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Derivation of Cardiomyocytes from Embryonic Stem cells and Development of Techniques to Study Cardiac Lineage

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ABSTRACT

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Embryonic stem cells have the potential to differentiate into ectodermal, mesodermal and endodermal derivatives. This property makes them a valuable source of tissue specific progenitor and differentiated cells that can be used for cell replacement therapy. The first goal of this thesis is to test if cardiogenesis from embryonic stem (ES) cells can be influenced by the overexpression of different proteins. We find that cardiogenesis is significantly enhanced by the overexpression of the c isoform of the transcription factor 'Paired-Like Homeodomain Transcription Factor (Pitx2c). This effect was verified morphologically by differentiating ES cells with Pitx2c and immunostaining for cardiomyocyte-specific proteins. Similarly, changes in RNA transcripts specific to cardiomyocytes during the process of differentiation were also recorded by means of Quantitative RT-PCR (QRT PCR). Finally, the contractile properties of these cells were measured by means of calcium imaging and their action potential characteristics were measured. We transplanted these cells into a mouse model of myocardial infarction and

found significant improvement of cardiac function. We subsequently, subjected Pitx2-ES cells to hypoxic conditions and observed that the supernatant also caused significant functional recovery when injected into infarcted mice. The second goal of this thesis is to study the development of cardiomyocytes from ES cells by utilizing a series of stage specific promoters to drive different fluorophores. We designed a system where three different constructs carrying the promoters of cardiac specific genes would express proteins which would heterodimerize and drive a response element expressing antibiotic resistance. This interdependence ensures the presence of the constructs in the non-silenced loci of the genome. We used this system to insert the promoters of NK2 transcription factor related, locus 5 (Nkx2.5), Myocyte-specific enhancer factor 2c (Mef2c), Alpha myosin Heavy Chain (α-MHC) and Myosin light chain2 – ventricular isoform (MLC2v) in murine ES cells and such that the cardiac differentiation of these cells could be visualized by the various fluorophores driven by these promotes. This system will be a powerful tool to study the effects of various morphogens on cardiac differentiation of ES cells and will also be useful for cell-transplantation studies.

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ABBREVIATIONS

α-MHC	Alpha myosin Heavy Chain
ANOVA	Analysis of variance
bFGF	Basic Fibroblast growth factor
bHLH	Basic helix-loop-helix transcription factors
BMP	Bone morphogenetic protein
BMPR1a, -1b	Bone morphogenetic protein receptor subtype-1a, -1b.
BSA	Bovine serum albumin
CCM	Cellular cardiomyoplasty
cDNA	Complimentary deoxyribonucleic acid
CFP	Cyan Fluorescent protein
CMV	Cytomegalovirus
DMEM	Delbecco's modified eagle medium
DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleic acid
DN-E Cadherin	Dominant negative E-Cadherin
EB	Embryoid body
ECM	Extracellular matrix
EDTA	Ethyldiammoium tetraacetic acid
EGF	epidermal growth factor
ES cell	Embryonic stem cell
FACS	Fluorescence Activated Cell Sorting

GATA 4	GATA binding protein 4
GFP	Green fluorescent protein
GSK-3b	Glycogen synthase kinase 3-b
HDAC	Histone deacetylase
HEK293	Human embryonic kidney 293 cells
HES cell	Human embryonic stem cell
HLA	Human leukocyte antigen
ICM	Inner cell mass
IGF	Insulin-like growth factor
Isl 1	Islet 1 (LIM/Homeodomain Islet 1)
JAK	Janus activated kinase
KDR	Kinase insert domain receptor (also known as Flk1)
LIF	Leukemia Inhibiting Factor
LRP	Lipoprotein related protein
MDR	Multidrug resistance
MEF	Mouse embryonic feeder cell
MEF 2c	Myocyte-specific enhancer factor 2C
mES cell	Mouse embryonic stem cell
MI	Myocardial Infarction
MLC2a	Myosin light chain2 – atrial isoform
MLC2v	Myosin light chain2 – ventricular isoform
mRNA	Messenger ribonucleic acid
n	Number of replicates

Nkx 2.5	NK2 transcription factor related, locus 5
PCR	Polymerase chain reaction
PDL	Poly-D-lysine
Pitx2c	Paired-like homeodomain transcription factor 2, c isoform
PGC	Primordial germ cell
RA	Retinoic acid
RAR	Retinoic acid receptor
RFP	Red fluorescent protein
RNA	Ribonucleic acid
RT-PCR	Reverse transcriptase polymerase chain reaction
RXR	Retinoid X receptor
Sca 1	Stem cell antigen 1
SCF	Stem cell factor
SEM	Standard error measurement
SHH	Sonic hedgehog
SSEA	Stage specific embryonic antigen
STAT	Signal transducer and activator of transcription
TCF/LEF	T-cell factor/lymphoid enhancing factor
TGF	Transforming growth factor
TSA	Trichostatin A
VEGF	Vascular endothelial growth factor
YFP	Yellow fluorescent protein

DEDICATION

This work is dedicated to Prof. James L. Sherley who inspired me with his intellect and encouraged me to study stem cell biology. I also would like to dedicate this work to the patients I had served in the Medical Oncology ward at the All India Institute of Medical Sciences.

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CHAPTER 1

General Introduction

The most important clinical manifestation of ischemic heart disease is myocardial infarction (MI). It is estimated that there are about 1.5 million cases of acute MI in this country every year [1]. The severity and duration of ischemia in MI causes a loss of cardiomyocytes, which cannot be adequately compensated for by replication of resident cardiac stem cells [2-4] or by terminally differentiated cardiomyocytes. The replacement of the damaged myocardium by fibrous tissue contributes to the resultant poor ventricular function [5]. One potential way to prevent this situation would be to provide a replacement for cardiomyocytes before the onset of remodeling.

Cellular cardiomyoplasty is a therapeutic approach in which various types of cells have been introduced at the site of infarction and an improvement in function has been attributed to the successful functional integration of these cells into the myocardium in animal models [6]. The variety of cells that have been used as candidates for cellular cardiomyoplasty includes skeletal myoblasts, bone marrow stromal cells, neonatal cardiomyocytes [7,8]. These studies have shown positive but limited results [9,10]. The main problems in these studies are: limited proliferation, incomplete differentiation and insufficient integration. Some of the undifferentiated Mesenchymal Stem cells (MSCs) when transplanted tend to become fibroblasts [11]. Unmodified embryonic stem cells (ESCs) when transplanted result in the formation of teratomas [12]. However, cells which were at an advanced stage of differentiation e.g. cardiac cells derived from ES cells with an alpha Myosin Heavy Chain (α-MHC) promoter driving an antibiotic resistance gene, formed stable intracardiac grafts [13]. This could possibly be due to the fact that cardiomyocytes derived from ES cells have been shown to have morphological and electrophysiological characteristics of fetal cardiomyocytes [14,15] and it is known that fetal cardiomyocytes have a superior engrafting ability [16]. Mesenchymal stem cells are attractive

candidates due to their convenient autologous source but their role in angiogenesis [17] has been more understood than in cardiogenesis.

Molecular pathways involved in the development of the heart

The heart is the first functional organ of the vertebrate embryo [18]. The lateral epiblast in the mouse embryo is the major source of precardiac mesoderm [19]. The induction of the heart mesoderm has been attributed to secreted factors like members of the Transforming Growth Factor-Beta (TGF-β) super family [20]. The cells of the cardiogenic mesoderm are specified to lineage-specific cardiac precursors by the anterior mesoderm. Bone Morphogenetic Protein (BMPs) from the endoderm in conjunction with Fibroblast Growth Factor-8 (FGF8) are critical for cardiac morphogenesis [21]. The effects of BMP2 are mediated by Nkx2.5 and the GATA family of transcription factors.

The BMPs exert their effect primarily through the smad pathway, but other pathways like Mitogen Activated Kinase (MAP Kinase), Extracellular Signal-regulated Kinase 1/2 (ERK1/2 and c-Jun N-terminal Kinase (JNK) are also involved. Nkx2.5 is known to be a coactivator of GATA4, which regulates several important cardiac specific genes like α-MHC, Atrial Natriuretic factor (ANF) and cardiac troponin C (cTnC). Wnts are a family of secreted proteins known to mediate diverse developmental processes. Wnts bind to a receptor complex (frizzled, Lipoprotein receptor-related peptides: Lrp5,6) and initiate a cascade which results in the phosphorylation of Dishevelled (Dsh). Dsh in turn inhibits the Glycogen Synthase Kinase (GSK-beta). This leads to an accumulation of beta catenin in the cytoplasm, which becomes available for signaling via

TCF/LEF in the nucleus. Beta catenin also serves the role of a structural component by binding to the cytoplasmic tail of Cadherins and the actin cytoskeleton. It has been shown that deletion of beta catenin in the endoderm causes the formation of multiple hearts along the antero-posterior axis of the mouse embryo [22] indicating a role in cardiogenesis for pathways that regulate beta catenin signaling such as Wnt and Cadherin pathways.

It is known that Wnt 1 and Wnt 3a inhibit cardiogenesis through the canonical Wnt pathway. Wnt11 on the other hand contributes to cardiogenesis through the non-canonical pathway [23]. Wnt 11 is required for the activation and maintenance of Nkx2.5 and murine Wnt11 conditioned medium triggers cardiogenesis in the mouse embryonic carcinoma stem cell line P19 [24]. Wnt 11 knockout mice do not have cardiac defects which points towards functional redundancy in the Wnt family [25]. The non-canonical Wnt signaling is mediated by JNK, protein kinase C (PKC) and calmodulin–dependent kinase II (CamKII). The upregulation of the eta isoform of protein Kinase C has been shown to promote cardiogenesis in ES cells [26]. The cytokines and growth factors implicated in cardiogenesis from ES cells are BMP-2 [27], Insulin like growth factor [28], FGF [29], Oxytocin [30], Erythropoietin [31], Ascorbic acid [32], Retinoic acid [33] and Dynorphin B [34]. Cytokines like Insulin-like growth factor (IGF-1) is known to improve the differentiation and engraftment ability of ES cells in an animal model of MI [35]. It has been shown that JAK2 and its target STAT3 direct cardiogenesis in murine ES cells [36].

Pitx2c is a transcription factor that is asymmetrically expressed in the heart and gut [37-39]. Pitx2 -/- mice develop nonseptated atrium, valvular deficiencies and outflow tract abnormalities like persistent truncus arteriosus [40] and ectopic expression of Pitx2c in vivo causes alteration

in organ situs [39,41]. It is not expressed in the adult cardiac tissue [42]. Thus in cardiomyocytes, beta catenin which binds to Pitx2 [43] is the common link between the Pitx2 pathway and cell adhesion molecules like Cadherins [44]. N-Cadherin is the only known Cadherin present in adult cardiomyocytes and is an essential part of adherens junctions [45,46]. The extracellular part is involved in calcium dependent homophilic intercellular contact and the intracellular part is connected to the cytoskeleton through beta catenin. Thus altering the levels of Pitx2, Cadherins and beta catenin might be helpful in understanding their role in the cardiomyocyte differentiation program.

The expression pattern of various cardiac specific proteins is useful to study the cardiac lineage. The restriction of cardiac lineage occurs during early gastrulation and the three main lineages atrial, ventricular and conduction arise from separate progenitors [47]. The cardiac lineage is initiated under the influence of the BMPs and one of the first transcription factors to be observed in this process is Nkx2.5 [48]. The promoter of Nkx2.5 contains several smad binding sites which drive its expression [49]. This transcription factor in turn activates the synthesis of a variety of other transcription factors. Irx4 is a member of the Iroquois family of homeobox genes, whose expression is restricted to the ventricle [50] and is useful as a marker for ventricular progenitor cells. It has been recently shown that there is a population of myocardial progenitors in the pharyngeal mesoderm which contributes to the right ventricular myocardium. This population resides in an area anterior to the early heart tube and is called the Anterior Heart Field or Secondary Heart Field [51]. Mef2c is another transcription factor which is expressed in the entire heart [52] and its enhancer element which is specific to the anterior heart field has been identified [53]. The enhancers of Nkx2.5 and Mef2c have been characterized and can potentially

serve as useful markers for early cardiac progenitors [54] in the primary and the secondary heart field. This approach has the advantage of cardiac specificity and minimal interference with the putative molecular homeostasis as compared to knocking in a fluorophore into the gene locus [55]. The expression pattern of Atrial Natriuretic Factor (ANF), however, changes at different stages of the development of the heart. It shifts from the ventricular myocardium to the atrial appendages and becomes restricted there [56]. Murine ES cells selected by transfecting them with an ANF promoter driving GFP, differentiated into pacemaker cells when treated with endothelin [57]. Genes specifically expressed in the conduction system like the Minimal potassium exchange channel (MinK) [58] and GATA 6 [59] can also be useful in studying cardiac lineage. The enhancers of MinK and GATA-6 have been used to study the formation of an in-vitro conduction system derived from ES cells [60]. The expression of structural genes such as α-MHC occurs after the expression of transcription factors [61]. Hence the enhancer/promoter elements of cardiac specific structural genes can serve as late markers for differentiation in the cardiac lineage. Myosin Light chain-atrial (MLC2a) is specific for atrial lineage in the embryonic and atrial heart [62]. MLC2v is a ventricular isoform of the myosin light chain and its promoter has been used as a marker for ventricular differentiation in-vivo [63-65] and in-vitro [66].

Cardiogenic potential of embryonic stem cells

The conventional way of studying cardiomyocyte differentiation was by inducing differentiation in immortalized cell lines like P19 with agents such as Dimethylsulfoxide (DMSO) [67]. The mechanism of action of agents like DMSO and 5-azacytidine has not been characterized well. However, microarray analysis in these studies has given considerable insight into the role played by various factors in the differentiation program [68]. For the purposes of transplantation immortalized cell lines cannot be used. Embryonic stem cells are derived from the inner cell mass of the embryo and can differentiate into cells of all the three germ layers. ES cells when induced to differentiate form structures called Embryoid Bodies (EBs). These EBs resemble an embryo but have disorganized germ layers due to the lack of organizers [69]. Cardiomyocyte progenitors form in an area between the outer epithelial layer and the inner mesenchymal layer [70]. The factors which influence the formation of ES-cell derived cardiomyocytes are the starting number of cells; the ES cell lines, the type of medium used and the time of embryoid body plating. In spite of the presence of distinct populations of chamber specific progenitors, the formation of chamber specific cardiomyocytes occurs in a time dependent manner. At least three populations are morphologically discernable: early which is pacemaker like, intermediate and terminal, which are more chamber-specific. The principal morphological difference between these stages is the organization of the sarcomeric apparatus which ranges from irregular organization to partial organization to well organized bundles [71].

The action potentials of terminally differentiated cardiomyocytes resemble that of postnatal cardiomyocytes. Another differentiating feature between terminally differentiated and early ES

cell derived cardiomyocytes is their responsiveness to beta adrenergic stimulation [71]. Thus the effect of various factors in the medium and the effect of genetic manipulation can be studied effectively using the cardiomyocyte differentiation protocol for ES cells. Gain of function studies involving the overexpression of the transcription factor, GATA-4 [72] in P19 cells have shown that it causes an increase in the number of EB outgrowths with beating cardiomyocytes. Similarly overexpression of noggin prevents differentiation into cardiomyocytes [73]. This effect was reversed by the addition of BMP2 to the medium. However transient inhibition of BMP signaling by noggin can have cardiogenic effects on murine ES cells [74]. Simultaneous overexpression of more than one transcription factor also gives insights into the differentiation program. It has been shown that over-expression of both Nkx2.5 and GATA4 is required for the cardiomyocyte differentiation of noggin over-expressing cells. However, all these studies have been performed in embryonic carcinoma stem cell lines (P19 and P19CL6**).** .

Conventionally cardiomyocytes were derived from embryonic stem cells by micro dissecting beating foci or by purifying cardiomyocytes by using various cardiac specific enhancers driving fluorophores or antibiotic resistance genes. The number of cells extracted with such techniques falls short of the quantity required for an effective replacement. We have developed a novel way of producing cardiomyocytes from ES cells by genetically altering factors known to be involved in cardiomyocyte differentiation. Pitx2c is transiently expressed and overexpression has been observed to enhance cardiogenesis. This is in contrast with Nkx2.5 which plays a central role in cardiac differentiation [75], but when overexpressed into the differentiated stage causes disruption of sarcomeric structures [76]. Overexpression of the dominant negative form of Cadherin possibly alters the level of beta catenin or other signaling molecules which may have a

housekeeping role. Investigating these two ends of the spectrum could possibly lead us to better molecular candidates for the in-vitro production of cardiomyocytes from ES cells.

