3 - Dissociation of 3-day old differentiating EBs:

EBs were dissociated after 3 days in suspension (termed 3-day old EBs) by using a commercially available non-enzymatic EDTA-based cell dissociation buffer (Gibco), for 15 minutes at 37^{o}C. Cells were then filtered using a 40 um sterile cell filter, and seeded onto the scaffolds or gelatin-coated dishes and examined 7 days later on day 10 of differentiation.

4.3.3 - Cellular Viability:

Live and dead cells were quantified by using Calcein AM and ethidium homodimer as per manufacturers instructions (Live/Dead viability and cytotoxicity kit, Invitrogen). Scaffolds and control gelatin coverslips were washed with 1x PBS and incubated for 30 minutes at 37°C with 2 uM Calcein AM, 4 uM ethidium homodimer, and 1:1000 Hoechst 33258. Fluorescent images of the collagen (+ and -RGD) scaffolds, and gelatin were obtained using a Nikon microscope. Eight images from each substrate were acquired and counted using ImageJ software (NIH) (n=3).

4.3.4 - Quantitative Polymerase Chain Reaction (QPCR):

QPCR was performed to quantify changes in gene expression of the cardiac muscle genes Nkx2.5, GATA4, MHC (myosin heavy chain), atrial natriuretic peptide (ANP), connexin 43, and cardiac α-actin, skeletal muscle precursor genes, Meox1 and Pax3, and the internal control GAPDH (glyceraldehyde 3-phosphate dehydrogenase). Primer sequences are given in Table 1. RNA was isolated and purified using the RNeasy mini RNA isolation kit (Qiagen), from cultures grown on scaffolds or controls until day 10. Cells were first liberated from the scaffolds by using Collagenase Type II (Roche) digestion for 30 minutes at 37°C. cDNAs were generated using the Quantitect Reverse Transcription kit (Qiagen),

including DNAse I treatment. QPCR was performed using FastStart SYBR Green with ROX (Roche Applied Sciences), following manufacturer's suggestions in a 25 ul final reaction volume. PCR amplification was performed using an ABI 7300 thermocycler (Applied Biosystems) as described within [39] The primers have been previously validated for their efficiency of amplification under these conditions, including a melt curve analysis and by comparison with results from Northern Blot analysis. Relative fold differences in expression of these genes between the collagen (+RGD or -RGD) scaffolds and the gelatin were calculated using the comparative CT method [40]. Briefly, the difference in cycle time ΔΔCT, was calculated by normalizing the values relative to the internal reference gene, GAPDH. ΔΔCT was obtained by finding the difference between the differentiated mES cells growing on the three different substrates (targets) and the control unaggregated mES cells (baseline). The equation ΔΔCT = ΔCT(target) - ΔCT(baseline) was used. Finally, the equation 2exp(-ΔΔCT) was used to calculate the fold change (RQ) value. Controls included a no cDNA (water only) control for each primer combination, and also a cDNA reaction for each RNA sample that did not contain the reverse transcriptase enzyme to ensure that there was no DNA contamination prior to cDNA synthesis. All reactions were performed in duplicate, and the results shown are the average +/- standard error of measurement (SEM) of three independent experiments.

Table 4.1 - Primers and their respective references used in QPCR reactions.

Gene of Interest	Forward Primer	Reverse Primer	Reference
MHC	ACAACCCCTACGATTATGCGT	ACGTTCAAAGGCACTATCCGTG	[56]
GATA4	AAACGGAAGCCCAAGAACCT	TGCTAGTGGCATTGCTGGAGT	[57]
ANP	ACTAGGCTGCAACAGCTTCC	TGACACACCACAAGGGCTTA	[58]
Cardiac α-actin	CTGGTATTGCCGATCGTATG	CTTGCTGATCCACATTTGCT	[56]
Connexin 43	CCTGCCGCAATTACAACAAG	AAGGTCGCTGATCCACGATA	[59]
GAPDH	AGGTCGGTGTGAACGGATTTG	TGTAGACCATGTAGTTGAGGTCA	[56]
Nkx 2.5	AAGCAACAGCGGTACCTGTC	GCTGTCGCTTGCACTTGTAG	NIH Pimer3

4.3.5 - Histology and immunofluorescence:

Scaffolds were fixed in PFA-PBS for 30 minutes at room temperature, washed two times with PBS and incubated in 20% sucrose overnight in preparation for cryopreservation. Scaffolds were snap frozen in OCT using isopentane and liquid nitrogen, and serial cryosections were processed for histological and immunofluorescence analysis. Hematoxylin and eosin (H&E) staining was performed as per standard protocol for morphological analysis of the constructs. Slides were dehydrated with alcohol, cleared in xylene, mounted with a coverslip, and visualized with a Nikon microscope. Serial cryosections of the scaffolds were fixed in -20°C methanol, and washed in stockholm's PBS (sPBS). Immunofluorescence was performed by incubating the sections with the hybridoma antibody anti-myosin heavy chain, MF20 (Developmental Studies Hybridoma Bank, University of Iowa), at room temperature for 1 hour. Sections were then washed with PBS and incubated in a 1:200 dilution of goat anti-mouse Cy3-linked secondary antibody in PBS (Jackson ImmunoResearch Laboratories, West Grove, PA) for 1 hour at room temperature. The coverslips were washed, mounted with 1:1000 Hoechst nuclear stain, and the fluorescence was detected using a Nikon microscope. For the gelatin controls, EBs growing on gelatin coated coverslips were fixed on day 10 using -20^{o}C methanol and stained for MHC using the MF20 hybridoma antibody as described above.

4.3.6 - Video analysis and calculation of the spontaneous beating area:

Spontaneous beating was recorded at 20 frames per second using phase contrast microscopy on a Nikon microscope. The number of spontaneous beats per minute were counted for cardiomyocytes grown on the scaffolds and the gelatin control. The beating

areas were measured in each field of view using ImageJ software (NIH), and the percentage of beating areas were calculated based on the total area in the field of view.

4.3.7 - Transmission Electron Microscopy (TEM):

Beating cardiomyocyte scaffolds were fixed using 2% glutaraldehyde in 1 x PBS for 24 hours, post-fixed in 1% osmium tetroxide, "en bloc" stained in 3% uranyl acetate, dehydrated in an ascending alcohol series, and embedded in Spurr epoxy resin. Thin sections were cut on Reichert OMU3 ultramicrotome and stained with lead citrate. Samples were examined and images were acquired using a JOEL 1230 TEM with Advanced Microscopy Techniques (AMT) software.

4.3.8 - Statistical Analyses:

Values are expressed as mean +/- standard error (SE) of the mean. Microsoft Excel was used to perform statistical analyses. Individual comparisons between two groups were performed using two-tailed Student's t-tests. $p < 0.05$ probability values were considered statistically significant.

4.4 - Results

4.4.1 - Assessment of cellular viability of mES cells in the collagen scaffolds:

To test the compatibility of the scaffolds as a biomaterial to sustain mES-derived cardiomyocyte cultures, live/dead staining was performed after 5 days of cell growth on the scaffolds. As shown in Figure 4.2, when 5-day old EBs were seeded onto the collagen (+RGD or –RGD) scaffolds or gelatin-coated control plates and grown until day 10, all three

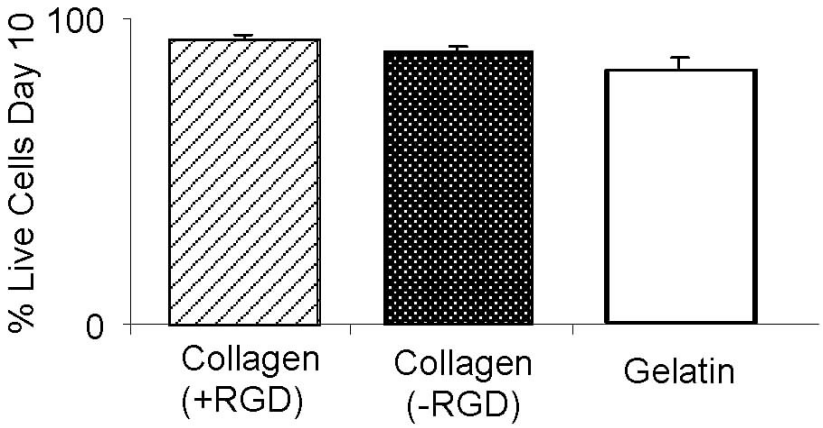

Figure 4.2 - Cells were viable on the Collagen (+ and - RGD) substrates, and cells growing on the RGD+ scaffolds had a higher viability compared to the gelatin controls.

Mouse ES cells were aggregated for 5 days and resulting EBs were seeded on either collagen scaffolds (+ or – RGD) or gelatin, and examined 5 days later to assess cell survival.

biomaterials were able to support mES cell survival. No significance differences were detected between the collagen +RGD, –RGD, or the gelatin controls. Cells growing on both types of scaffolds sustained a high percentage of viable cells.