Cardiogenic potential of resident cardiac stem cells

Postnatal cardiomyocytes have long been believed to be terminally differentiated [77]. However, studies examining the rate of apoptosis and cell turnover of cardiomyocytes in the heart have pointed towards the possibility of the presence of stem cells which act as a source for newly formed cardiomyocytes. The contribution of these cells has been postulated to be minimal since hypertrophy is the chief compensatory mechanism for augmented function rather than hyperplasia [78]. Recently, different cell populations indigenous to the heart have been shown to have stem cell properties. These tissue-specific stem cells bear different markers and have been shown to contribute to the formation of new cardiomyocytes to different degrees in both in-vitro and in-vivo conditions. Based on the presence of markers these cells can be classified into five different populations: Isl1 +ve cells, Sca1 +ve cells, cKit +ve cells, cardiac Side Population and Cardiosphere-forming cells. The extent of overlap of markers among these populations has not been completely studied. Isl1 +ve cells are also positive for Nkx 2.5 and Gata 4 and are negative for Sca 1, cKit, CD 31, and sarcomeric proteins [79]. These cells arise from the secondary heart field and Wnt/beta catenin pathway has been shown to be involved in their self renewal [80]. Differentiation assays by co-culturing neonatal cardiomyocytes with Isl1 +ve cells give rise to cardiomyocytes, smooth muscle cells and endothelial cells. The cardiac phenotype of the differentiated cells was verified by immunostaining far cardiac Troponin T and alpha actinin.

These cells also exhibited action potentials and calcium transients. The utility of these cells for cell transplantation has not been tested. Sca 1 +ve cells are positive for Gata 4, Mef2c, CD 31 and CD38 [81]. These cells are negative for Nkx 2.5, cKit, Flk 1, vWf, CD 34, CD 45 and sarcomeric proteins. The origin of these cells has not been investigated and their self-renewal properties are unknown. Sca 1 +ve cells differentiate into cardiomyocytes when treated with 5-Azacytidine. The cardiac phenotype of the differentiated cells can be seen by the presence of Nkx 2.5, sarcomeric alpha actin and cardiac Troponin I. These cells have been shown to engraft into infarcted myocardium when detected two weeks post infarct. Cell fusion was also detected in a small number of cases in the engrafted areas and has cast doubt on the validity of these experiments. cKit +ve cells have a low positivity for Nkx2.5, Gata 4 and Mef 2c [2]. These cells are negative for Lin, CD 8, CD 20, CD 34, CD 45 and sarcomeric proteins. The origin of these cells is unknown but cKit +ve cells have been shown to self renew and give rise to clonal progeny. Differentiation assays on these cells show their ability to give rise to cardiomyocytes, smooth muscle cells and endothelial cells. The cardiac phenotype of the differentiated cells was verified by the presence of Connexin 43, N-Cadherin and alpha actinin. cKit +ve cells when injected into infarcted myocardium contribute to functional improvement when measured 3 weeks post infarction. Cardiac side population cells derive their name from their ability to exude a dye (Hoechst 3342, Rhodamine 123) by a membrane pump and be sorted as a low staining population during flow cytometry. These cells are positive for ATP-binding Cassette transporter G2 (Abcg 2), Stem Cell Antigen 1 (Sca 1), cKit, Mef 2a, Mef 2c, CD 34 and CD 45 [82]. They are negative for Nkx 2.5, Gata 4, CD31 and sarcomeric proteins. The origin of these cells is unknown and their self renewal properties have not been demonstrated. Differentiation by co-culture with adult cardiomyocytes causes them to express alpha actinin. These cells have not

been shown to form functional sarcomeric apparatus and do not exhibit calcium transients. Their utility for cell transplantation has not been demonstrated. Cardiosphere-forming cells derive their name from their ability to form spherical floating masses of cells that are enriched in cardiomyocytes. This ability is analogous to the neurosphere forming ability of neural stem cells. These cells are positive for cKit, Sca 1, CD31, CD34, Flk 1, vWf, cardiac Troponin T and myosin heavy chain [3]. On differentiation, the cardiac phenotype is seen by the presence of cardiac Troponin I, alpha sarcomeric actin, connexin 43 and the formation of sarcomeric apparatus. The engraftment ability of these cells has been tested and the have been shown to form bands of regenerated myocardium in infarcted tissue when detected 18 days post infarction. Recently, a more primitive set of cells called Uncommitted Cardiac Precursor cells (UPCs) have been isolated. These cells are positive for SSEA 1 and Oct 4 in the neonatal heart and additionally expressed Nkx 2.5, Gata 4 and myosin heavy chain in the adult heart. They are negative for CD 31, CD 45, and VE-Cadherin. These cells when differentiated formed spontaneously contracting clusters. However, they transiently express Flk 1, Sca 1, Abcg2, Kit and Isl 1 during differentiation [83]. It is possible that other resident cardiac stem cells bearing the above markers are more differentiated progeny of these cells. However, none of the above cells have been shown to specifically form pure populations of left ventricular cardiomyocytes. It is possible that there are yet undetected populations of resident cardiac stem cells distributed sparsely in the left ventricular tissue that give rise to new cardiomyocytes and help replace senescent ventricular cardiomyocytes.

Cardiogenic potential of extra-cardiac stem cells

Transdifferentiation of extra cardiac stem cells has been a controversial issue [84]. The chief arguments against transdifferentiation have been the paucity of in-vitro evidence and presence of cell fusion events [85]. The majority of extra-cardiac cells used were derived from the skeletal muscle and the bone marrow. Skeletal myoblasts have been attractive candidates since they represent the stem cell compartment in skeletal muscles and have been shown to possess Connexin 43 and N-Cadherin in in-vitro culture conditions. These cells have been shown to electrically couple with cardiomyocytes in-vitro [86]. No transdifferentiation has been reported in these cells after co-culture with rat cardiomyocytes, however cell fusion between the two cell types has been documented [87]. Clinical trails suggest feasibility with arrhythmogenic potential [88,89]. Skeletal-based Precursors of cardiomyocytes (SPOC) are negative for Pax 7 (as compared to skeletal myoblasts), Sca 1, cKit, MyoD, Myf 5, Myogenin, CD 34 and CD 45 [90]. These cells when differentiated in a medium supplemented with EGF and bFGF differentiate into a spontaneously contracting cardiac phenotype. The cardiac nature of these cells can be demonstrated by the presence of transcription factors like Nkx 2.5, Gata 4, calcium channels and structural proteins like cardiac Troponin T, myosin light chain 2v, alpha and beta myosin heavy chain, and Connexin 43. They also exhibit organized sarcomeric apparatus, calcium transients and pharmacologic responsiveness to chronotropic reagents. However, these cells exhibit asynchronous contractions in in-vitro conditions and take a prolonged time period for differentiation. SPOC cells have been transplanted into infarct models and have been shown to engraft but electromechanical coupling with the host myocardium has not been demonstrated. Cells derived from the bone marrow can be divided into hematopoietic and mesenchymal cells.

Earlier reports of transdifferentiation of hematopoietic cells into cardiomyocytes [10] have been vigorously contested [91] and cell fusion has been shown to be the predominant factor [92] responsible for the observed effect. Hematopoietic cells positive for cKit and Lin –ve were shown to acquire cardiac markers like Nkx2.5, Mef2c, Gata 4, and connexin 43 when injected into the infarcted myocardium. These cells caused an improvement in the functionality of the infarcted myocardium, reduction in the infarct size when detected ten days post infarction. However there is no in-vitro evidence that these cells have the ability to differentiate into cardiac lineage. The same group [93] has used cKit +ve and CD 45 cells and obtained similar results. It was observed that co-culture with rat cardiomyocytes or injection into infarcted myocardium provided these cells with the cues necessary to differentiate into cells that were positive for Connexin 43, alpha sarcomeric actin laminin and N-Cadherin. The functional improvement in this case can be explained by the formation of new capillaries and improved electromechanical coupling with the host myocardium. Bone marrow side population cells have also been used to study transdifferentiation into cardiomyocytes. These cells are positive for cKit, CD 31, Tie 2, and negative for CD 34, Flk 1, alpha actinin, ICAM 2, and vWf [17]. When differentiated these cells acquire alpha actinin and are negative for CD 45. However, due to low engraftment of these cells in cell transplantation studies it has been difficult to determine if the observed phenotype was an artifact. Mesenchymal stem cells derived from term placental membrane are positive for CD 29, CD 44, CD 73, CD 166 and are negative for CD 14, CD 34 and CD 45. Differentiation in medium supplemented with ester of hyaluronan, butyric and retinoic acid causes these cells acquire cardiac markers such as Nkx 2.5, Gata 4, cardiac Troponin I, Connexin 43, alpha actinin and myosin heavy chain. They also express other markers such as vWf, VEGF A, and HGF [94]. Sarcomeric organization is seen in these cells in in-vitro studies, however upon transplantation in

infarct model, these cells tend to have reduced sarcomeric organization. They also contribute to reduced scar formation and formation of new blood vessels when observed four weeks post infarction. Chorionic plate cells (CPCs) are mesenchymal stem cells derived by culture of the fetal part of the chorionic plate on tissue culture dishes [95]. They are positive for Nkx2.5, Gata 4, cardiac Troponin T, brain natriuretic peptide, myosin light chain–atrial isoform, CD 59, CD 73, CD 90, CD 105 and are negative for CD 14, CD 31, CD 34, and CD 45. Differentiation with co-culture with fetal cardiomyocytes for 4-5 days results in cells positive for cardiac actin, cardiac Troponin I, alpha actinin, and Connexin 43 [96]. These cells exhibit spontaneous synchronized contractions and when transplanted show a reduction in cardiac Troponin I expression. Stem cells derived from the amniotic fluid have also been investigated for transdifferentiation into cardiomyocytes [97]. These cells are positive for Oct 4, SSEA 4, cKit, Flk 1, vWf, CD 29, CD 44, CD 73, CD 90, CD 105, CD 129, CD 144, CD 146 and are negative for CD 31, CD 34, CD 45, CD 117, CD 133 and sarcomeric proteins [98,99]. These cells when differentiated by co-culture with neonatal cardiomyocytes for 4-9 days acquire markers like Nkx 2.5, Gata 4, cardiac Troponin T and I, beta myosin heavy chain, myosin light chain-ventricular isoform and sooth muscle alpha actin. They also form organized sarcomeric apparatus in in-vitro culture conditions. The chief problem with these cells is immunorejection in animal models and very low frequency of cardiomyocyte differentiation. Germline stem cells (mGSs) derived from neonates and adults have been shown to be multipotent in nature [100]. Multipotent adult germline stem cells are positive for Nanog, SSEA 1, Oct ¾, Esg 1, Str 8, Rex 1, Ras 1 and are negative for cKit, Thy 1, and CD 34 [101]. These cells are differentiated by the formation of embryoid bodies. Cardiac specific cells that are formed during the process of differentiation are positive for Nkx 2.5, Isl 1, Gata 4, Mef 2c, connexin 43, alpha myosin heavy chain, myosin light

chain-ventricular isoform, cardiac Troponin T and cardiac actinin. Spontaneous contractions are observed in these cells and action potentials and calcium transients can be recorded. These cells have a very similar differentiation spectrum as that of embryonic stem cells and also carry the risk of teratoma formation when impure cell populations are used for cell transplantation studies. Germline stem cells derived from the neonatal testes are positive for Rex 1, E-Cadherin and are negative for FGF 5, Gata 4, Bry, PDGFRa, Flk 1 and VE-Cadherin. Co-culture with OP9 stromal cells for 5 days differentiates these cells into cardiac phenotype which is positive for Nkx 2.5, Gata 4, Connexin 43, alpha and beta myosin heavy chain, atrial ventricular isoforms-myosin light chain, SERCA2, and cardiac Troponin I [102]. These cells express calcium, sodium and potassium channels and have measurable action potentials. The disadvantage of these cells is the prolonged differentiation time required and the lack of organized sarcomeric structures in engrafted cells. Thus, there are a wide variety of extra cardiac stem cells which could potentially be a source for cardiac cell replacement. However, most of these cell types seem to contribute to reduced scar formation by the formation of new blood vessels and by secretion of pro-survival paracrine factors [103]. Extra cardiac stem cells which are more pluripotent (Germline Stem cells) as compared to multipotent tissue specific stem cells are more efficient in generating bonafide cardiomyocytes.

In this work the more stringent criterion of automaticity or spontaneous contractions in conjunction with biochemical markers has been used for determination of the formation of cardiomyocyte population. This study is the first study to quantify the spatio-temporal evolution of the cardiac lineage in mouse and ES cells. It also uses genetically modified ES cells, whose differentiation is diverted preferably towards the cardiac lineage. The insights gained in these

studies are very important for the design of optimal culture conditions for growing chamber-specific cardiomyocytes in-vitro. Cardiomyocyte differentiation in HES cell cultures is observed when the medium was supplemented with factors like EGF, BMP-4 and basic FGF [104]. However there is heterogeneity in the cardiomyocytes produced [105]. Using a variety of combinations of cardiac specific enhancers driving fluorophores reveals details of cardiac lineage in an EB and also enables us to use it as a system to study the effects of pharmacological agents like Retinoic acid on cardiac lineage. The identity of the chamber specific cell populations can be verified by electrophysiology and biochemical analysis. The stage of differentiation, which is optimal for transplantation, can be potentially determined such that the cells are specified and yet have enough proliferative potential to adequately colonize an infarct. The various chamber-specific cell populations studied here are marked by fluorophores such that they can be purified by FACs and would be the starting point for other studies, which require transplantation like myocarditis, myocardial infarction etc.

Hypotheses and Specific Aims

This study seeks to define mechanisms that regulate commitment of embryonic stem (ES) cells to the cardiomyocyte lineage, and to develop techniques for efficiently generating cardiomyocytes from ES cells. The long term goal is to develop techniques for using ES cells to repair the damaged or diseased heart. We hypothesize that overexpression of factors such as Pitx2c and DN-E-Cadherin in ES cells enhances cardiogenesis in-vitro, and to examine the effects of signaling molecules such as retinoic acid on the differentiation pathway. The stage-specific effects of chemical agents like retinoic acid can be quantified by using stem cells that express fluorophores under the control of different cardiac specific enhancers. This approach has the added benefit that it should ultimately enable us to use FACs sorting to purify specific populations of cardiac progenitors and to use them for transplantation in various disease models in murine and porcine systems.

Specific Aim 1: To overexpress Pix2c and DN-E Cadherin in ES cells, characterize the extent of cardiogenesis and to test their utility in disease models.

The transcription factor, Pitx2c, is transiently expressed by developing cardiomyocytes, and preliminary studies suggest that overexpression of Pitx2c in ES cells fosters commitment to the cardiomyocyte lineage. Conversely, β-catenin signaling blocks generation of cardiomyocytes, and we find that sequestration of β-catenin by a dominant negative form of E-Cadherin (DN-E Cad) enhances differentiation of ES cells into cardiomyocytes. This part of the study seeks to

characterize the stage-specific effects of Pitx2 and DN-ECad and to define the mechanisms mediating their effects on cardiomyocyte lineage commitment.

1. Define the effects on cardiomyocyte differentiation of overexpression of Pitx2c and DN-E Cadherin in mouse embryonic stem (mES). The expression of various stage-specific cardiac markers as well as the development of contractility will be monitored and collaborative studies will examine the electrophysiological characteristics of the cells.

2. Transplant cardiomyocytes derived from Pitx2c overexpressing ES cells into a mouse model of myocardial infarction and study functional changes.

Specific Aim 2: To study different stages of cardiomyogenesis in ES cells by stage specific promoters driving fluorophores.

Embryoid bodies contain a heterogeneous group of cardiac progenitors. Retinoic acid treatment reportedly shifts cardiac lineage species towards a ventricular phenotype. In this part of the proposal we will quantify the pattern of differentiation of cardiac progenitors and we will define culture conditions that enhance the production of ventricular cardiomyocytes.

1. Create murine ES lines that express different fluorophores under the control of stage-specific cardiac lineage markers and validate the fidelity of expression of the fluorophores with respect to the marker proteins.