4.4.2 - Determination of the ability of the scaffolds to support cardiomyocyte development from unaggregated mES cells or 3-day old EBs.

The ability of the scaffolds to replace the requirement for 5 days of EB formation for efficient cardiomyogenesis was examined. Unaggregated (Day 0) cells or 3-day old EBs, which are partially differentiated but not yet expressing high levels of cardiac muscle-related genes, were dissociated and seeded onto the scaffolds or gelatin-coated control dishes. On day 10 of differentiation, spontaneous beating was not observed in any samples (data not shown). RNA was extracted and analyzed for the presence of Nkx2.5, GATA4, cardiac α actin, MHC, Atrial Natriuretic Peptide (ANP), and Connexin 43. On day 10 of differentiation, neither the unaggregated cells nor the 3-day old EBs growing on scaffolds showed any significant changes in the level of cardiac muscle-related transcription factors as compared to cells grown on the gelatin controls (Figs. 4.3 and 4.4, respectively). Notably, although neither the collagen scaffolds nor the gelatin coating was sufficient to induce differentiation to functional cardiomyocytes, there was an up-regulation by day 10 of differentiation of GATA4, MHC, and cardiac α actin from 3-day old EBs compared to the unaggregated Day 0 cells. Substrates examined on day 10 after seeding with 3-day old EBs showed an average increase of 4 +/- 2 fold in transcript levels of GATA4, MHC and cardiac α-actin compared to substrates seeded with unaggregated cells (Fig 4.4). Therefore, the

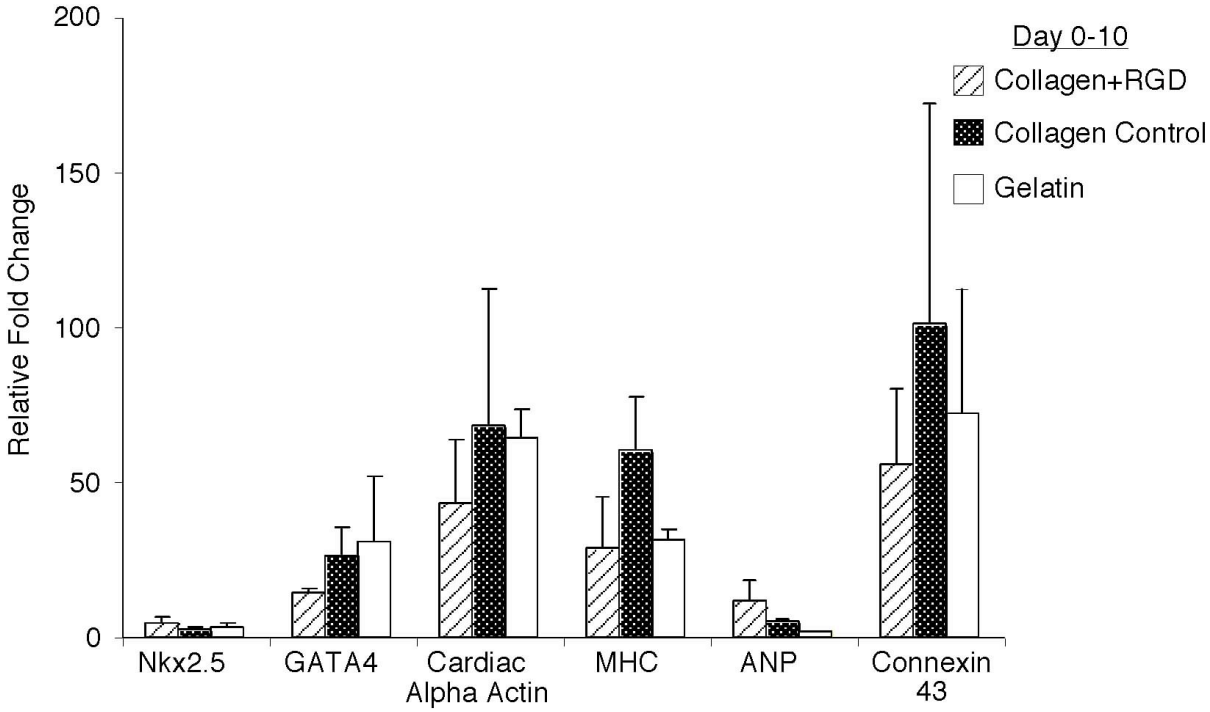

Figure 4.3 - Undifferentiated cells (Day 0) seeded on the scaffolds expressed low levels of cardiac muscle genes.

Proliferating mES cells were dissociated and seeded onto the collagen scaffolds on Day 0 (undifferentiated cells), in the absence of LIF. RNA was extracted on Day 10 of differentiation to assess the cultures for the presence of cardiac muscle gene expression by QPCR, using the primers indicated (n=3). Results are presented as fold change relative to undifferentiated cells. A two-tailed Student's t-test was performed and no significant change in transcript level was detected for samples grown on the scaffolds compared to unsorted control cells ($p>0.05$).

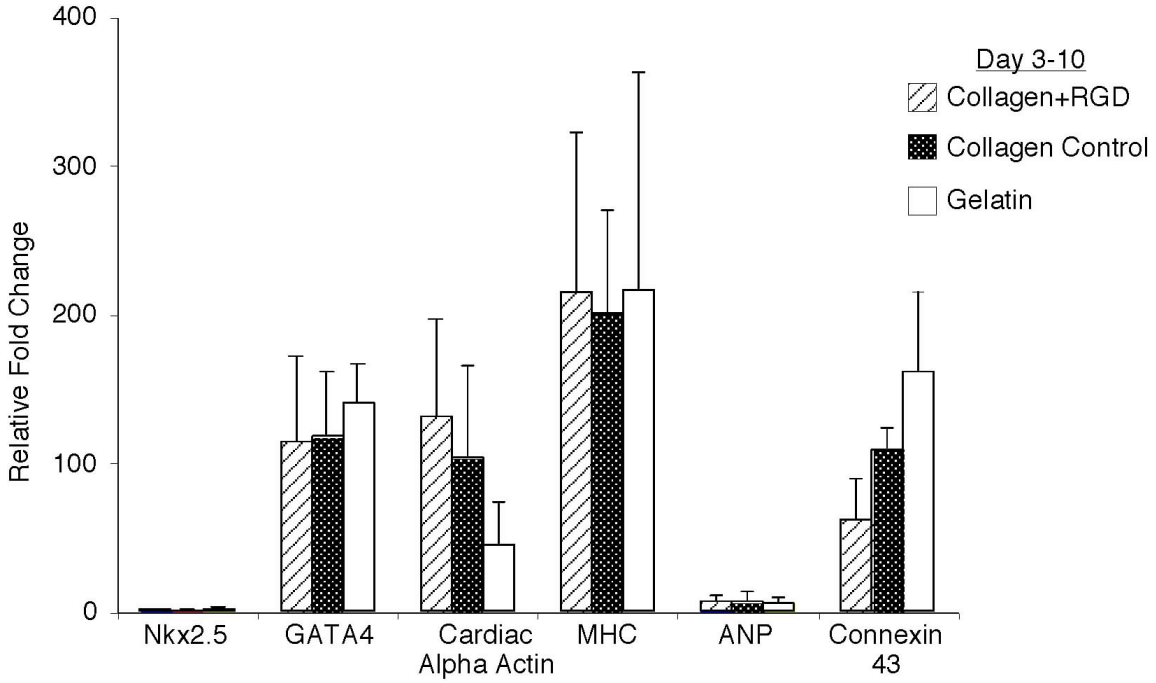

Figure 4.4. - Scaffolds and controls seeded with 3-day old EBs expressed low levels of cardiac muscle genes.

MES cells were differentiated in suspension culture conditions until Day 3 of differentiation and seeded onto the Collagen (+ RGD) and (– RGD) scaffolds or the gelatin control. RNA was extracted on Day 10 of differentiation and examined for the expression of cardiac muscle genes by QPCR, as indicated (n=3). Results are presented as fold change relative to undifferentiated cells. A two-tailed Student's t-test was performed and no significant change in transcript level was detected for samples grown on the scaffolds compared to unsorted control cells ($p>0.05$).

collagen scaffolds and the gelatin control substrates were not able to support the efficient differentiation of mES cells, aggregated for 3 days or less, into beating cardiac muscle.