2. Examine the effects of retinoic acid treatment on cardiac lineage commitment by cardiac progenitors in embryoid bodies derived from wild type ES cells.

These studies thus have the dual goals of defining mechanisms underlying cardiomyocyte differentiation and providing methods for repair of the damaged or diseased heart.

Specific Hypotheses:

1. **Overexpression of Pitx2c in mES cells enhances cardiomyocyte lineage commitment.**

2. **Overexpression of Nkx2.5 mES cells enhances cardiomyocyte lineage commitment.**

3. **Overexpression of the dominant negative form of E-Cadherin (DN-ECad) in murine ES cells enhances cardiomyocyte lineage commitment.**

4. **Transplantation of Pitx2c overexpressing cells contributes to the functional recovery of myocardial infarction in mice.**

5. **Pitx2c overexpressing cells secrete paracrine factors in hypoxic conditions that contribute to the functional recovery of myocardial infarction in mice.**

6. **Multiple constructs interdependent on each other for antibiotic resistance can be used to integrate stage specific promoters driving fluorophores to study differentiation patterns in ES cells.**

7. **Retinoic acid alters the differentiation pattern of differentiating ES cells and enhances the production of ventricular cardiomyocytes.**

CHAPTER 2

Effect of Overexpression of Transcription Factors on Cardiogenesis from ES cells

Rationale:

Cardiomyocyte lineage commitment by ES cells is enhanced by a wide variety of factors including transcription factors such as GATA-4, signaling molecules such as phosphokinase C and morphogens such as Wnt11. We have observed that overexpression of the transcription factor, Pitx2c or of DN-ECad in mES cells enhances cardiomyocyte differentiation.

Transplantation strategies for repairing the damaged heart have employed a number of different cell types including skeletal muscle progenitor cells, fetal derived cardiomyocytes, mesenchymal stem cells, and embryonic stem (ES) cells [106]. Although there are problems with each of these cell types, ES cells show particular promise as a source of cells for myocardial repair [107,108]. ES cells can be generated in large quantities, and ES cell derived cardiomyocytes [109] possess the phenotypes of cardiomyocytes, such as morphology, electromechanical coupling, and the presence of gap junctions [70,110,111]. ES cell differentiation into cardiomyocytes is facilitated by formation of embryoid bodies (EBs), aggregated clusters of cells that form cystic structures with layers of ectodermal, mesodermal, and endodermal lineages [69]. However the heterogeneous mixture of cells present after EB formation yields only about 1-3 % cardiomyocytes, and the generation of beating EBs is not reliable [112]. Multiple protocols have been devised to increase the efficiency of cardiomyocyte differentiation and to obtain purified preparations of cardiomyocytes by genetic selection or mechanical dissection of beating clusters of EBs [13,55]. Such ES cell-derived cardiomyocytes can integrate into infarcted myocardium after transplantation and improve ventricular function [13,113]. However the protocols for generating ES cell-derived cardiomyocytes are still cumbersome and relatively inefficient, and this limits the potential for using these cells for transplantation. Thus, the creation of a reliable

method for generating large numbers of cardiomyocytes would be an important step towards development of methodologies for using transplanted cells to regenerate damaged myocardium.

Cardiomyocyte differentiation of ES cells can be fostered by manipulating the expression of transcription factors and their signaling cascades. For example, ES cells deficient for RBP/J, a protein involved in the transduction of the notch signaling cascade, show enhanced cardiomyocyte differentiation [114]. Also, expression of GATA-4, a Zn++ finger transcription factor and Tbx5, a T-box transcription factor involved in heart development, increases cardiomyocyte differentiation of P19 embryonic carcinoma cells [72,115]. Myocyte differentiation of P19 cells is also fostered by treatment with DMSO which leads to an increase in canonical wnt signaling. Conversely, inhibition of endogenous wnt signaling by treatment with frizzled receptor body inhibited DMSO induced myogenesis [116]. In this study, we direct ES cells to differentiate into the cardiomyocyte lineage by expression of the bicoid related homeobox gene pitx2c, a wnt/β-catenin target gene [40,117].

Pitx2c plays a crucial role in the establishment of left-right asymmetry in the developing heart [39,118]. Pitx2c expression begins in the left cardiac crescent, a population of cardiac progenitor cells, and in E8 mouse embryos (tubular heart stage), pitx2c expression is restricted to the left side of the tubular heart. By E10.5, expression of pitx2c is observed in the left inflow tract, the left atrial appendage myocardium, and the left atrioventricular canal myocardium. Loss of pitx2c function leads to atrial isomerism, double inlet left ventricle, transposition of the great arteries, persistent truncus arteriosus, and abnormal aortic arch remodeling [118,119]. Patients

suffering from Axenfeld-Rieger syndrome, an autosomal dominant disease resulting from mutations in the homeodomain of pitx2c, can suffer from defects in cardiac development [120]. Because of its role in cardiac development, we investigated the potential of pitx2c in inducing cardiac differentiation of ES cells to form functioning cardiac myocytes. It has been shown that Pitx2c overexpression in skeletal myoblasts promotes cell proliferation and decreases differentiation [121]. In our study we find that expression of pitx2c induces cardiomyocyte differentiation. Cardiomyocytes generated from pitx2c overexpressing ES cells possess pacemaker activity and are electrophysiologically indistinguishable from fetal cardiomyocytes. Further, they engraft in infarcted mouse myocardium leading to functional recovery of contractility and blood pressure. We have also found that hypoxic conditions cause Pitx2c overexpressing cells to secrete paracrine factors which also contribute to functional recovery in a mouse model of myocardial infarction. We conclude that pitx2c enhances cardiomyocyte differentiation of ES cells, and that transplants of such ESC-derived cardiomyocytes can be used to repair damaged myocardium.

Results:

Effect of overexpression of Pitx2c in embryonic stem cells:

ES cells stably transfected with pitx2c were induced to differentiate by seeding the cells at 2-5 X 10^5 cells/ml in induction media to form EBs which were grown in suspension for 8 days. Cells transfected with an empty vector were used as controls. On the 8^{th} day of the induction, the cells were dissociated and plated onto either laminin-1 or fibronectin coated glass coverslips in induction media. Maintenance in induction media promoted cardiomyocyte differentiation. Spontaneously contracting cell clusters were observed 2 days after EB dissociation in the pitx2c transfected cell line but only rarely in the control cell line, and clusters continued to beat spontaneously for over 30 days. The generation of spontaneously contracting foci in the culture dishes was not homogeneous, and we routinely observed that distinct foci began beating at different times. This suggests that cardiomyocyte differentiation did not occur at the same rate in all of the EBs in spite of the homogeneity in their size. Furthermore, beating cells originated in the center of large clusters, suggesting that pitx2c did not induce cardiomyocytes alone and that other factors such as cell-cell contact may be important in cardiomyocyte differentiation of ES cells in vitro. Cells were fixed and stained 5-7 days post EB dissociation. Cardiomyocyte differentiation was confirmed by immunostaining the cells for the transcription factors GATA-4 and Nkx2.5 and cardiac specific troponin-T, proteins that are all expressed in cardiomyocytes [122]

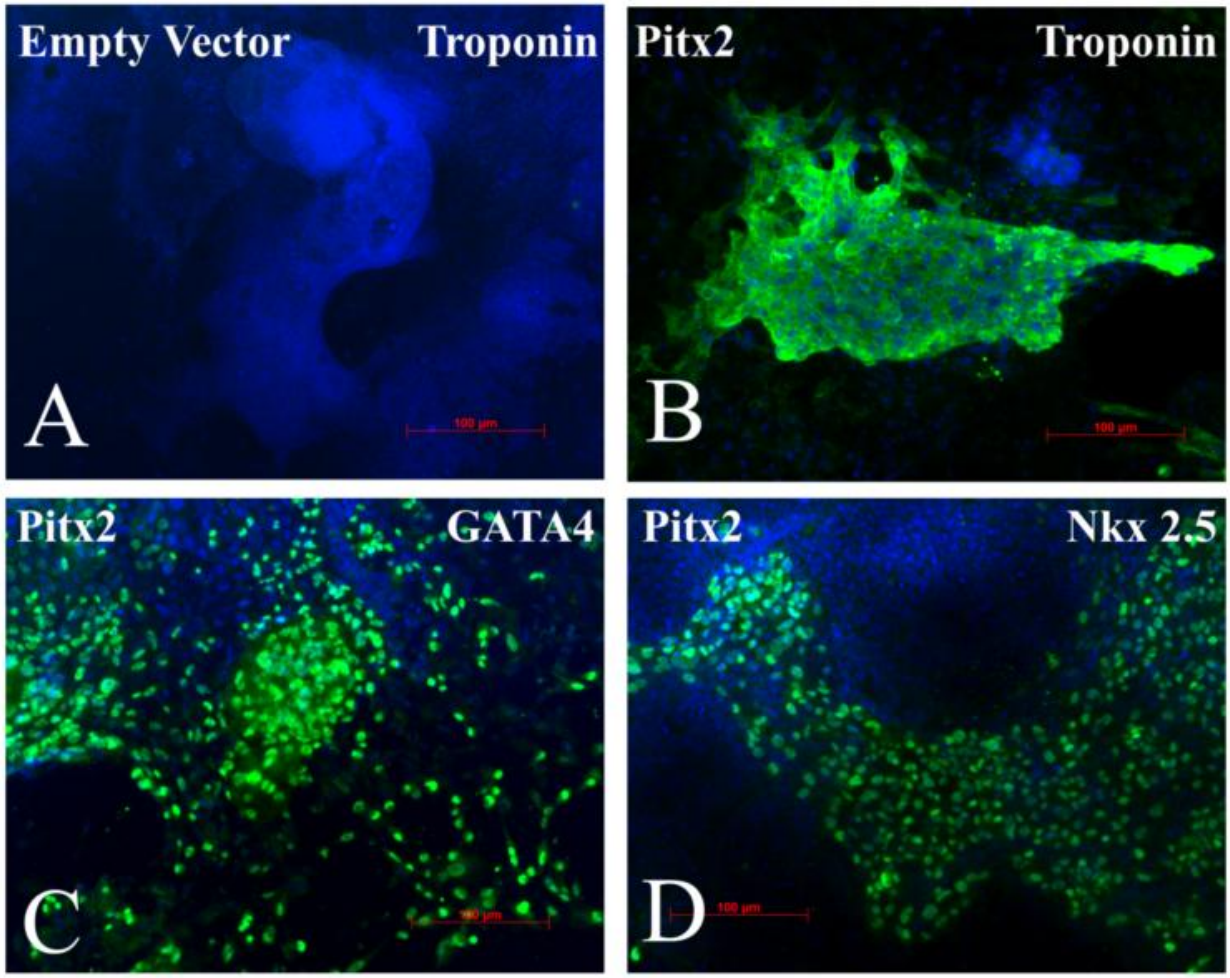


Figure 1: Immunostaining of Pitx2c overexpressing cells.

Panel A: Cardiac Troponin T immunostaining of EB of ES cells transfected with empty vector.

Panel B: Cardiac Troponin T immunostaining of EB of ES cells transfected with Pitx2c.

Panel C: GATA 4 immunostaining of EB of ES cells transfected with Pitx2c.

Panel D: Nkx 2.5 immunostaining of EB of ES cells transfected with Pitx2c.

The extent of differentiation induced by pitx2c was measured by quantifying the number of cardiac troponin positive EBs of different clones. Approximately 30% (29.33 $\pm$ 8.4 %) of the pitx2c overexpressing EBs were found to contain more than 50% of cardiac troponin T positive cells compared to less than 8% of the control group of EBs consisting of empty vector transfected ES cells.

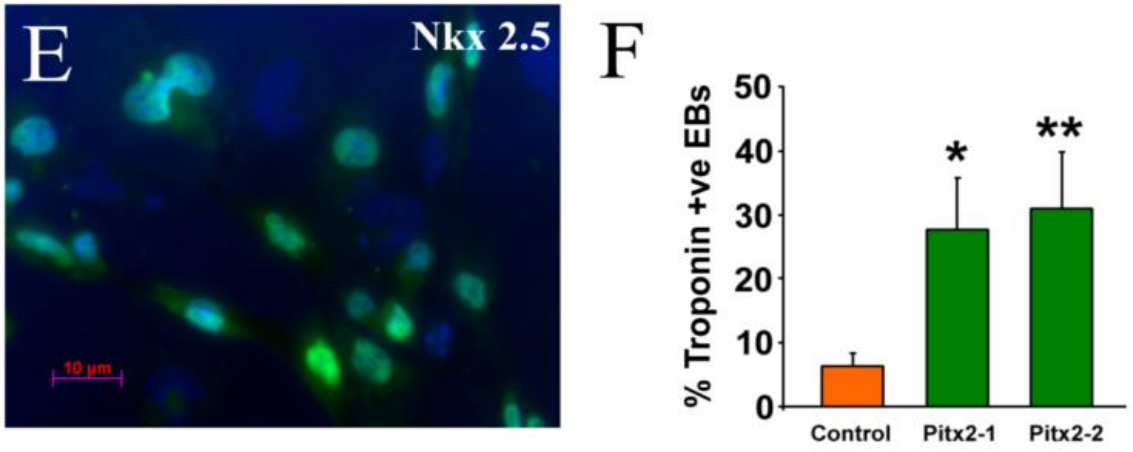


Figure 2: Quantification of Troponin T positive Pitx2c cells.

Left panel: High magnification of Nkx 2.5 immunostaining in Pix2c positive cells.

Right panel: Quantification of troponin positive embryoid bodies from two different Pitx2c overexpressing cell lines. Quantification of cardiac Troponin T positive EBs in different clones of pitx2 transfected cells shows a significant (* and ** $p<0.05$ compared to control group by ANOVA) increase compared to control empty vector transfected cells.

The time course of cardiac specific differentiation was studied by performing a time series analysis of various cardiac specific mRNA levels of pitx2c transfected cells and control cells with real time quantitative PCR.. Transcription factors known to be involved in cardiogenesis, Nkx2.5, Mef2c, Tbx5 and Mef2c were significantly upregulated in the pitx2c transfected cells compared to empty vector-transfected controls.

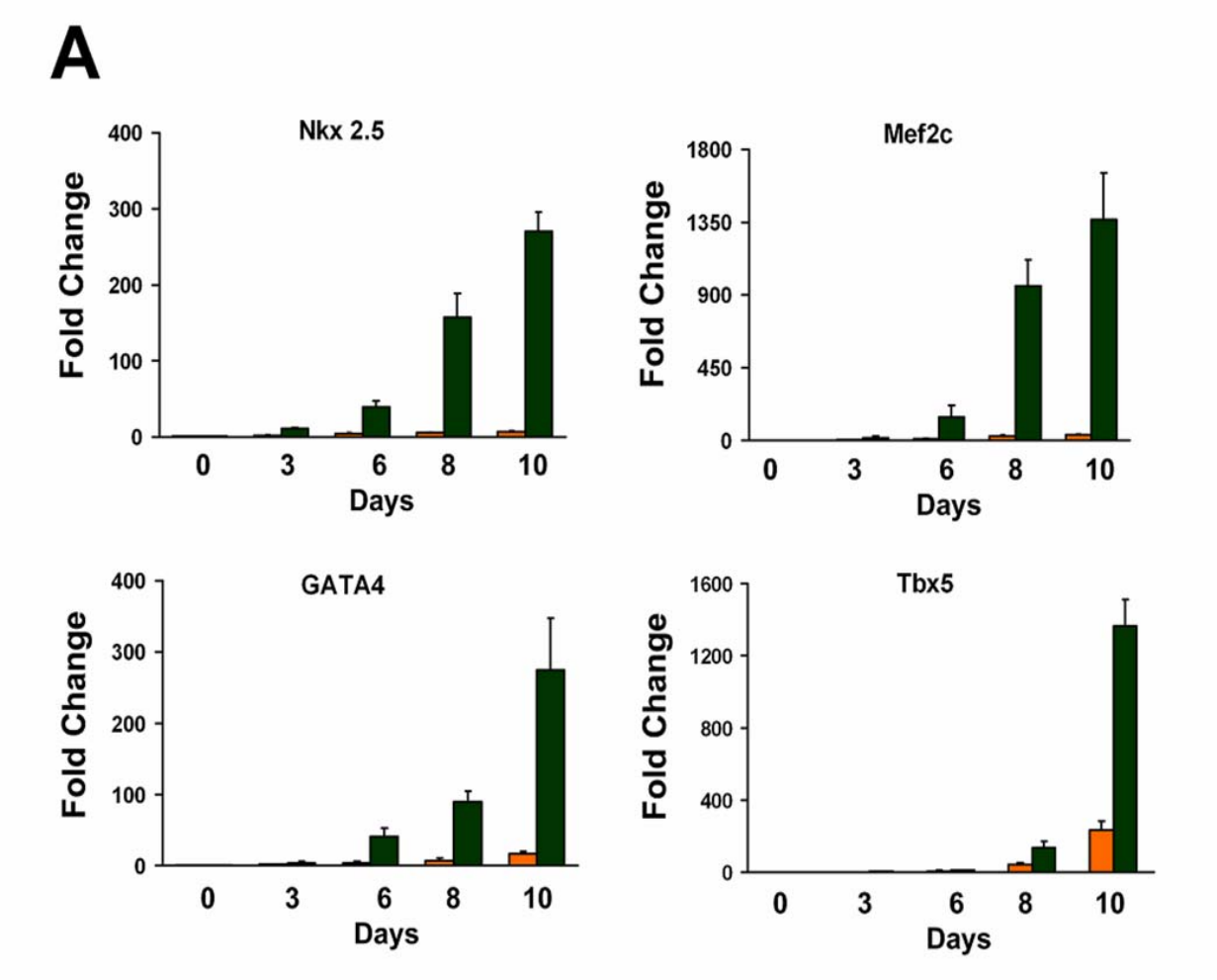


Figure 3: Quantification of mRNA of transcription factors in Pitx2c overexpressing cells. Green bars indicate Pix2c overexpressing cells and orange bars indicate ES cells transfected with empty vector.