4.4.3 - Seeding 5-day old EBs onto collagen scaffolds resulted in efficient cardiomyogenesis:

Following 5 days in suspension culture, EBs were seeded directly onto the scaffolds without dissociation. H&E staining was performed on cryosectioned scaffolds and gelatin-coated control plates on day 10 of differentiation, to qualitatively assess the morphology of the cells growing on the scaffolds (Fig. 4.5 panels A-E). The cells adhered and migrated within the collagen scaffold pores, showing that, similar to gelatin, the cells did not remain as aggregates once plated onto the collagen scaffold

RNA was harvested and analyzed on day 10 of differentiation, from the 5-day old EBs that were seeded on scaffolds or gelatin control plates. The collagen +RGD scaffolds maintained a similar high level of MHC, Nkx2.5, GATA4, and cardiac α-actin transcripts compared to –RGD collagen scaffolds and the gelatin control plates (Figure 4.6, panel A). When compared to the scaffolds seeded with unaggregated mES cells (as reported in 3.2), there was a 12 +/- 6 fold increase in the transcript levels for MHC, cardiac α actin, and Nkx2.5. However for ANP, GATA4, and Connexin 43, there was no enhancement (results are plotted together for comparison of the three cell seeding protocols and presented in Supplementary Figure 4.1). Surprisingly, GATA4 transcript levels were notably higher in the 3-day aggregation protocol. Therefore, similar increases in cardiac muscle-related gene expression were identified for 5-day EBs plated onto the collagen scaffolds, with or without RGD, and the gelatin-coated control plates.

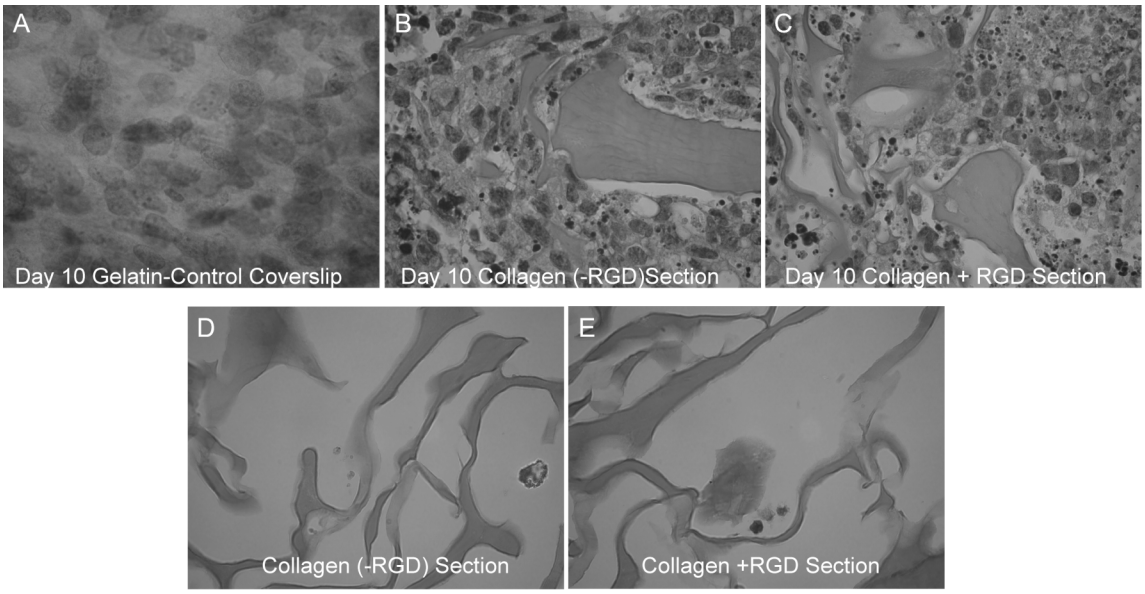

Figure 4.5 - Cells from 5-day old mEBs were able to migrate and integrate within Collagen (+ and - RGD) scaffolds.

Scaffolds and gelatin were seeded with 5-day old mEBs. Gelatin coverslips were fixed on day 10 and H&E staining was performed (A). Scaffolds were cryosectioned on day 10, and the morphology of the cells was analyzed by H&E staining (B,C). In order to identify the collagen scaffolds, scaffolds that did not contain cells were sectioned and stained as well (D,E).

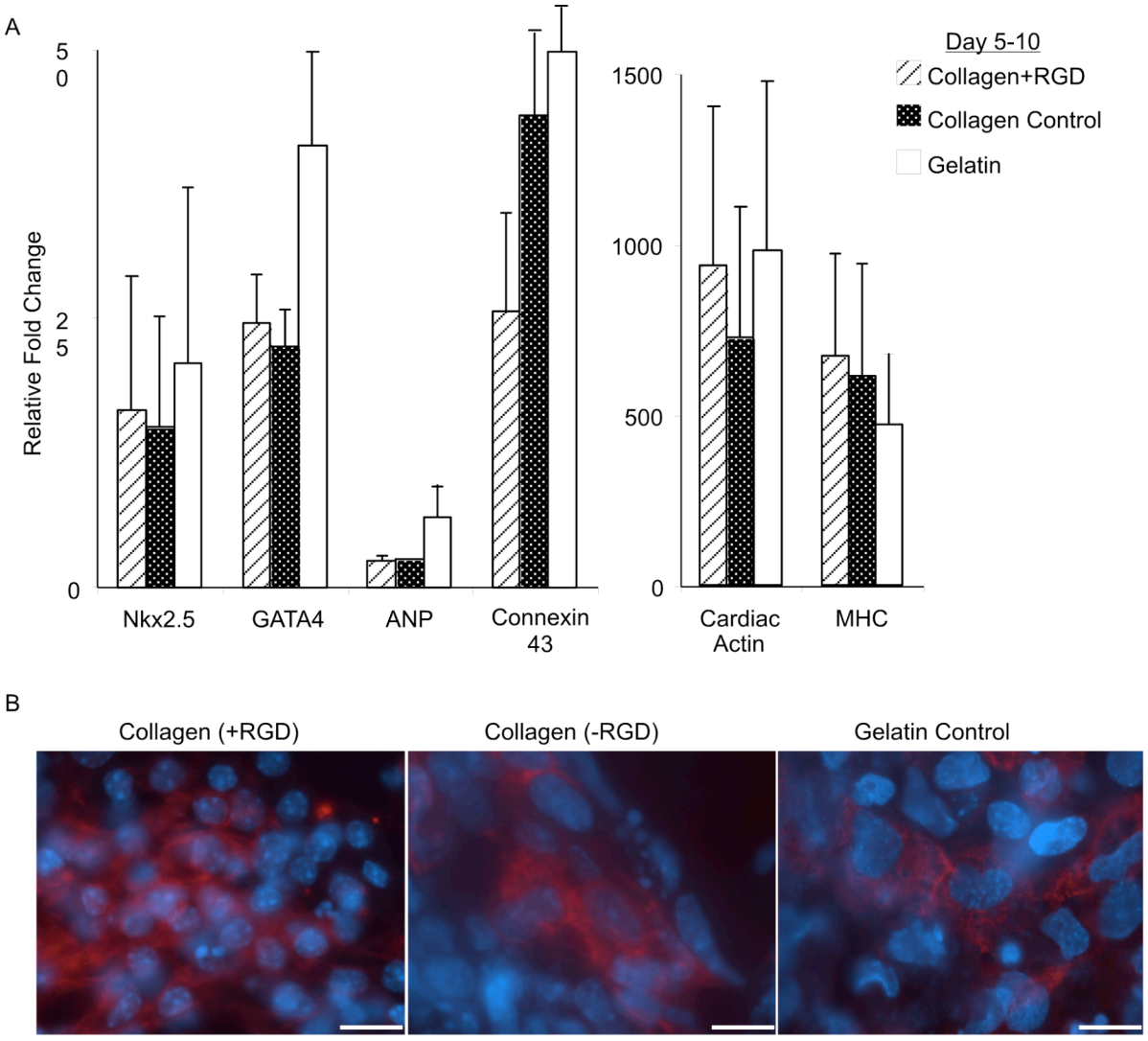

Figure 4.6 - Collagen (+ and - RGD) scaffolds seeded with 5-day old EBs maintained their level of cardiac muscle development compared to gelatin controls.

The three substrates were seeded with 5-day old EBs and analyzed on Day 10 of differentiation by QPCR with the indicated genes (Panel A, n=3). QPCR results are presented as fold change relative to undifferentiated cells. A two-tailed Student's t-test was performed and no significant change in transcript level was detected for samples grown on the scaffolds compared to unsorted control cells ($p>0.05$). Panel B. Immunohistochemistry with an anti-MHC antibody (red) and Hoechst nuclear stain (blue) was performed on cryosections of the scaffolds and control gelatin coated coverslips, revealing MHC-positive clusters of cells growing within the scaffolds and controls. Scale bars = 20μm.

Immunofluorescent staining on day 10 of differentiation revealed that the 5-day EB-derived cardiomyocytes growing on the scaffolds expressed MHC (Fig. 4.6, Panel B), and displayed a rounded or elongated mononuclear morphology with large nuclei. The 5-day EB-derived cardiomyocytes grown on the +RGD scaffold were further assessed at the ultra structural level by TEM. The cardiomyocytes contained sarcomeric components, including organized bundles of myofibrils and Z bands (Figure 4.7, panel A-E), as expected for ES cell-derived cardiomyocytes as demonstrated previously in the literature [41]. Cardiomyocytes growing on the scaffolds appeared to have a varying degree of maturity of sarcomeric structures. Myofiber organization in the mES-derived cardiomyocytes ranged from disorganized myofibers (Fig. 4.7 Panel A, B), to more organized myofibers and Z bands (Fig 4.7. Panel C-E), and this is also consistent with published observations of mES-cell derived cardiomyocytes growing in culture [41]. An electron microscopic image of the collagen I/III scaffold alone (Collagen) is shown in Figure 4.7, Panel F.

In summary, 5 day-old EBs growing on the scaffolds until Day 10 of differentiation integrated into the scaffolds and developed into embryonic cardiomyocytes, as assessed by H&E staining, QPCR analysis of the cardiac-related gene up-regulation, MHC immunohistochemistry, and the presence of organized myofibers and Z bands by TEM.

4.4.4 - Cardiomyocytes beat slower on collagen scaffolds compared to gelatin control substrates

Spontaneous beating of the mES-derived cardiomyocytes was observed on the scaffolds and the gelatin control cultures starting on Day 7-8 of differentiation. The mES-derived cardiomyocytes on the collagen (+RGD and –RGD) scaffolds had a significantly

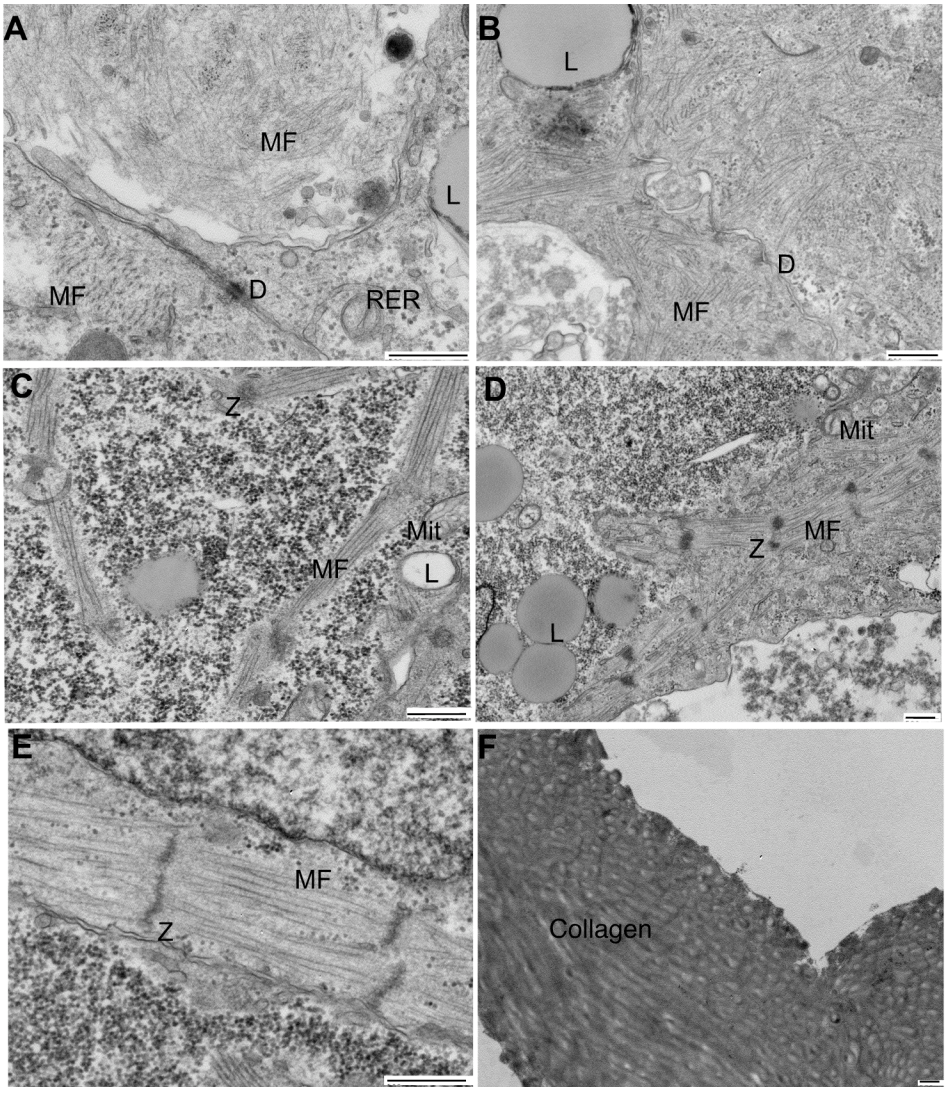

Figure 4.7 - Cardiomyocytes growing on collagen scaffold contained muscle fiber bundles and Z bands.

The ultrastructure of the developing cardiomyocytes was examined using an electron microscopy on day 10 after seeding 5-day EBs onto scaffolds. Cardiomyocytes growing on the collagen scaffolds contained organized bundles of myofibers (MF), premature sarcomeric components including Z bands (Z), and cell-cell junction desmosomes (D). Cardiomyocytes growing on the scaffolds were found to have a varying degree of maturity, from disorganized myofibers (Panels A, B), to more organized myofibers with Z bands (Panels C ,D, and E). Also labeled in the images are mitochondria (Mit), rough endoplasmic reticulum (RER), and lipid droplets (L). An electron microscopic image of the collagen I/III scaffold alone (Collagen) is shown in Panel F. Scale bar = 500nm

slower spontaneous beating rate than those grown on gelatin (Fig. 4.8, panel A). As shown in Figure 4.8, Panel B, the collagen (+RGD) scaffold had a 10-fold larger beating area than the gelatin samples. No significance was detected between the collagen scaffolds with or without RGD. Therefore, while a similar extent of cardiomyogenesis was observed in gelatin-coated plates versus the collagen scaffolds, the beating rate was significantly slower and the beating area larger when cells were grown on scaffolds.

4.5 - Discussion:

This study revealed that mES cells grown for 5 days in suspension to form EBs are able to integrate within collagen scaffolds, with or without RGD, and subsequently differentiate into beating cardiomyocytes. The scaffolds themselves were not sufficient to bypass the requirement for cellular aggregation to induce efficient cardiomyogenesis. The mES-derived cardiomyocytes growing on the collagen scaffolds contained larger beating areas with a slower beating rate, compared to those grown on gelatin. These results show that this substrate supports cardiomyogenesis in mES cells grown as EBs, warranting further *in vitro* and *in vivo* characterization using cardiomyocytes derived from both mouse and human ES cells.

Our study is the first to examine the effect of RGD on cardiomyogenesis using a mES EB model. No major effect of the presence of an RGD sequence on cardiomyocyte formation was identified, as shown by QPCR analysis of cardiac-related transcripts, the presence of spontaneous beating, and MHC immunofluorescence. These results are in contrast to a previous report that the addition of an RGD ligand to polyethylene glycol (PEG)-based hydrogels promotes cardiomyogenesis in P19 EC cells [21]. P19 EC cells were