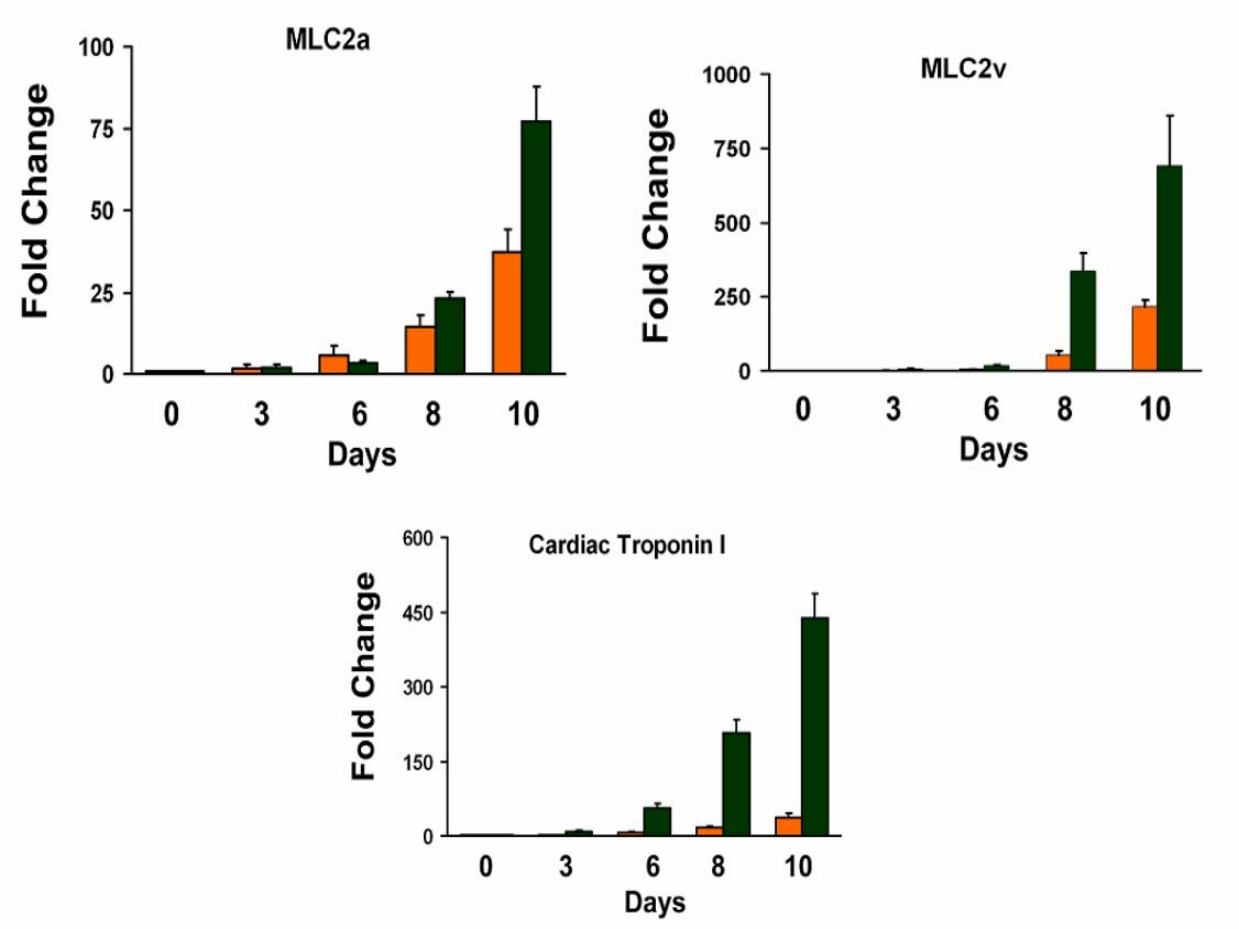


Figure 4: Quantification of mRNA of structural proteins in Pitx2c overexpressing cells. Green bards indicate Pix2c overexpressing cells and orange bars indicate Es cells transfected with empty vector.

Transcripts for structural proteins like MLC2a, MLC2v and Cardiac Troponin I were also significantly elevated during a differentiation protocol of 10 days. Similarly, RT-PCR of control and pitx2c transfected cells showed a clear decrease in Oct3/4 by day 3 of differentiation and a

complete loss by day 8 which indicates that most of the cells are differentiated. Connexin 43 also increased significantly providing evidence of the potential for formation of gap junctions. The alpha and beta isoforms of myosin heavy chain were also upregulated along with cardiac actin which points towards the production of proteins necessary for the contractile apparatus. Pitx2c derived cardiomyocytes possess electrophysiological properties of ventricular cardiomyocytes.

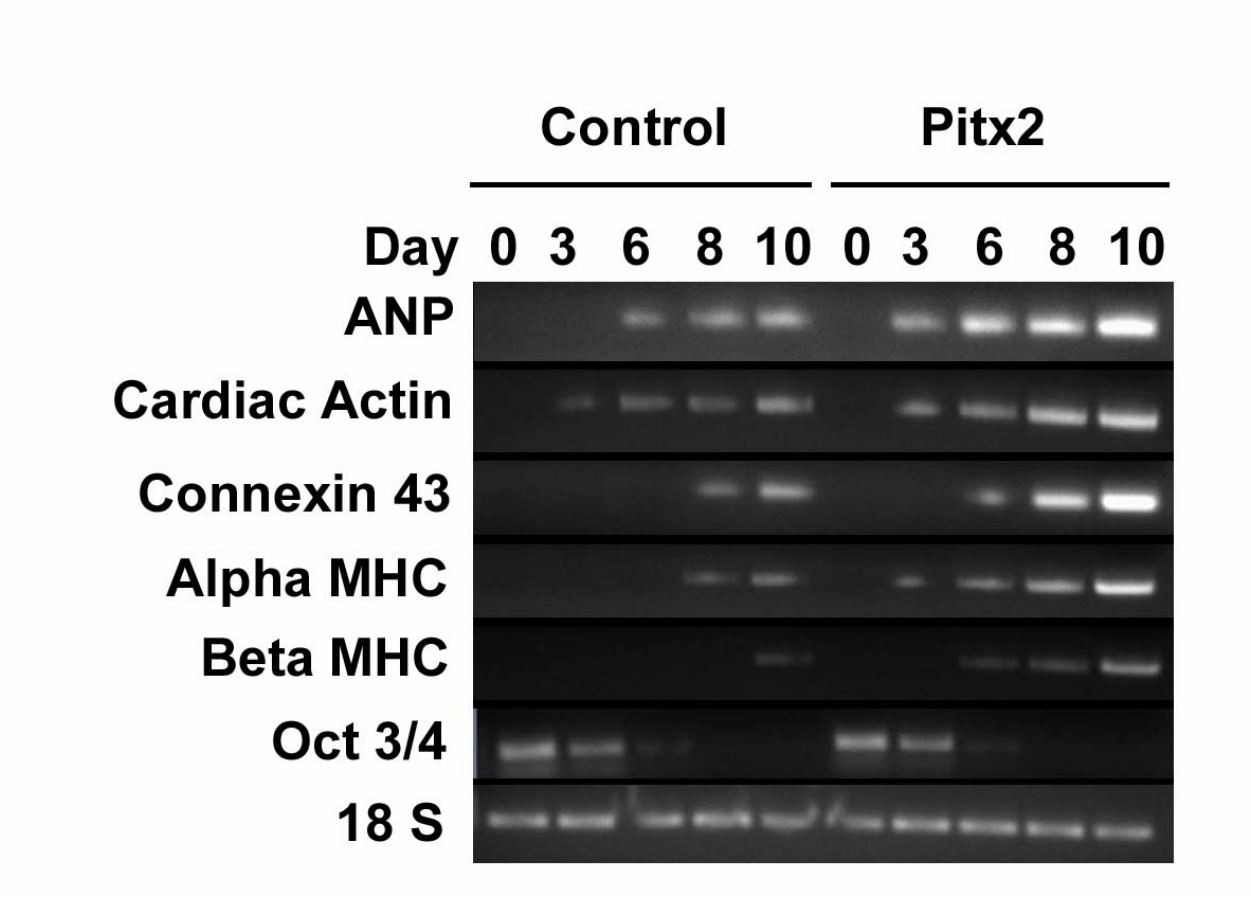


Figure 5: Temporal changes in cardiac specific transcripts in Pitx2c overexpressing cells.

The functional properties of these cultured cardiomyocytes were assayed by fluorescent calcium imaging. Recordings of fluorescence intensity were taken across a pitx2c overexpressing EB using fluo-4AM. Cells were sampled repetitively to build linescan images. There were 4 cells in which Ca^{2+} transients were visible in this EB. Fluo-4AM-loaded cells within this EB generate spontaneous and coordinated increases in cytoplasmic [Ca^{2+}] (fluorescence intensity). Ca^{2+} increased abruptly reaching a maximum within a few milliseconds before declining, and the cycle of Ca^{2+} transients was repeated at a frequency of about 1.25/sec. A video of the fluorescent signal propagating in a plated EB consisting of pitx2c transfected cells shows the wave originating from one side of the EB and propagating towards the other parts. During field stimulation (2Hz), the cells were able to follow the external stimulus indicating that excitation of these cells could be evoked by external activation of sarcolemmal ion channels. These Ca^{2+} transients from both spontaneous and stimulated pitx2-ES cell-derived cardiomyocytes resemble those found in ventricular myocyte-like cells derived from wild type ES cells [123].

Recordings of action potentials from differentiated EBs after 10 days of beating in culture were taken. In this cell, the maximum diastolic potential was –57mV and overshoot was 16mV, with action potential durations at 50% and 90% (APD50 and APD90) of repolarization of 121 and 170msec, respectively. The action potential is very much like that of neonatal rather than adult ventricular myocytes[124], showing a rapid phase of early depolarization (phase 0), an early phase of partial repolarization (phase 1) and a relatively long plateau phase terminated by rapid repolarization. Overall, maximum diastolic potential was -56.0$\pm$3.6mV, overshoot was 9.4$\pm$2.7mV, amplitude was 65.4$\pm$4.2mV, APD50 was 120.4$\pm$16.5msec and APD90 was

181.0±18.9msec (mean±SEM, n=7). No action potentials typical of atrial and nodal cardiomyocytes were detected in any of the recordings which is consistent with our observation that each EB usually has only one predominant type of cardiomyocytes. These findings indicate that pitx2c expression is capable of inducing ventricular-like cellular electrophysiological properties in differentiating mouse ES cells.

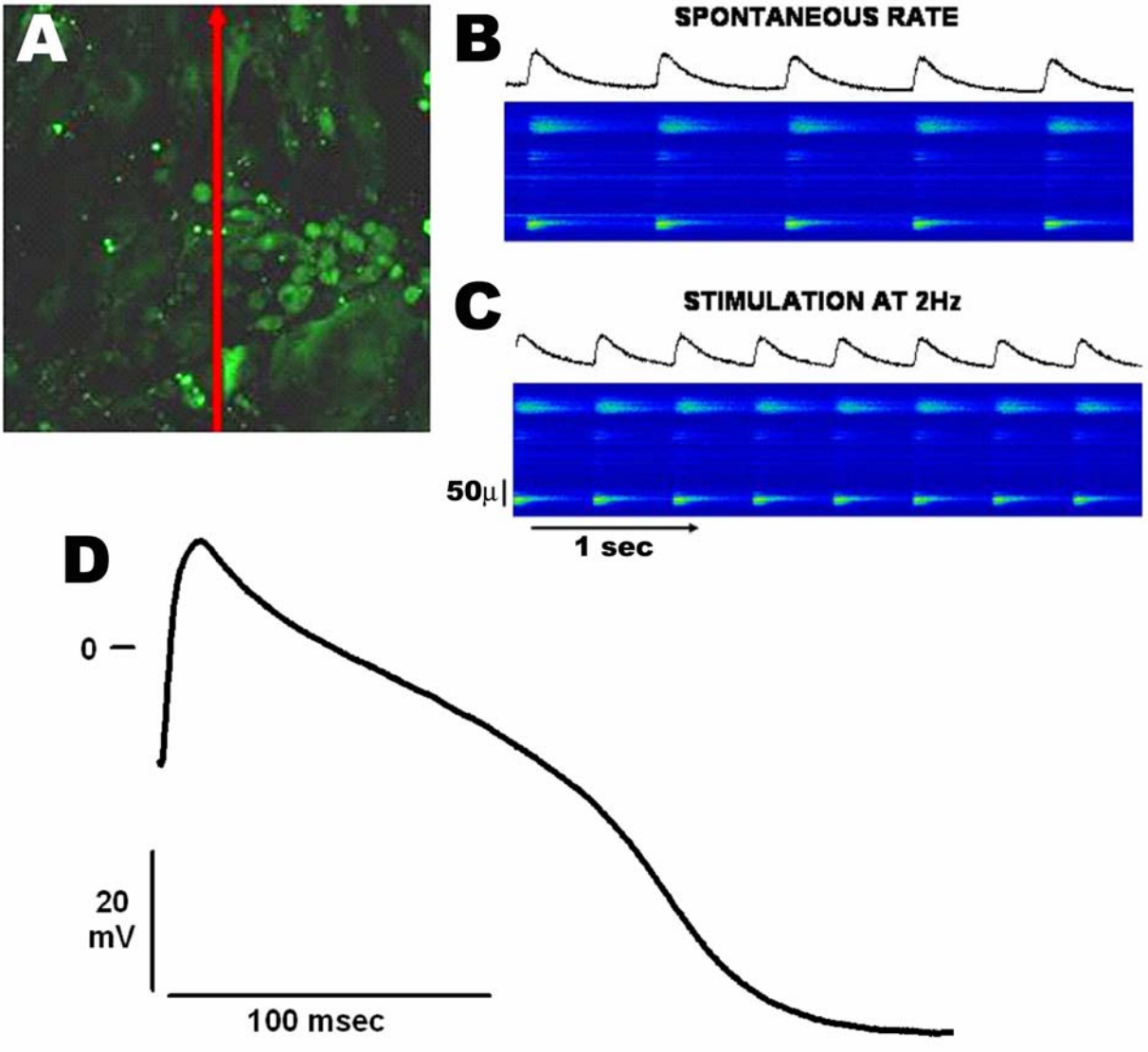
Figure 6: Electrophysiological characteristics of pitx2c overexpressing cells.

Panel A shows a 2-D image of Pitx2c expressing cells in an 8 day EB after loading with fluo-4AM. The red line shows the site of repetitive scanning used to record linescan images in subsequent panels. The scale of the image is 340μ square.