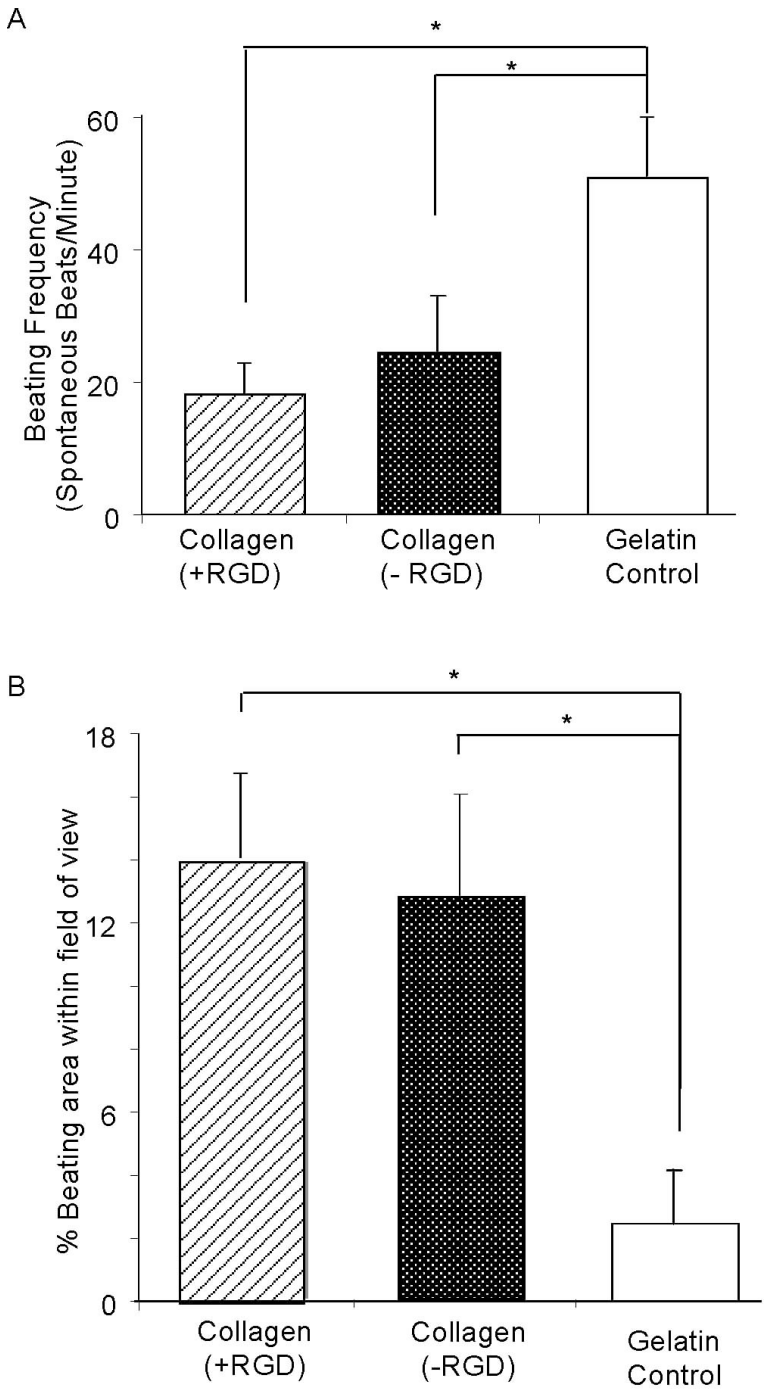

Figure 4.8 - Cardiomyocytes growing on the collagen scaffolds had a slower beating rate and a larger beating area compared to the gelatin control substrate.

Beating was recorded using a Nikon microscope. The beating rate was recorded per minute and compared between the three substrates (A). The size of the beating area relative to the field of view was calculated using ImageJ software (B) (*=$p<0.05$, n=3 using a Student's t-test comparing scaffold grown cells to gelatin controls).

seeded within the PEG +/-RGD hydrogel as undifferentiated single cell suspensions, which formed large aggregates, independent of the presence of the RGD sequence. Although spontaneous beating was not observed, 6-fold and 3-fold increases in MHC expression were observed compared to cells grown as EBs or within a hydrogel lacking RGD, respectively [21]. There are several possible explanations for the differences between our results and those in P19 cells. The non-cell adhesive nature of the PEG-based hydrogel allowed for P19 aggregation formation within the scaffold, whereas the 5-day old EBs adhered to and migrated within the collagen scaffold pores. Furthermore, slightly different RGD sequences were used (RGDSP compared to GRGDS). Other studies have shown that the context of the RGD sequence itself can have a major impact on its biological function [18]. It is possible that P19 and ES cells could have a different ECM, creating different sensitivities to an RGD sequence. Finally, there are differences in the scaffold elasticity, resulting in different intrinsic abilities to differentiate into beating cardiomyocytes [42].

In agreement with our results, a study using human ES cell aggregates, encapsulated in a dextran-based hydrogel with and without RGD peptides and vascular endothelial growth factor (VEGF), showed that RGD did not increase cell viability and did not contribute significantly to the upregulation of VEGF receptor expression. [43]. Thus it appears that RGD functions differently in distinct contexts, both in terms of the sequence in which RGD is used and in terms of the specific cells and individual differentiation programs [18]. Importantly, 5-day old EBs were not dissociated prior to seeding directly onto the scaffolds, due to the high level of cell death and cardiomyocyte loss caused by dissociating ES-derived cardiomyocytes (unpublished results from our group and discussed in [44-47]. However, although the cells within the 5-day old EBs do spread out and migrate into the scaffolds, with and without RGD, in our study, we cannot rule out the possibility that cell-cell interactions or

the presence of a primitive ECM basement membrane within the EBs [48], may overshadow a potential enrichment gained by the addition of RGD.

Studies using primary cultured rodent neonatal cardiomyocytes have shown that immobilizing RGD on tissue engineered 3D or 2D substrates positively influences neonatal-derived cardiomyocyte cultures [24-26]. In all these studies, the neonatal cardiomyocytes were seeded onto the various scaffolds as single cell suspensions. Although human and mouse ES-derived cardiomyocytes do share many characteristics with neonatal cardiomyocytes, there are also slight differences in the proteonomic profile [49], and neonatal cardiomyocytes generally show a different degree of organization and coupling when compared to mES cardiomyocytes [41]. Furthermore, it is difficult to compare the maturation of neonatal cardiomyocytes to the differentiation of ES cells.

The collagen scaffolds were not sufficient to overcome the requirement for EB formation, and did not induce differentiation of unaggregated mES cells into functional cardiomyocytes. Notably, the 3-day EBs induced an upregulation of GATA4 transcripts, but not MHC, cardiac α-actin or NKX2.5 when plated onto scaffolds, suggesting that GATA4 induction could be due to the formation of other tissue types, such as endoderm [50]. There are several excellent protocols that use bone morphogenic protein (BMP) 2/4 and/or activin A to enhance cardiomyogenesis under ES monolayer conditions [4, 5, 51, 52]. We cannot rule out the possibility that RGD would have an effect on the ability of ES cells to differentiate in monolayer using a monolayer-induction protocol. Furthermore, it would be interesting to include known inducers of cardiomyogenesis in future scaffold designs.

Spontaneous beating in the mES-derived cardiomyocytes was observed on the collagen (+RGD and –RGD) scaffolds and gelatin substrates when cells were grown and seeded as EBs. We hypothesize that the larger beating area and slower rate of beating

observed in the collagen scaffolds compared to the gelatin coating may be due to the elasticity of the collagen scaffolds compared to monolayer gelatin coatings [42]. In addition, since no large differences were observed in the level of cardiomyocyte differentiation, the larger beating area observed on the scaffolds may be due to contracting cells pulling surrounding areas of the scaffold as they beat in culture. Other possible contributing factors that may affect the beating characteristics include, the triple helical conformation of collagen, differences in the rate of hydrolysis of gelatin compared to collagen [53], and differences in the dynamic storage modulus of gelatin and collagen [54].

At this time, there is no evidence in the field of cardiac tissue engineering indicating the ideal beating rate of an *in vitro* developed cardiomyocyte patch. It is our hypothesis that implanting slowly beating cardiomyocytes may be more favourable for integration than a faster beating patch of muscle. Dysrhythmias resulting from implanted cardiomyocytes are a major concern with the tissue engineering approach, and implanted stem cells exhibiting a rapid beating rate are thought to induce cardiac muscle dysfunction when coupled with slower host cardiomyocytes [55].

In summary, the collagen (+RGD and –RGD) scaffolds both support embryonic cardiomyocyte growth and function. Scaffolds seeded with 5-day old mES EBs were beating spontaneously in culture, and were enriched for Nkx2.5, GATA4, cardiac α-actin, and MHC to similar levels as the EBs grown on the gelatin control 2D cultures. This collagen scaffold, with or without RGD, does not bypass the requirement for the formation of EBs, and is not sufficient to induce cardiomyogenesis in unaggregated mES cells. Addition of the adhesion peptide arginine–glycine–aspartate–serine did not improve cardiomyocyte differentiation compared to gelatin. This study is the first to look at the effect of RGD on cardiomyogenesis from aggregated mouse ES cells, showing that this collagen scaffold is a suitable substrate

for the *in vivo* delivery of ES-derived cardiomyocytes.

4.6 - Acknowledgments:

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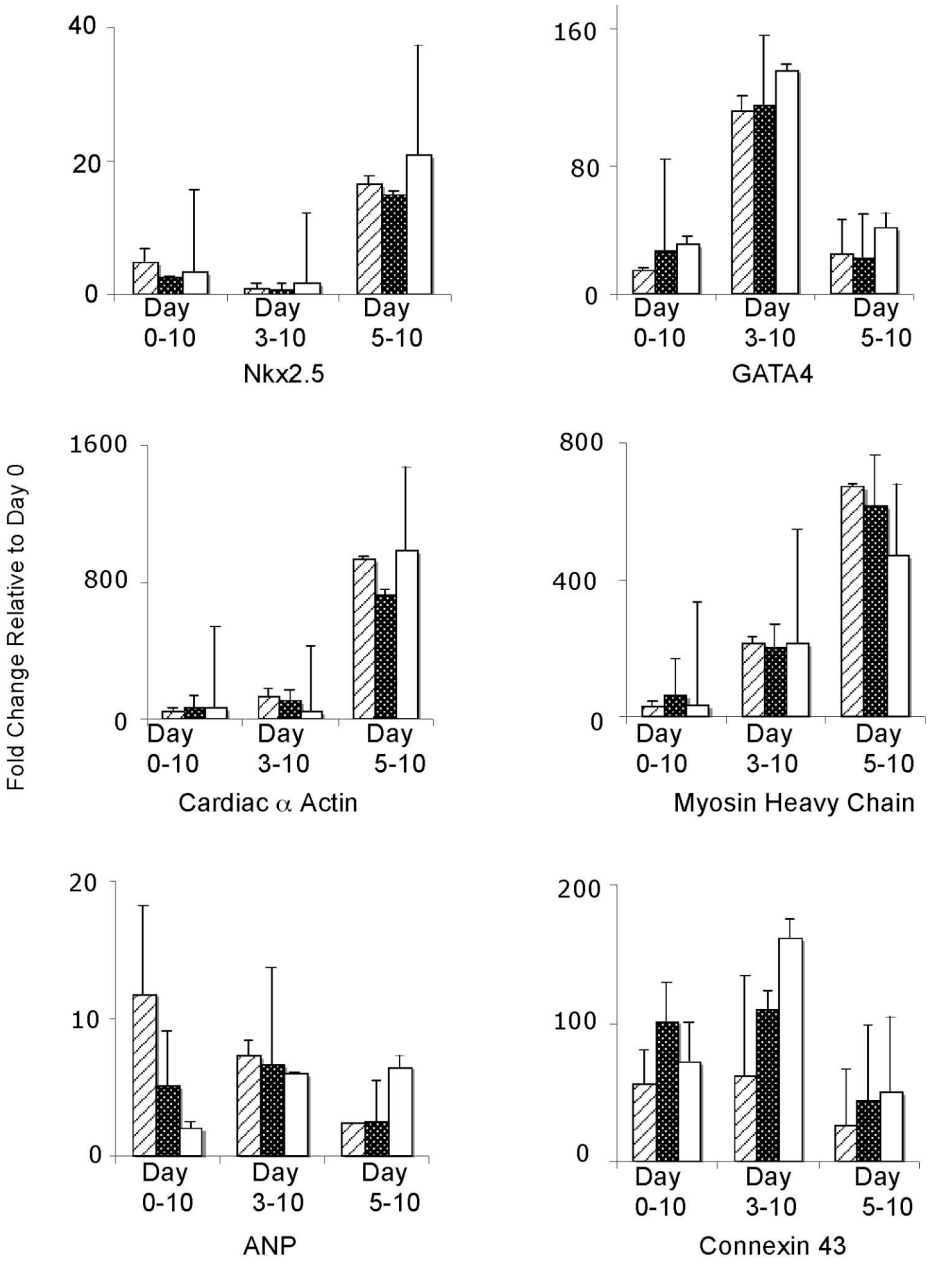
4.7 – Supplementary Figure



Supplementary Figure 4.1 - 5-day old EBs grown until day 10 on the collagen (+ and - RGD), and gelatin substrates differentiated more efficiently into beating cardiomyocytes than 3-day old EBs or unaggregated cells.

Higher levels of MHC, cardiac α actin, and Nkx2.5 transcripts were observed in cardiomyocytes aggregated for 5 days, when compared to unaggregated cells and 3-day old mEBs grown on the substrates until day 10. Results are expressed relative to undifferentiated mES cells grown in monolayer, n=3.

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CHAPTER 5: General Discussion

Before ESC-derived cardiomyocyte replacement therapy can become a safe and effective method to treat heart disease in humans, several technical challenges must be overcome. Two of the most important challenges are:

1. Obtaining a lineage-defined or pure population of mature donor cardiomyocyte cells that is free from teratoma-forming ESCs and/or unwanted lineages.
2. Developing an efficient cell delivery system or tissue engineered substrate that will minimize cell loss in order for sufficient numbers of donor cardiomyocytes to graft and integrate into the host and elicit clinically significant improvements in myocardial function.

5.1 – Significant Scientific Contribution

In Chapters 2, 3, and 4, I have addressed the above challenges and have contributed to the growing body of knowledge that will progress cardiomyocyte stem cell delivery nearer to the clinical testing stage. My specific contributions will be discussed in more detail below.

5.1.1 - Purifying ES-derived Cardiomyocytes to a clinically relevant population

As indicated in chapters 2 and 3, two different strategies were utilized in order to purify ES-derived cardiomyocytes. Chapter 2 describes the use of P19 cells as a model cell line to develop techniques for creating a stable cardiomyocyte selectable cell line. This transgenic approach towards purification of cardiomyocytes or progenitors from differentiating ES cells is not novel. As discussed in chapter 1 and summarized in Table 1.1,

hES and mESC models have shown that, using similar techniques, one can obtain a nearly pure population of ES-derived cardiomyocytes (Anderson et al., 2007; Kita-Matsuo et al., 2009; Xu et al., 2008b). Linking the cardiac α-actin gene promoter with a gene for antibiotic resistance, allows for the purification of cardiomyocytes without the need for dissociation and FACS. This enriched cardiomyocyte EC model was sufficient for evaluating the effectiveness of the collagen biomaterial's ability to sustain cardiomyocytes without disrupting cell-cell interactions by dissociation. Although P19(CA-puro) differentiation and antibiotic selection did not yield 100% pure cardiomyocytes; significant cardiomyocyte enrichment of the cultures was achieved, providing a useful progenitor-derived cardiomyocyte model for biomaterial testing. Discrepancies between this study and the high purity reported in previous studies may be explained by differences in the promoter specificity due to the site of integration or differences in the construct transfected into the P19 cells. The differences between this study and other transgenic studies related to the integration site or construct may be resolved by re-designing the construct and by screening new stable cell line clones. For the purposes of chapter 2, however, the P19(CA-puro) cell line cardiomyocyte purity was sufficient to test the collagen biomaterial.

Chapter 3 describes ES-cardiomyocyte enrichment obtained using selection with the cell surface protein DHP-R. I demonstrated that this surface marker is an excellent candidate for further study and will benefit from further optimization to minimize cell death associated with cardiomyocyte dissociation and FACS. Similar to the enrichment obtained using the cardiac α-actin transgenic approach in P19 cells, however, DHP-R sorting also did not lead to a 100% pure population of cardiomyocytes. The lack of purity in the DHP-R positive

mES cells may be explained by the fact that DHP-R, although lineage restricting, is not cardiac specific, as it is also expressed in other excitable cells (Moosmang et al., 2005).

At this time, there is no published cardiac-specific cell surface marker sorting technique that yields 100% pure cardiomyocytes from ESC cultures. Cardiac or progenitor-related proteins used for surface marker sorting, such as ALCAM and N-Cadherin, yielded enriched, but not pure populations of cardiomyocytes (Honda et al., 2006; Rust et al., 2009). The most successful method for purifying cardiomyocytes based on the cell surface phenotype has been by taking a lineage restricting approach using Flk-1 (Kdr) positive cell populations, with Flk-1 positive cell populations differentiating into 57% cardiomyocytes, along with endothelial and vascular smooth muscle cells (Yang et al., 2008). Flk-1 positive sorted mESCs improve cardiac function in a cardiomyopathic mouse model and do not lead to teratoma formation in mice (Baba et al., 2007b). Transplanting a mixed cell population containing vascular progenitor cells can support the donor cardiomyocytes and improve graft survival (Stevens et al., 2009a), however the need for further purifying cardiomyocytes has been demonstrated by studies indicating that Flk-1 positive cardiomyocytes are superior to Flk-1 positive cells of vascular lineages at improving cardiac function following muscle damage (Adler et al., 2010). The use of DHP-R sorting, along with other lineage restricting approaches, for selecting cardiomyocytes may improve the purity obtained, and is a future direction for this project.

A recent focus of the scientific community for improving the yield of cardiomyocytes obtained from ESCs has been in manipulating the signals involved with cardiomyogenesis to direct the differentiation of the ESC cultures towards cardiomyocytes lineages. As discussed in chapter 2, recent successes of 60-80% pure cardiomyocytes have been reported using BMP4 and Activin A treatment in defined growth factor media on hESC and IPS cultures

(Kattman et al., 2011). A future direction for this project is to combine DHP-R sorting with a directed differentiation approach, such as BMP4 / Activin A treatment to increase cardiomyocyte yield prior to / after sorting the differentiating ESC cultures in order to improve the purity of the cultures and remove unwanted lineages.

As described in chapter 2, P19 cells were utilized as a model to enhance cardiomyocyte purity and test biomaterials. The P19 cell model was chosen for this study due to the cell's ease of expandability in culture and relative amenability of performing genetic manipulations. The P19(CA-puro) cell line is not clinically appropriate due to the tumorigenic origin, and the transgenic modification of cells. Chapter 3 describes the use of mESCs in order to test an ESC-derived cardiomyocyte source. Similar to the clinically relevant hESCs, mESCs are derived from the blastocyst and exhibit pluripotency without being transformed or derived from tumours.

The death of cardiomyocytes upon dissociation is a common observation in the literature (Robey et al., 2008). As discussed in chapter 2, the challenge of dissociating cardiomyocytes for purification was circumvented by designing a model cell line in which antibiotic selection was used to enrich the cultures for cardiac α–actin expressing cells.