Panel B shows a linescan image recorded by repetitive scanning at site indicated by the red line in Panel A at a rate of 2msec/line. Spontaneous Ca2+ transients were activated simultaneously at a number of cells throughout the EB. The integrated profile across the entire image is shown above.

Panel C shows Ca2+ transients that were activated in response to field stimulation (2Hz), indicating the ability of these same cells within the EB to react to external stimulation and the likely presence of sarcolemmal ion channels.

Panel D shows a ventricular-like action potential in a cell from an EB induced by Pitx2c. The action potential closely resembles that reported by others to be characteristic of left ventricular myocytes from neonatal mouse [124]. Action potentials were recorded by delivery of a current pulse through the microelectrode for 2msec at 1.2X threshold at 2Hz.


A
B
SPONTANEOUS RATE
C
STIMULATION AT 2Hz
50μ
1 sec
D
0
20
mV
100 msec

The efficiency of cell transplantation is often limited by poor cell survival [125,126]. It is possible that stressors like hypoxia, mechanical damage loss of extra-cellular matrix environment contributes to enhanced activation of apoptotic pathways. Studies have shown that cells genetically modified to express pro-survival genes contribute to better outcomes in terms of functional recovery as compared to unmodified cells [127]. The question of cell survival and clearance by the host immune system was addressed by transplanting differentiated ES cells into healthy myocardial tissue in syngeneic mice. Half a million cells were injected into the left ventricle of healthy 129SvJ mice and after 37 days, the mice were sacrificed and their hearts examined for cell engraftment. Mice which received cell transplants had a significant collection of cells at the level of injection as compared to mice which received vehicle (PBS). Given the time frame of 37 days, it is unlikely to be an inflammatory response.

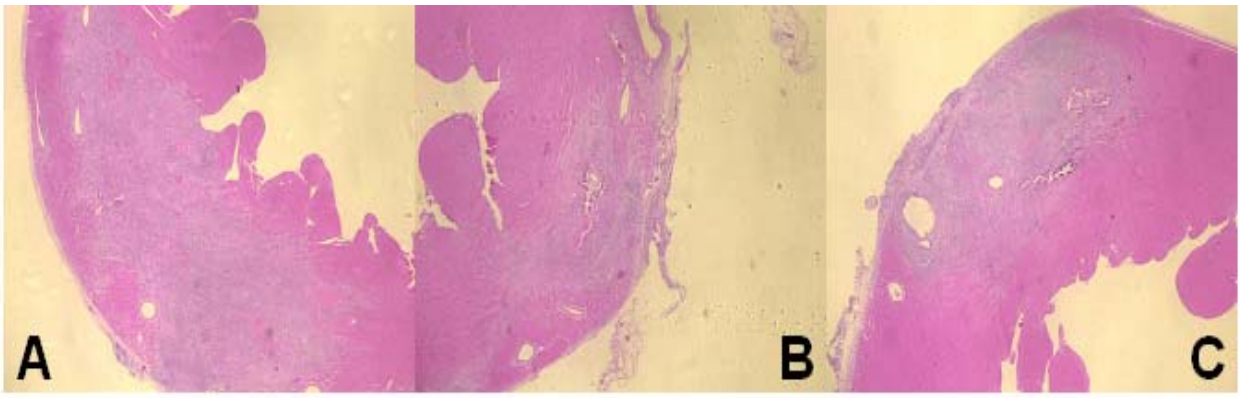


Figure 7: Persistence of transplanted cells in healthy myocardial tissue after one month.

A, B, C are H & E stains of ventricular myocardium with significant areas of cell collections.

ES cells are known to express some class I major histocompatibility antigens (MHC 1) and very few MHC 2 molecules [128]. However, upon differentiation they are upregulated and can cause immunorejection [129]. The low immunogenicity contributes to the unchecked growth of teratomas that form upon transplantation of undifferentiated ES cells [130]. However, the question of the critical amount of cells required for that effect is not known. Transplantation of as few as 1000 cells resulted in significant growth and invasion into the surrounding healthy myocardium.

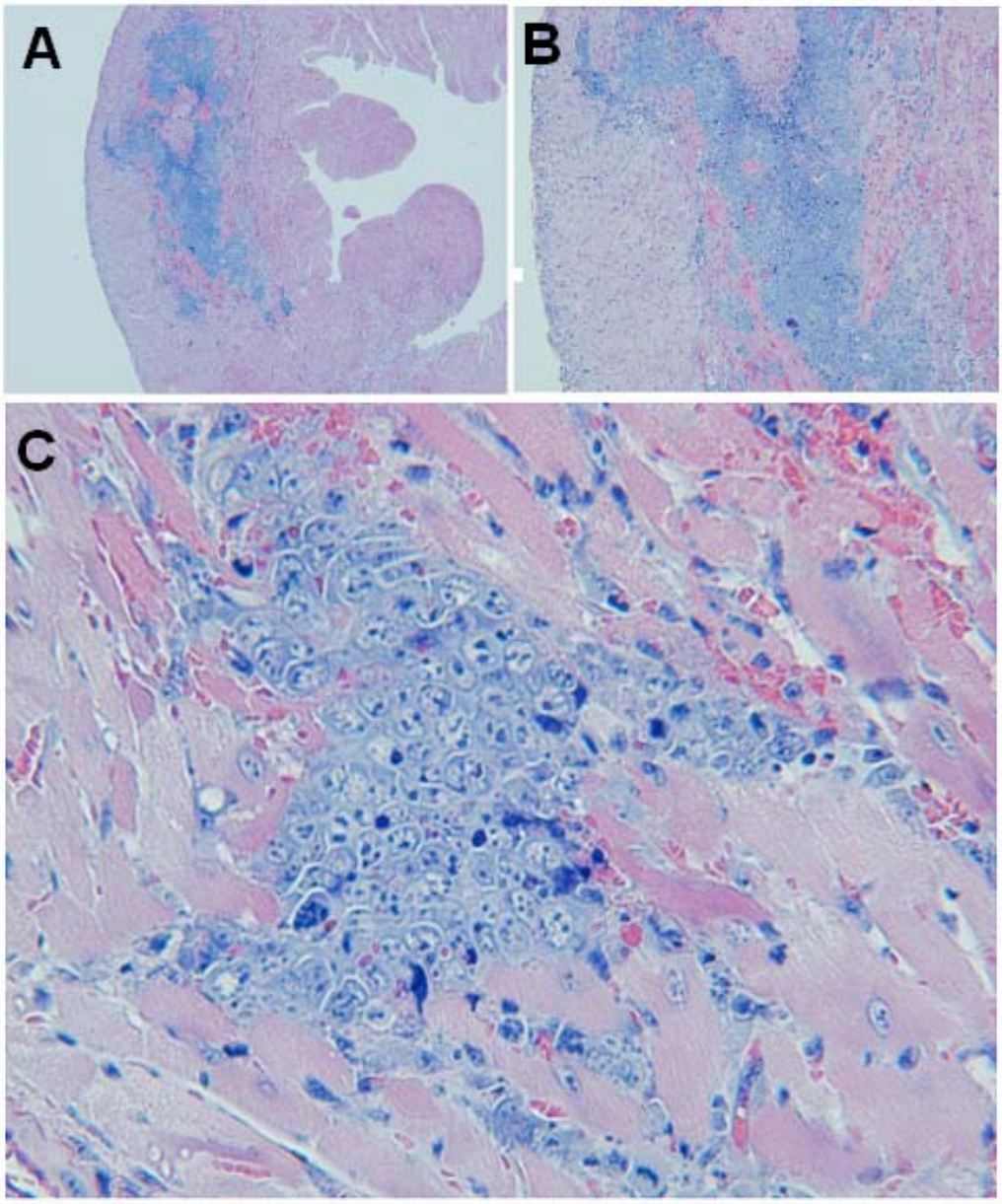


Figure 8: Growth of undifferentiated ES cells in healthy myocardium.

Panel A: The extent of growth of undifferentiated ES cells at the site of injection in the left ventricular wall can be seen.

Panel B: Magnification of panel A and shows the paucity of cytoplasm in these cells.

Panel C: Magnification of panel B and shows mitotic figures.

An animal model of myocardial infarction requires that there is minimal or no sparing of the myocardium in the infarct scar. Engrafted cells in the infarct can be clearly demonstrated by a sex-mismatch transplantation. However, if there is functional improvement even after minimal detection of engrafted cells, it would make sparing a most likely explanation. It is also possible that there is significant remodeling of the scar tissue. The infarcted hearts were sectioned and stained for cardiac troponin T to demonstrate that the infarct is transmural and there is no sparing of the myocardium. High magnification pictures also showed that scar tissue did not have any dense collections of inflammatory cells.

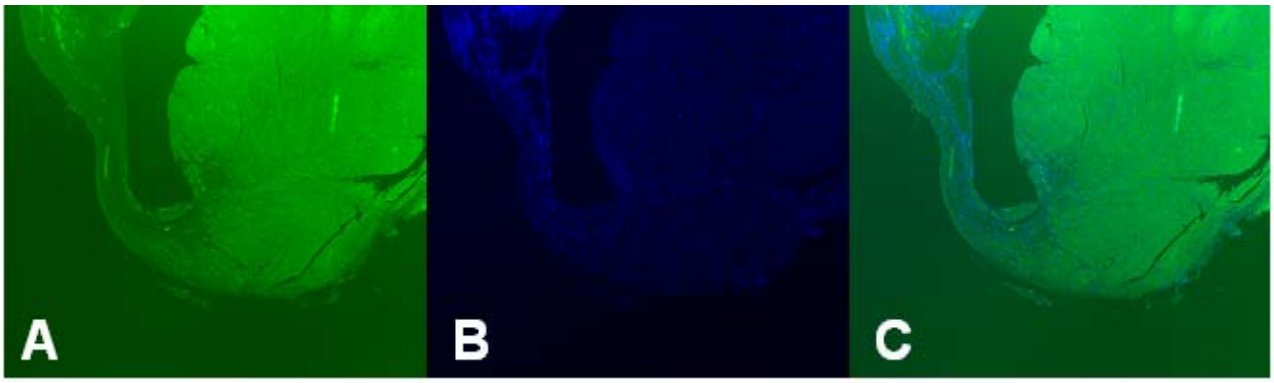


Figure 9: Troponin T staining of Infarct junction

Panel A: Troponin T staining of infarct junction. The infarcted myocardium has no spared tissue and has significantly thinned.

Panel B: DAPI staining of nuclei in the tissue seen in panel A.

Panel C: Merge of Troponin T and DAPI staining seen in panels A and B.

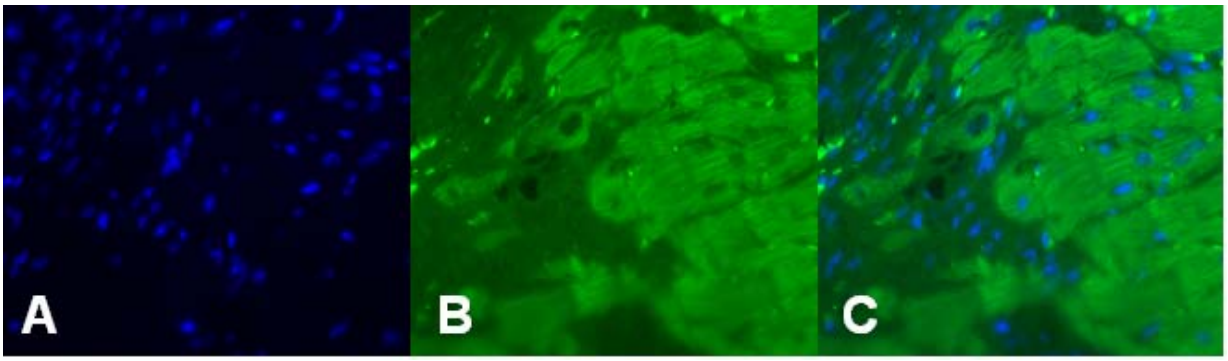


Figure 10: High magnification of Troponin T staining of Infarct junction.

Panel A: DAPI staining of nuclei in the infarct junction.

Panel B: Troponin T staining of infarct junction. There is a clear loss of staining at the junction of the infarcted and normal tissue.

Panel C: Merge of Troponin T and DAPI staining seen in panels A and B.

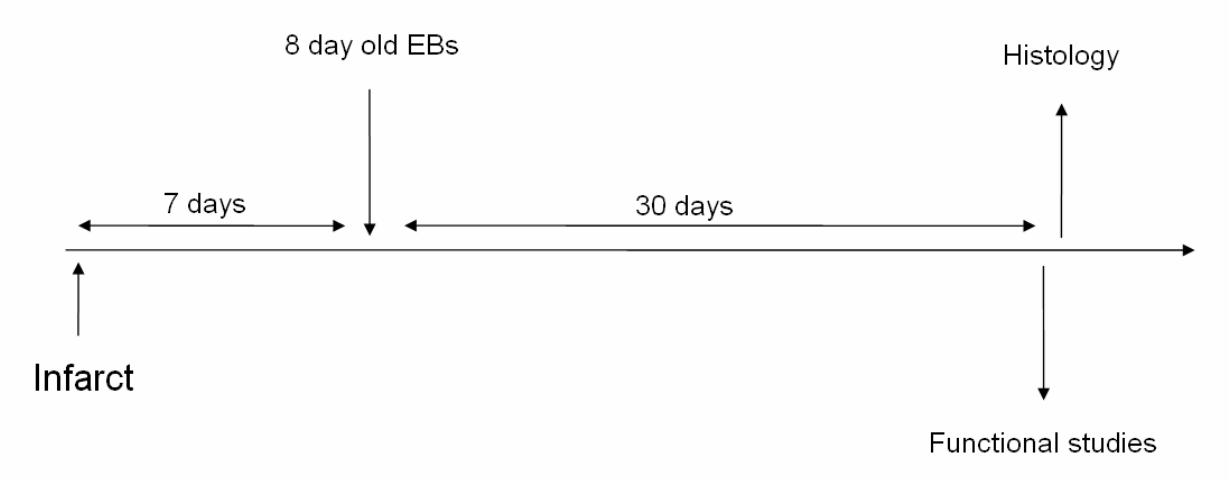


Figure 11: Protocol used for cell transplantation experiments.

Myocardial infarction was induced in female syngeneic (129SvJ) mice by left anterior descending artery ligation. One week later pitx2c overexpressing ES cells that had been differentiated for eight days as embryoid bodies were injected directly into the infarcted ventricular wall of some animals. Others received injections of similarly differentiated wild type R1 ES cells, and others received no injection (myocardial infarction only). Histological examination of the hearts injected with pitx2c overexpressing cells demonstrated the presence of transplanted cells in the scar region that were not present either in the hearts injected with differentiated wild type ES cells or in hearts that received no cell injections.

Some of the transplanted cells located in the middle of the scar tissue had a positive immunostaining for cardiac troponin T. The presence of transplanted cells in the infarcted tissue was verified by FISH analysis for Y chromosome positive pitx2c overexpressing cells in the XX genetic background of host myocardium. In addition, we also observed transplanted (Y chromosome positive) cells in the scar tissue which formed rosette-like structures which had not differentiated into cardiomyocytes.