In the experiments described in chapter 3, cardiomyocytes were enriched using a clinically relevant novel surface marker purification strategy. In order to sort the mESCs, cells were dissociated, stained with the antibody to DHP-R, and processed by FACS. Despite measures to optimize the dissociation protocol, an average of 50% of cardiomyocytes were lost in the cell dissociation step alone. It has been reported that an additional 65-92% of total ES cells are not recoverable following FACS (Emre et al., 2010). Cardiomyocyte loss specifically related to FACS was not assessed in these studies. These examples demonstrate the need for improving cardiomyocyte viability following dissociation and sorting. Attempts

to improve cardiomyocyte survival by applying two previously reported cell survival strategies to the mES model in chapter 3 were unsuccessful. By optimizing the cell survival strategies for these particular applications, these findings can be applied to future experiments involving DHP-R co-sorting with lineage restricting surface markers.

As mentioned above, studies in chapter 3 reported that an average of 50% of MHC-positive cardiomyocytes are lost in the dissociation stage. This cardiomyocyte loss is associated with a corresponding loss in the transcript levels of cardiac α-actin and α and β isoforms of MHC. Interestingly, the cell loss is not accompanied by similar decreases in the early cardiomyocyte transcription factors Nkx2.5 or GATA4. This observation warrants further investigation into the possibility that early cardiomyocytes, or cardiomyocyte progenitor cells, are surviving the dissociation step and may be more viable following FACS. In Chapter 3, DHP-R transcript levels increased prior to the development of functional beating cardiomyocytes, and Flk-1 has been shown to be expressed on cardiac progenitors (Kattman et al., 2006; Yang et al., 2008). In addition, a recent study using monkey ESCs containing Mesp1 positive cardiomyocyte progenitors sorted using SSEA-1 surface marker staining, found that this progenitor population survived the dissociation and sorting steps, and differentiated into ventricular cardiomyocytes that reconstituted 20% of a myocardial scar in a non-human primate model (Blin et al., 2010). Thus, a future direction of this project is to further investigate the viability of cardiomyocyte progenitor cells following dissociation, and apply these findings to purification strategies targeted at progenitor populations, including the use of DHP-R cell surface purification strategies.

5.1.2 - Tissue Engineered Cardiac Scaffolds for Sustaining Functional ES-derived Cardiomyocytes

In chapters 2 and 4, two collagen-based biomaterials were shown to sustain stem cell-derived cardiomyocytes. The first biomaterial is a type I collagen hydrogel scaffold. The use of transglutaminase as a natural cross-linking reagent to increase the collagen scaffold strength for cardiomyocyte applications was investigated. The biomaterial tested in chapter 4 was a commercially available hemostat, a collagen I and III-based scaffold that was modified by adding the RGD motif. For both the collagen I hydrogel and the collagen I/III scaffolds (+ RGD and –RGD), the biomaterials sustained cardiomyocyte growth, differentiation, and spontaneous beating.

There are conflicting reports in the literature regarding the ability of the RGD motif inserted into scaffolds to improve ESC differentiation. The addition of RGD to PEG scaffolds improved P19 cardiomyocyte differentiation compared to scaffolds lacking RGD (Kraehenbuehl et al., 2008). In contrast, RGD did not improve endothelial differentiation or viability of human ES cells growing on dextran scaffolds (Ferreira et al., 2007). In chapter 4, using mESC EBs differentiated into cardiomyocytes, no differences in cell viability, cardiomyocyte differentiation, or function was detected between the +RGD and –RGD modified collagen I/III scaffolds. Notably, a previous report using neonatal cardiomyocytes growing in the same collagen I/III +RGD and –RGD scaffolds demonstrated RGD enhanced contractility and survival compared to the –RGD collagen scaffolds (Schussler et al., 2009). Although neonatal cardiomyocytes are a useful cardiomyocyte model, the maturation and function of neonatal cardiomyocytes cannot be directly compared to stem cell-derived cardiomyocyte differentiation. In my study, the possibility that ECM basement membranes and cell-cell interaction within the EBs may conceal scaffold-ECM

interaction and diminish the enhancement potential of the scaffolds cannot be ruled out. Further studies confirming the lack of improvement provided by the addition of the RGD motif are necessary to confirm these results, including testing using purified ES cardiomyocytes.

In summary, both collagen-based scaffolds can sustain ES-derived cardiomyocyte growth and are suitable candidates to move forward with further ES-cell *in vitro* and *in vivo* testing in combination with FACS purified ES-derived cardiomyocytes. My results indicate that the addition of the RGD motif to the collagen scaffold does not enhance ES-derived cardiomyocyte differentiation, survival or function.

5.2 – Summary of the Limitations of the Work Presented in this Thesis

The following limitations should be noted and many can be addressed as future directions for this project:

1. The P19 cardiac selectable stable cell line designed in Chapter 2 can only be used as an *in vitro* model to test potential substrates for cardiac tissue applications. Due to the cell line's tumorigenic origin (carcinoma stem cells) and the genetic modification performed, this cell line and approach is not suited for *in vivo* or clinical applications.
2. DHP-R positive cell sorting did not yield a 100% pure population of cardiomyocytes, however may be suitable as a lineage restricting approach or in combination with a directed differentiation approach.

5.3 – Overview of Future Directions

Future directions for this project involve further optimizing the conditions surrounding ES-cell sorting and purification, such as determining the ideal developmental

stage to purify ES-derived cardiomyocytes, and reducing cell-death by finding the optimal combination of cell survival approaches. These findings can next be combined with directed cardiomyocyte differentiation and DHP-R sorting and characterization of the enriched cardiomyocyte populations to determine a suitable regime for seeding the cardiomyocytes onto the ECM-based biomaterials. Particular attention will be paid to the level of maturation of the cardiomyocytes. The defined population of cardiomyocytes can be then tested on biomaterials for *in vivo* assessment of their abilities to repair injured rodent hearts.

5.4 – Summary of My Contribution to the Field of Cardiac Tissue Engineering

My specific contributions to the field of cardiac tissue engineering from my thesis work are as follows:

1. I have developed and tested an EC-derived line of P19 cardiomyocytes that can be utilized for demonstrating the feasibility of using tissue engineered substrates for enhancing differentiation of progenitor cells into cardiomyocytes.
2. I then followed up with the characterization of a cell surface protein, DHP-R, as a potential cell surface marker that can be used for enrichment of ES cells that will differentiate along the cardiomyocyte lineage.
3. I have shown that collagen-based matrices (ranging from hydrogels to foams) serve as effective scaffolds for cardiomyocyte differentiation from EC and ES cells. Addition of the RGD cell adhesion peptides to the collagen supports does not appear to enhance cardiomyocyte survival, differentiation, or functions. However, transglutaminase serves as an effective, biocompatible crosslinker to stabilize the collagen matrices that are used as scaffold.

5.5 –Conclusions

Both the risks of tumour formation due to transplantation of undifferentiated ES cells into animals, and cardiomyocyte graft survival issues due to cardiomyocyte death and cell dissipation have been well documented (Fong et al., 2011; Robey et al., 2008). They justify the approaches taken in my studies to contribute to these areas of research. Although significant achievements have been reported from transplanting ES-derived cardiomyocytes onto animal hearts (Adler et al., 2010; Blin et al., 2010; Christoforou et al., 2010; Laflamme et al., 2007; Lu et al., 2010a; Menard et al., 2005; Stevens et al., 2009a; van Laake et al., 2009), the lack of a fully characterized cardiomyocyte purification protocol that can ensure a defined population of donor cells, 100% free of cells capable of forming tumours, has stalled progression of applying these findings to humans. In these studies, the use of DHP-R as a cardiomyocyte enrichment strategy may prove to be a valuable tool to purify ES-derived cardiomyocytes.

Many challenges still surround the use of cell therapies to treat cardiovascular disease. However, *in vitro* characterizing ECM-based tissue engineered scaffolds to deliver the cells to the heart, and preliminary characterization of a novel method to purify ES-derived cardiomyocytes, contributes to growing research in this area. As summarized in Figure 5.1, the combination of a purified or defined population of cardiomyocytes derived from ES cell sources, combined with an ECM-based scaffold to deliver mature cardiomyocytes in an organized and functional manner for integration into the heart, is an important milestone for progressing cell therapy options for cardiac patients to the clinical trial stage.

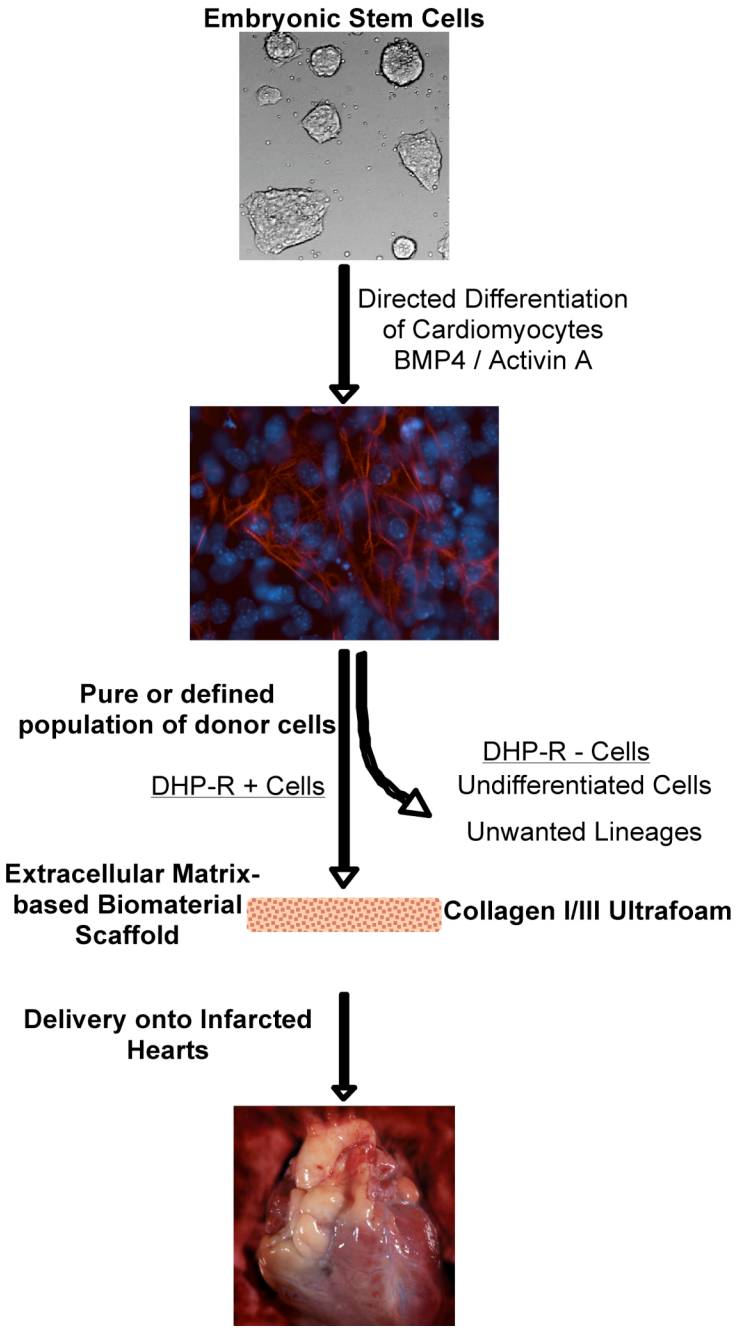

Figure 5.1 – Summary of my Proposed Strategy for Delivering ESC-derived Cardiomyocytes to Infarcted Hearts.

I propose that a lineage-defined population of donor cardiomyocytes, obtained by directing ESCs to form high percentages of cardiomyocytes and sorting the cultures with DHP-R, seeded on an ECM-based tissue engineered substrate, such as the clinically approved Collagen I/III ultrafoam, will elicit clinically significant improvements in myocardial function when transplanted onto an injured heart.

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Appendix I: Curriculum Vitae

Jennifer Elizabeth Dawson, BSc, MSc.

SUMMARY OF STRENGTHS

- Communication (oral presentations, technical written reports)
- Teaching and Presentation Skills: Medical students and Undergraduate teaching, Toastmasters
- Leadership experience in the lab and beyond.

EDUCATION

2005- present **Graduate Student**, PhD Candidate, Cellular and Molecular Medicine. University of Ottawa. *Supervisor: Dr. I. Skerjanc, Biochemistry*

2004 **Masters of Science**, Pharmacology and Toxicology. Queen's University. *Supervisor: Dr. L. Winn, Pharmacology and Toxicology.*

2002 **Bachelor of Life Science**, Research Stream, Queen's University.

EMPLOYMENT

2006-2009 **Neuroanatomy Demonstrator**, Faculty of Medicine, University of Ottawa -2nd year medical school neuroanatomy teaching assistant (contract)

2005-2008 **Teaching Assistant**, Department of Chemistry, University of Ottawa -Undergraduate General Chemistry laboratory teaching assistant (contract)

2002-2004 **Teaching Assistant and Tutor,** Department of Pharmacology, Queen's University –Undergraduate, medical students, and nursing students teaching assistant (contract)

Summer 2002 **Laboratory Technician,** Dept of Pharmacology, Queen's University

2000-2001 **Aquatics Director,** City Of North Bay Tourism and Leisure Services -Direct supervision and training of 22 aquatics staff (spring/summers contract)

PUBLICATIONS

Articles Published in Refereed Journals:

- **Dawson, J.E**., Boisvenue, S., Skerjanc*, IS, and Griffith*, M. A P19 Cardiac Cell Line as a Model for Evaluating Cardiac Tissue Engineering Biomaterials. *The Open Tissue Engineering and Regenerative Medicine Journal*. 2, 53-62 (2009)
- **Dawson, J.E.,** Winn, L.M. (2006). Folic Acid and Human Health. Queen's Health Science Review.

- **Dawson, J. E.**, Raymond, A.M, and Winn, L.M. (2005). Folic acid and pantothenic acid protection against valproic acid induced neural tube defects in CD-1 mice. Toxicology and Applied Pharmacology. 212(2):124-32.

Article Submitted at Refereed Journals:

- **Dawson, J.E**., Schusser, O., Al-Madhoun, A., Menard, C., Ruel, M., Skerjanc, IS. Collagen (+RGD and –RGD) scaffolds support cardiomyogenesis after aggregation of mouse embryonic stem cells. Submitted February 15, 2011

Non-Refereed Contributions:

- **Dawson, J.E.,** and Winn, L.M. (2004). Valproic Acid Induced Neural Tube Defects. Society Toxicology Canada Spring 2004 Newsletter.

SCHOLARSHIPS AND AWARDS

Academic:

- Ontario Graduate Scholar Award December 2006-2010
- Ontario Graduate Scholarships in Science and Technology Award, January 2005-07
- University of Ottawa Admissions Scholarship, January 2005
- Eldon Boyd Fellowship, Dept of Pharmacology and Toxicology, Queen's University, 2004
- Deans Honor List, Queen's University 2002
- Undergraduate Entrance Scholarship, North Bay Ontario, September 1998

Research and Communication:

- Health Science Research Trainees Meeting, Queen's University, 2004. Best oral presentation.
- CANTOX award, Society of Toxicology of Canada, 2003. Best MSc poster presentation.
- Society Toxicology Canada Travel Award, March 2004.

Leadership and Athletics:

- Campbell University, North Carolina. Full Athletic Scholarship, September 1998-2002 *(declined)*
- North Bay Athlete of the Year, 1997-1998

CONFERENCES, POSTERS AND ORAL PRESENTATIONS:

- **Dawson, J.E.,** Al-Madhoun, A., Skerjanc, I.S. (2009). Surface marker purification of mouse embryonic stem cells. Weinstein Cardiovascular Development Conference, San Francisco, (PhD. Work)
- **Dawson, J.E.,** Skerjanc, I.S., Griffith, M. (2007). P19 Cells as a Model for Evaluating Tissue Engineered Substrates. Canadian Stem Cell Network AGM, Ottawa, Ontario. (Poster presented orally, PhD work)
- **Dawson J.E.**, Griffith, M. (2007) Progress in Cardiac Tissue Engineering. Ottawa Health Research Institute Day (Poster presented orally, PhD. Work)
- **Dawson, J.E.,** Skerjanc, I.S., Griffith, M. (2006). Comparison of P19 embryonic carcinoma cells, neonatal mouse cardiomyocytes, and HL1 atrial cells for Cardiac Tissue Engineering. Cdn Stem Cell Network, Ottawa, Ontario. (Oral Presentation, PhD work)
- **Dawson J.E.**, Griffith, M. (2006) Cardiac Tissue Engineering. Ottawa Health Research Institute Day (Poster presented orally, PhD. Work)

- **Dawson J.E.**, Griffith, M. (2006) Myocardial Scaffolds for Tissue Engineering. Ottawa Eye Institute Research Day (Poster presented orally, PhD. Work)
- **Dawson, J.E.,** Griffith, M. (2005). Myocardial regeneration and tissue engineered scaffolds. Stem Cell Network Annual Meeting, Calgary Alberta. (Poster presented orally, PhD. Work)
- **Dawson, J.E**.,and Winn, L.M. (2004) Folic Acid Protects Against Valproic Acid Induced Neural Tube Defects. Society of Toxicology Annual Meeting, Baltimore Maryland. (Oral and Poster Presentation, MSc.)
- **Dawson, J.E.** (2004) Folic acid protects against Valproic Acid Induced Neural Tube Defects. Health Science Research Trainees Meeting, Queen's University. (Oral presentation, MSc work)
- **Dawson, J.E.** (2004) The Etiology of Neural Tube Defects. Department of Pharmacology and Toxicology, Queen's University, 2004 (Oral presentation, MSc work)
- **Dawson, J.E.** (2004) Chemical Induced Teratogenesis. Guest Lecturer, School of Environmental Studies Queen's
- **Dawson, J.E.,** and Winn, L.M. (2003) Valproic Acid Induced Neural Tube Defects in CD-1 Mice. Society of Toxicology Canada, Montreal. (Abstract and poster presented orally)