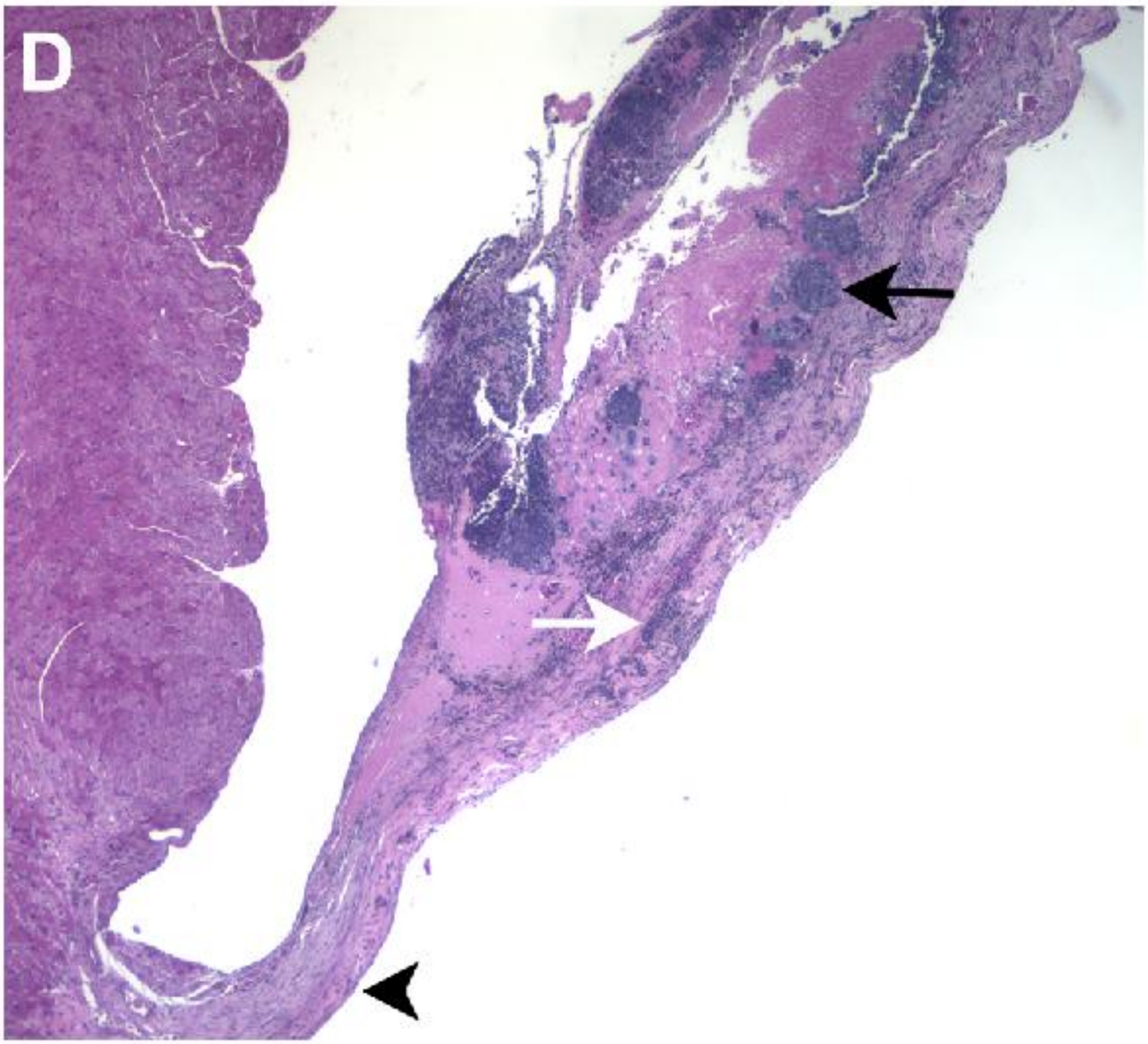


Figure 12: H&E stain of the cross section of the infarcted myocardium injected with Pitx2c overexpressing cells. The relatively acellular nature of the infarct is evident at the junction of the infarct (black arrowhead) in an H&E stain, and the transplanted cells can be seen as in laminar arrangement (white arrow) or as rosettes (black arrow).

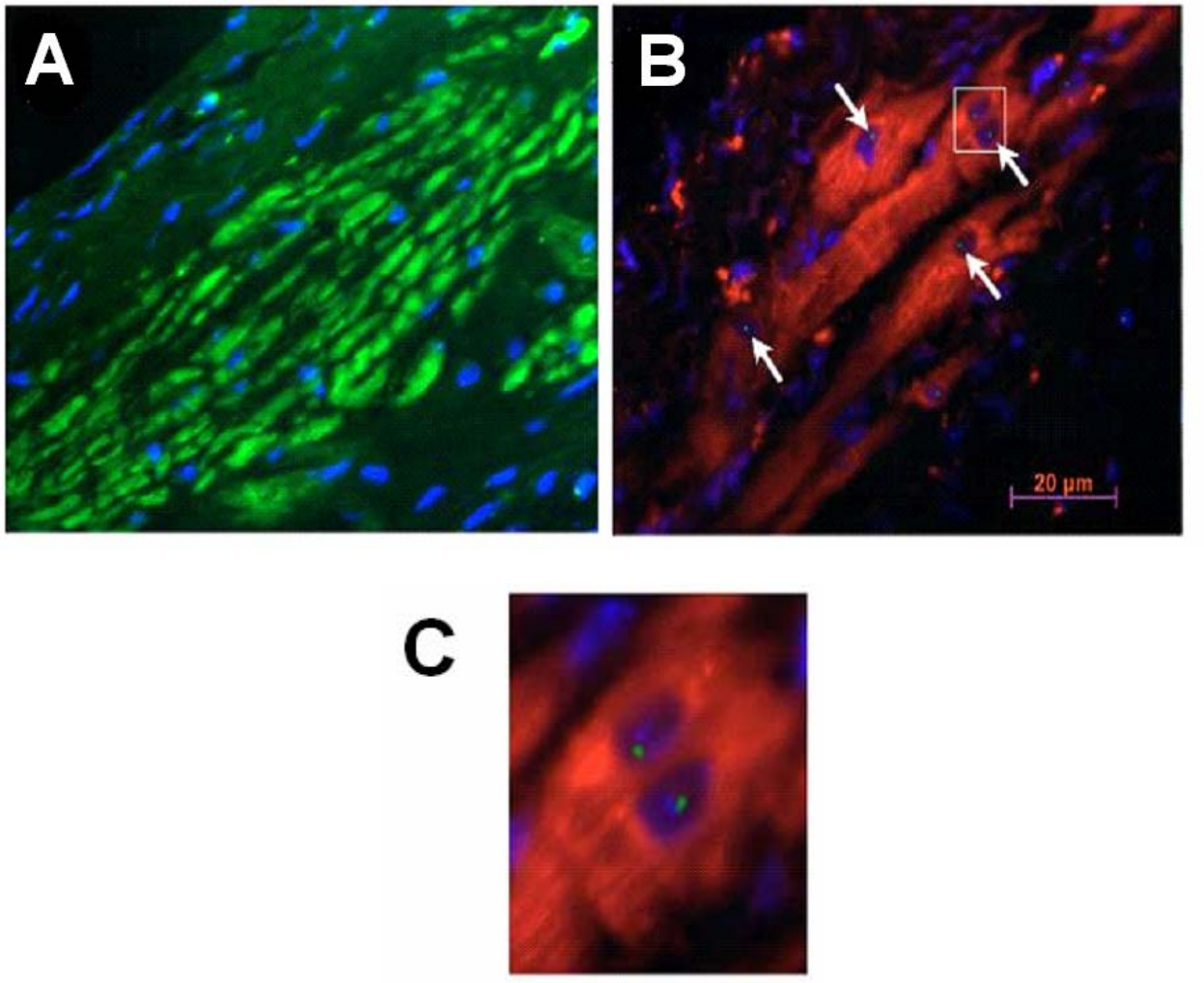


Figure 13: Troponin T staining of engrafted cells in the infarcted myocardium.

Panel A: The section shown in the previous figure was stained for cardiac differentiation of transplanted cells with cardiac troponin T in the scar (20x).

Panel B and C: The presence of transplanted cells was confirmed by FISH analysis of the Y chromosome with an FITC conjugated probe (white arrows) and cardiac differentiation by cardiac troponin T immunoflourescence (red) (40x).

Transplanted cells also differentiated into non-cardiac phenotype with scant cytoplasm and high nucleus to cytoplasm ratio. Their non-cardiac nature was confirmed by staining them with cardiac troponin T.

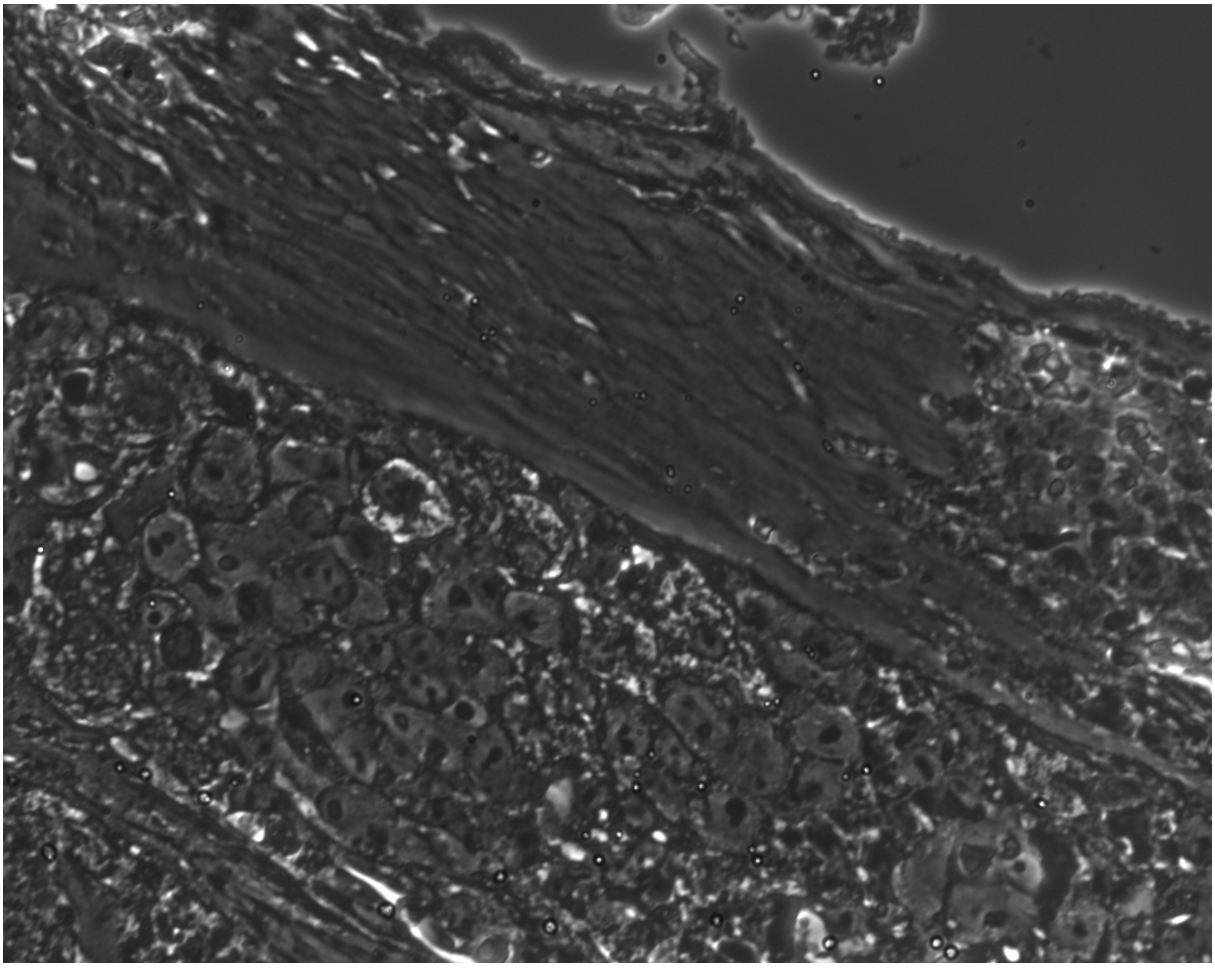


Figure 14: Phase contrast picture of non-cardiac engrafted cells.

These cells are conspicuous for the lack of sarcomeric structures and exhibit a large nucleus to cytoplasm ratio.

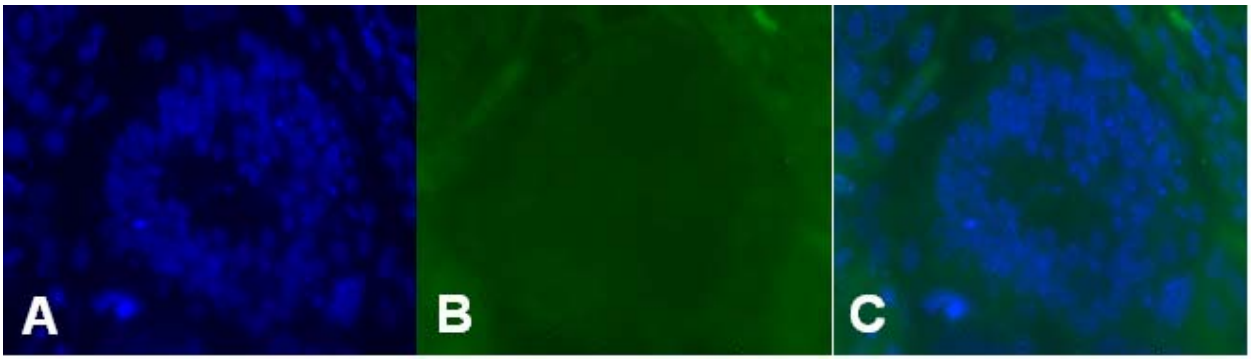


Figure 15: Troponin T staining of engrafted cells in the infarcted myocardium.

Panel A: DAPI staining of nuclei. The cells are arranged in a rosette fashion which is different from the laminar arrangement of troponin T positive engrafted cells.

Panel B: Cardiac troponin T staining.

Panel C: Merge of panels A and B.

Functional effects of Pitx2-ES cells in MI disease model:

Transplantation of differentiated ES cells has been shown to improve myocardial function in rats [131] Mice receiving pitx2c overexpressing cells had a significant 57% improvement in contractility, 59% improvement in relaxation, and 56% improvement in blood pressure as compared to the uninjected (myocardial infarction only) group when observed one month after cell transplantation. By contrast, injection of wild type ES cells produced insignificant changes in any of these measures of cardiac function. All the mice involved in this experiment were sacrificed after one month; however it is possible that this beneficial effect may not be sustained in the long term. There have been reports of such transience of benefits in similar studies [132]. It is unlikely that the cells which stained for troponin T had electromechanically integrated with the host myocardium.

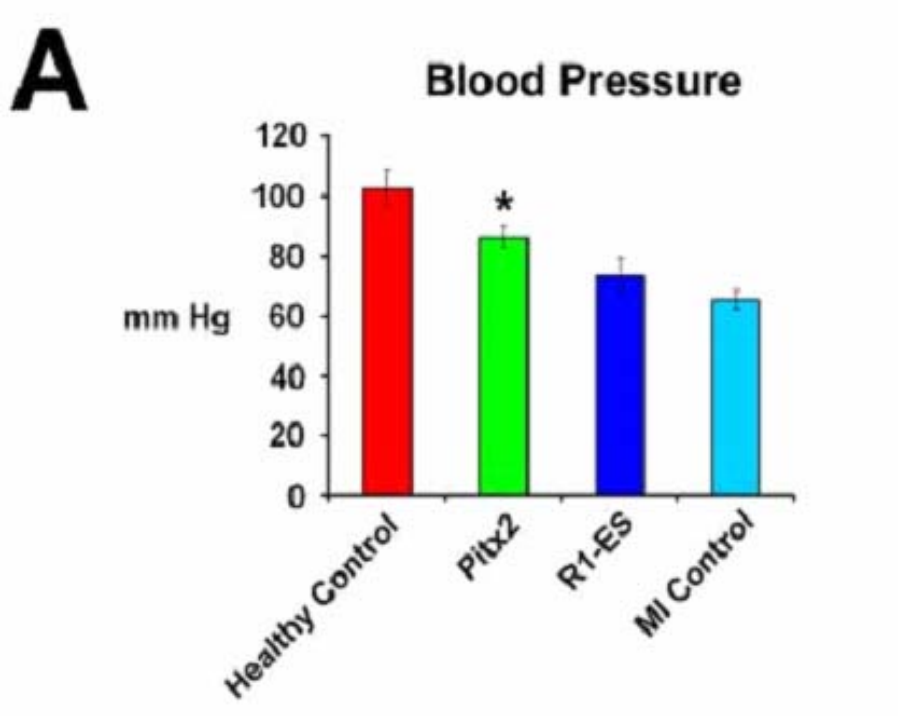
Figure 16: Functional measurements of cell transplanted mice.

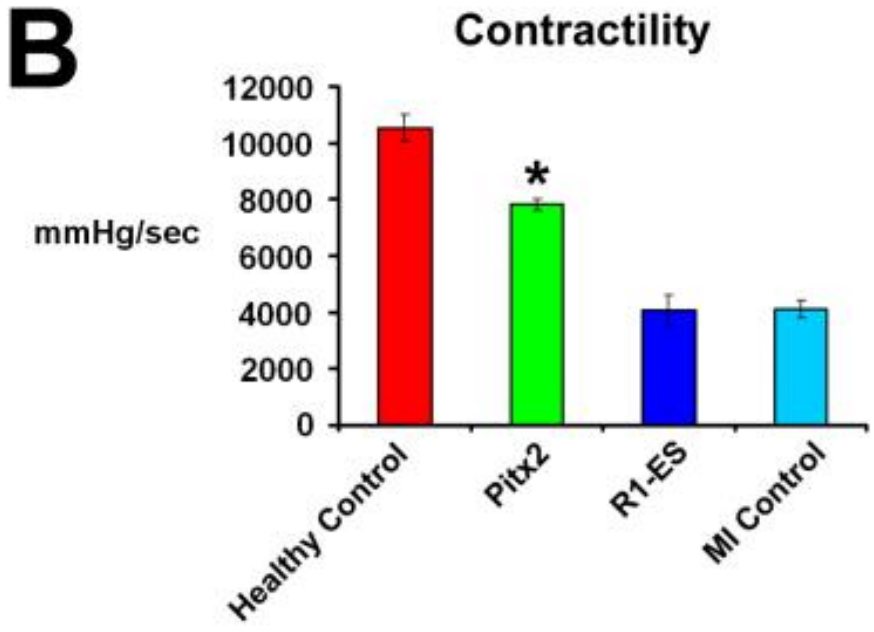
Panel A: Blood pressure measurements.

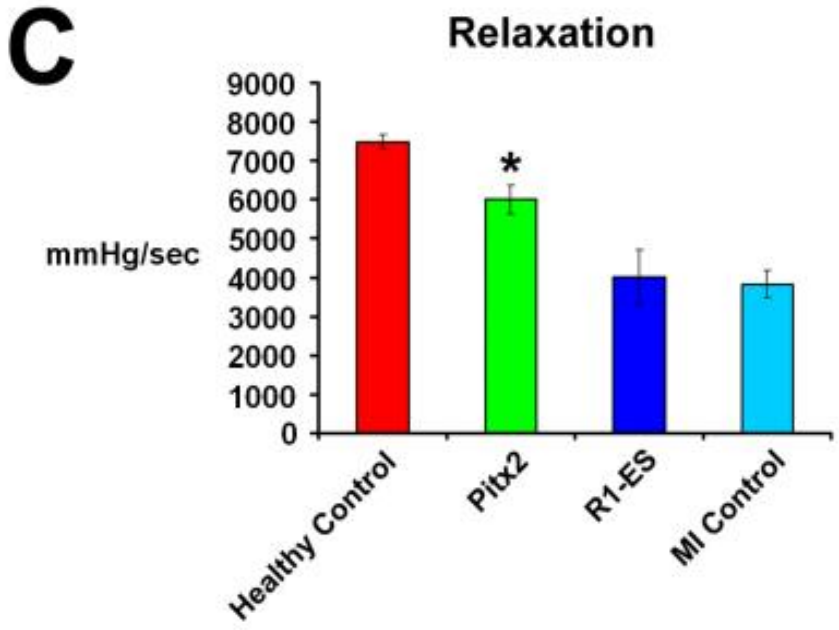
Panel B: Contractility measurements (+ve dp/dt).

Panel C: Relaxation measurements (-ve dp/dt).

(* $p<0.05$ compared to all other groups by ANOVA, R1-ES cell group and MI control group do not significantly differ from each other).


A
Blood Pressure
mm Hg
120
100
80
60
40
20
0
*
Healthy Control
Pitx2
R1-ES
MI Control


B
Contractility
mmHg/sec
12000
10000
8000
6000
4000
2000
0
*
Healthy Control
Pitx2
R1-ES
MI Control


C
Relaxation
mmHg/sec
9000
8000
7000
6000
5000
4000
3000
2000
1000
0
*
Healthy Control
Pitx2
R1-ES
MI Control

Paracrine effects of Pitx2-ES cells in MI disease model:

To investigate the other possible mechanisms that might contribute to functional recovery, Pitx2c overexpressing cells were subjected to hypoxic conditions for 24 hours in a serum free medium and the supernatant was incubated with a biocompatible gel. Subsequently the gel was injected into the infarcted myocardium. The gel used was a self assembling amphophile which contained heparin [133]. Control experiments involved the usage of unconditioned medium with the gel. Similar studies have directly injected hypoxic medium from mesenchymal stem cells and have shown functional recovery in rodent model of myocardial infarction [127,134,135].

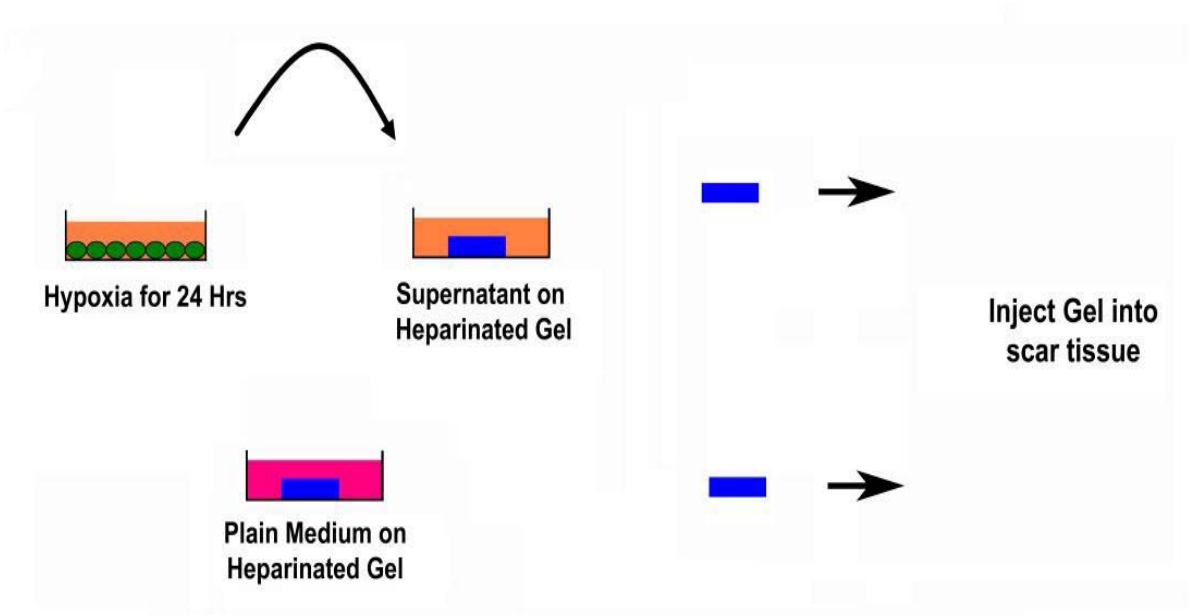


Figure 17: Schematic of the experiment for testing paracrine factors expressed by pitx2c overexpressing cells.

The protocol used for these experiments was the same as that of the cell transplantation experiment. Functional measurements were done one month after the gel injections and showed similar functional recovery comparable to cell transplantation experiments.

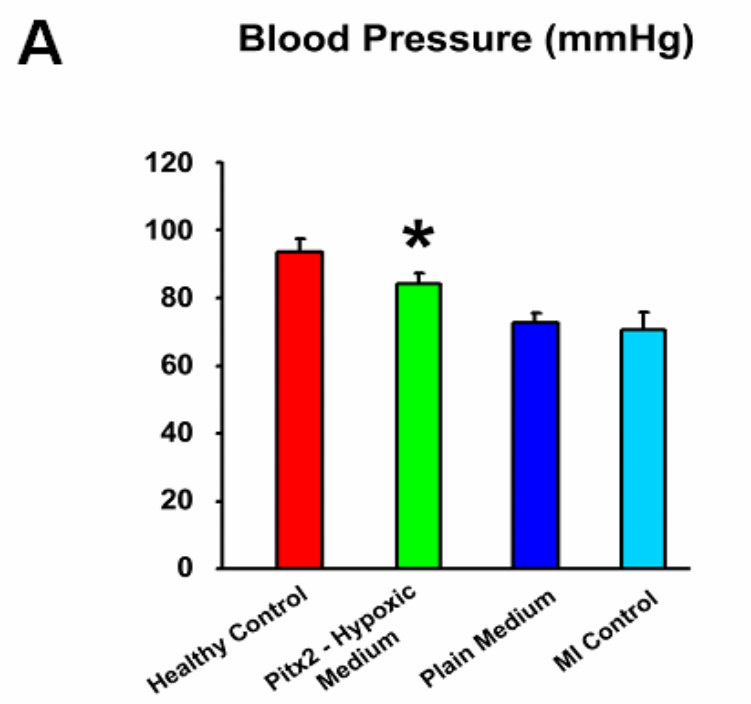
Figure 18: Functional measurements of gel injected mice.

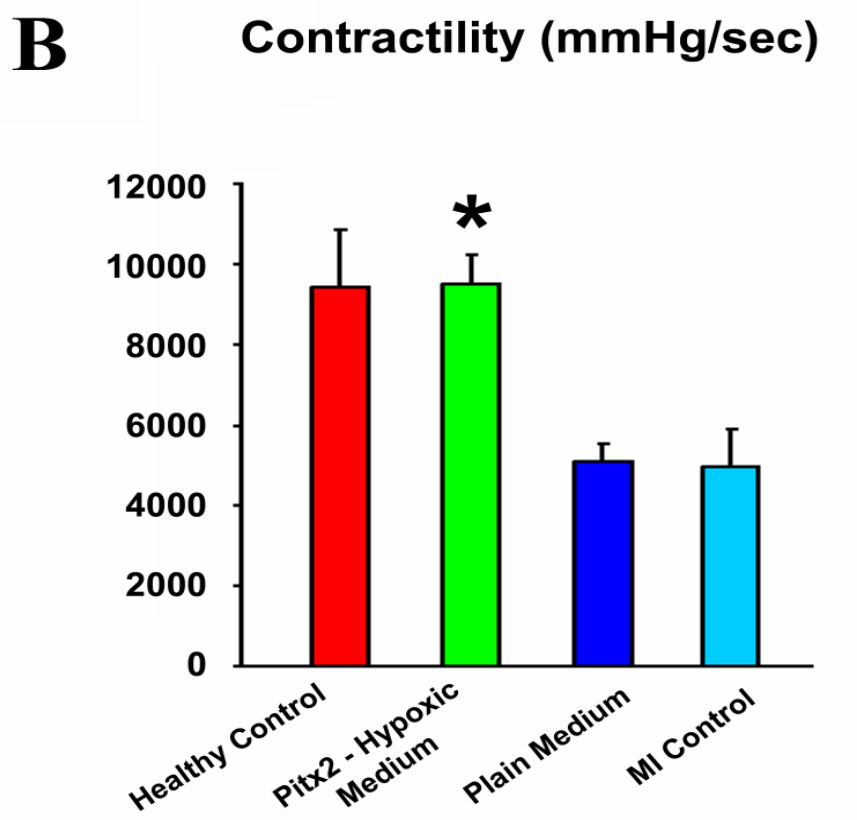
Panel A: Blood pressure measurements.

Panel B: Contractility measurements (+ve dp/dt).

Panel C: Relaxation measurements (-ve dp/dt).

(* $p<0.05$ compared to all other groups by ANOVA, plain medium group and MI control group do not significantly differ from each other).


A
Blood Pressure (mmHg)
120
100
80
60
40
20
0
*
Healthy Control
Pitx2 - Hypoxic Medium
Plain Medium
MI Control


B
Contractility (mmHg/sec)
12000
10000
8000
6000
4000
2000
0
*
Healthy Control
Pitx2 - Hypoxic Medium
Plain Medium
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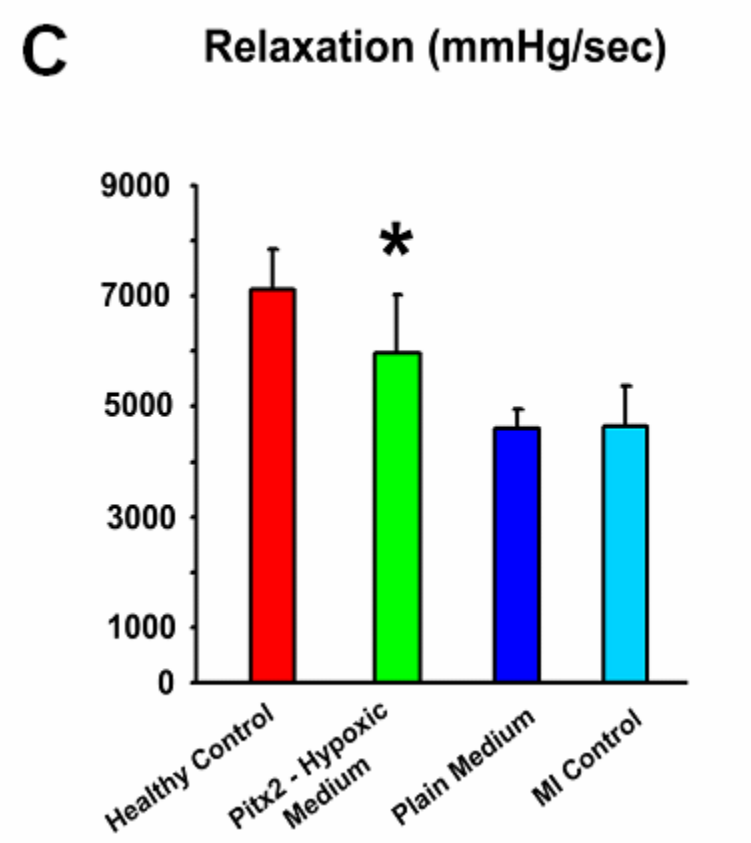
C
Relaxation (mmHg/sec)
9000
7000
5000
3000
1000
0
*
Healthy Control
Pitx2 - Hypoxic Medium
Plain Medium
MI Control

Similar cell transplantation experiments were attempted in mouse model of Chagas disease in collaboration with Dr. David Engman. Due to contamination of the transplanted cells with some undifferentiated cells, extensive teratoma formation was observed.

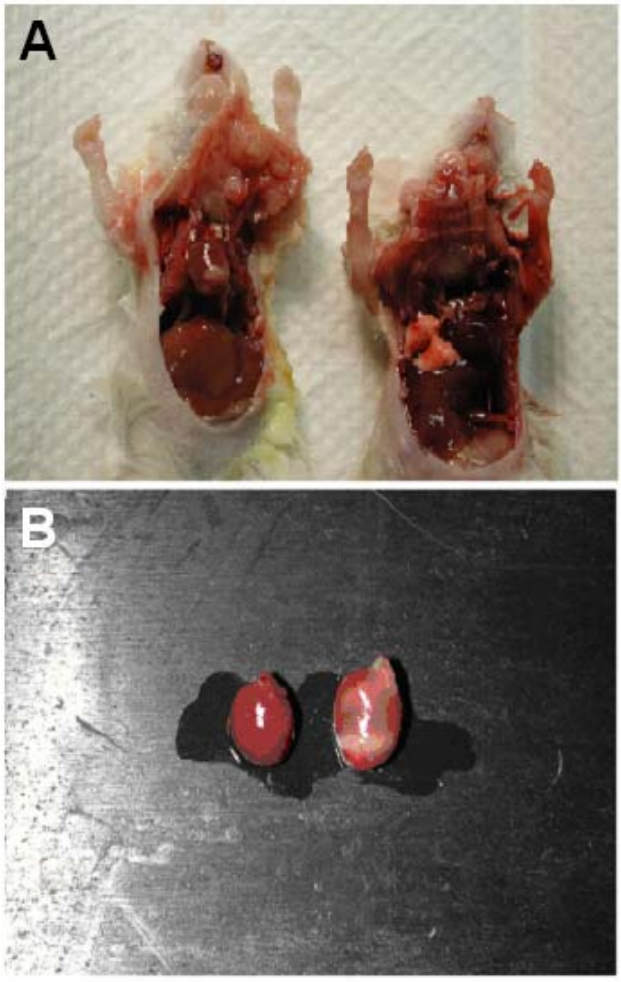

Figure 19: Cell Transplantation in mouse model of Chagas disease.

Panel A: Tumors are visible in the thoracic cavity of chagasic mice injected with Pitx2c overexpressing cells (left side) as compared to mice with PBS injection (right side).

Panel B: Gross morphology of cell-injected heart (right) and PBS injected heart (left).

Effect of overexpression of dominant negative E-Cadherin on cardiogenesis in ES cells:

Overexpression of Nkx2.5 in ES cells did not have any cardiogenic effects. Nkx 2.5 tagged with CFP at the C terminus showed nuclear localization but differentiation of Nkx 2.5 overexpressing cells does not show a higher incidence of cardiomyocyte formation in the differentiated cells. Deletion of beta catenin in the embryonic endoderm causes the formation of multiple hearts [22]. It is known that stabilization of beta catenin by a Wnt-independent mechanism affects cardiomyocyte growth [136]. The effects of beta catenin have been shown to be biphasic; effects in the early stages of differentiation of ES cells is pro-cardiogenic and beta catenin is anti-cardiogenic in the later phase of differentiation [137]. Beta catenin signaling also affects the renewal and differentiation of Isl 1 +ve cardiac progenitors [80]. Antagonizing beta catenin signaling in mouse embryonic stem cells with binding partners of beta catenin also promotes cardiogenesis [138].

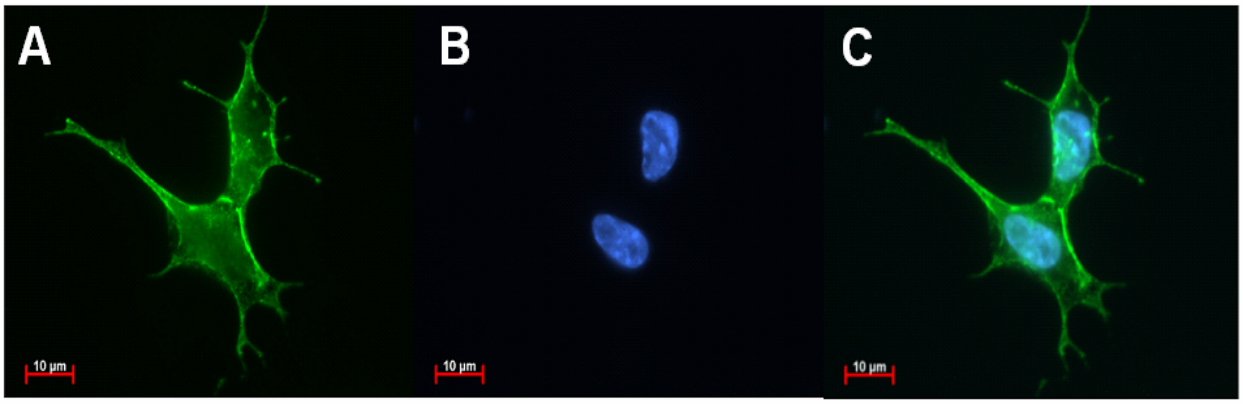

Figure 20: Beta catenin staining of DN E-Cadherin expressing cells.

Panel A: Beta Catenin (FITC) showing a membranous distribution.

Panel B: DAPI staining. Panel C: Merge of panels A and B.

Beta catenin signaling can also be altered by using other proteins which bind to it. Dominant negative E-Cadherin has a beta catenin binding domain but no extracellular domain. It can sequester beta catenin and promote terminal differentiation in human epidermal keratinocytes [139]. Overexpression of DN E-Cadherin in ES cells promotes cardiogenesis. ES cells when stably transfected with DN E-Cadherin exhibit an increased tendency to acquire cardiac markers. The number of spontaneously beating clusters also increases. The DN E-Cadherin molecule has an externally engineered epitope: 'H2kd'. It is possible to check the interaction between DN E-Cadherin and beta catenin by performing a co-immunoprecipitation using antibodies against beta catenin and H2kd.

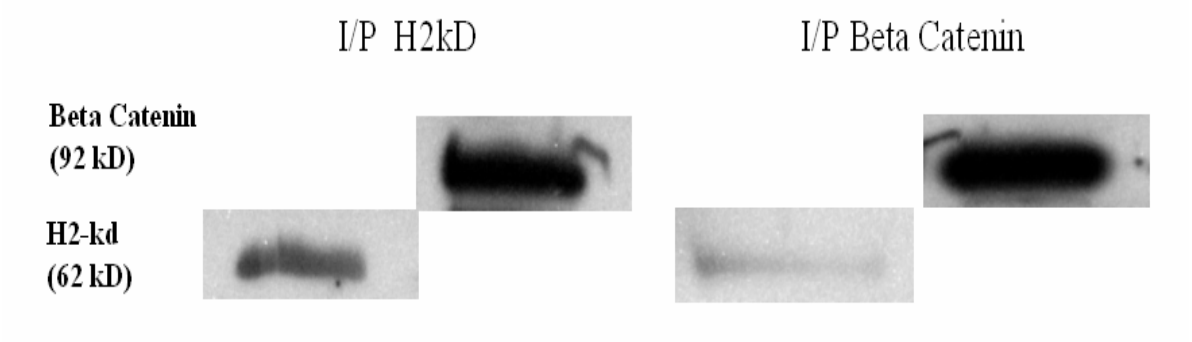

Figure 21: Co-immunoprecipitation of beta catenin and H2kd.

The cardiac nature of the differentiated cells derived from ES cells overexpressing DN E-Cadherin were confirmed by immunostaining them with Nkx 2.5, Gata 4, cardiac troponin T , myosin heavy chain

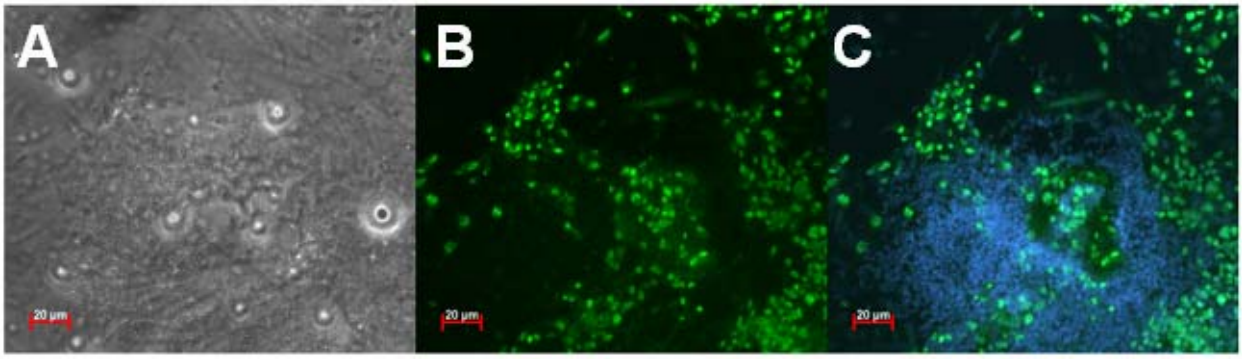

Figure 22: Nkx 2.5 staining of DN E-Cadherin overexpressing cells.

Panel A: Phase contrast picture of EB.

Panel B: Nkx 2.5 (FITC) showing a punctate distribution.

Panel C: Merge of DAPI and Nkx 2.5.

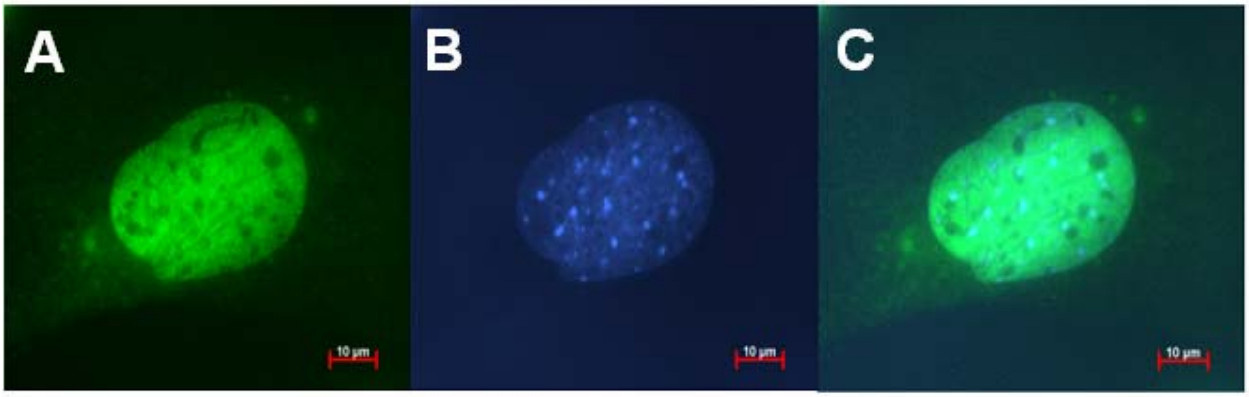

Figure 23: High magnification of Gata 4 staining of DN E-Cadherin overexpressing cells.

Panel A: Nkx 2.5 (FITC) showing a nuclear distribution.

Panel B: DAPI staining of the nucleus.

Panel C: Merge of panels A and B.

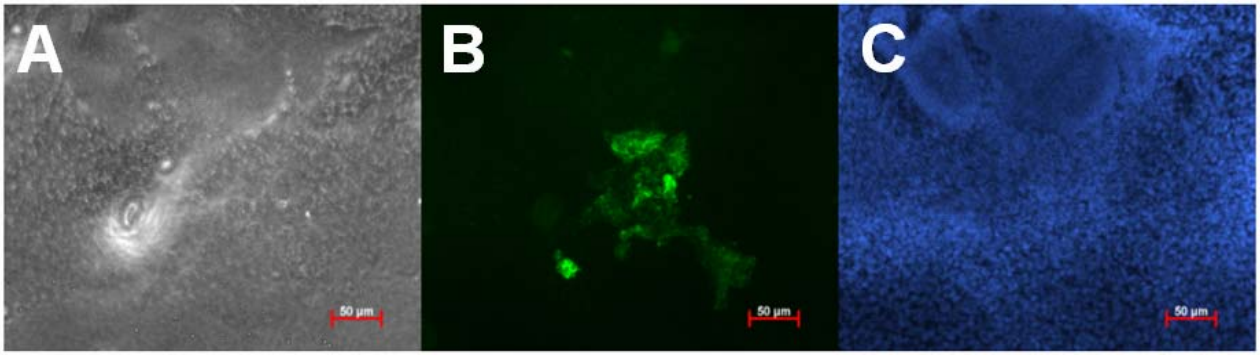

Figure 24: Troponin T staining of DN E-Cadherin overexpressing cells.

Panel A: Phase contrast picture of EB.

Panel B: Troponin T (FITC) showing a nuclear distribution.

Panel C: DAPI staining of nuclei.

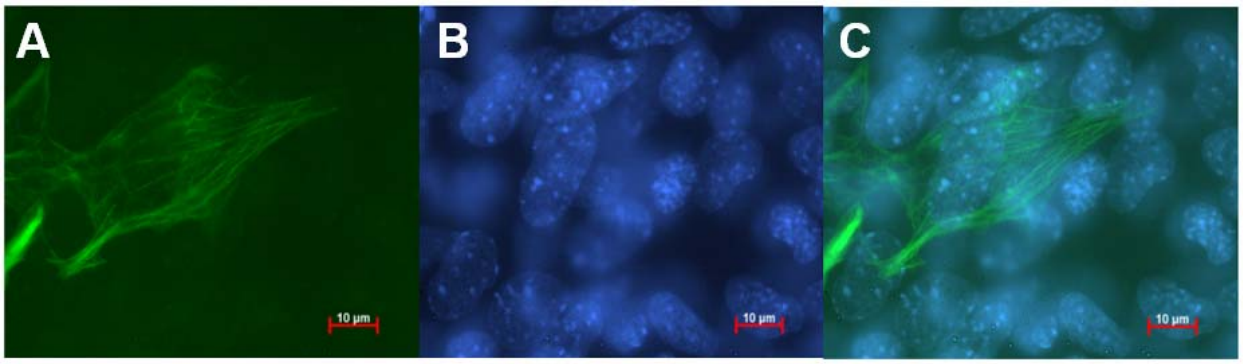

Figure 25: High magnification of Troponin T staining of DN E-Cadherin overexpressing cells.

Panel A: Troponin T (FITC) showing sarcomeric distribution.

Panel B: DAPI staining of the nucleus.

Panel C: Merge of panels A and B.

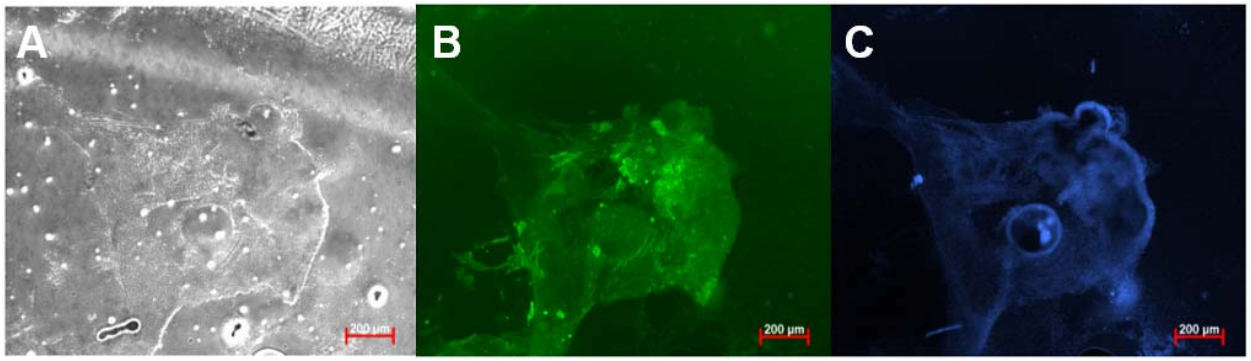

Figure 26: MHC staining of DN E-Cadherin overexpressing cells.

Panel A: Phase contrast picture of EB.

Panel B: Alpha Myosin Heavy Chain (FITC).

Panel C: DAPI staining of nuclei.

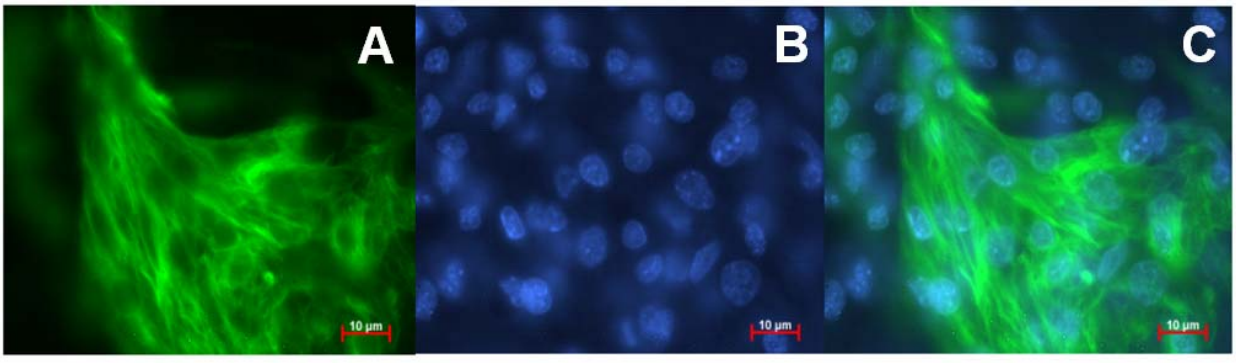

Figure 27: High magnification of MHC staining of DN E-Cadherin overexpressing cells.

Panel A: Alpha Myosin Heavy Chain (FITC) showing a sarcomeric distribution.

Panel B: DAPI staining of the nucleus.

Panel C: Merge of panels A and B.

Discussion:

A number of techniques have been described for enhancing the differentiation of ES cells into cardiomyocytes [32,67,114,122,140,141], but the low efficiency and reliability of these techniques has limited their utility for myocardial regeneration. In this study we have found that we can reliably and reproducibly induce cardiomyocyte differentiation of ES cells by overexpressing pitx2c, a bicoid related homeobox transcription factor involved in the generation of left-right asymmetry of the developing heart. Pitx2c expression enhanced the expression of GATA-4 and Nkx2.5 [142], both of which have been implicated in the development of cardiomyocytes [59,75,143-145]**.** However overexpression of GATA-4 or Nkx2.5 alone in murine ES cells is insufficient to induce cardiomyocyte differentiation [72,75,76,146], suggesting that pitx2c acts through other pathways as well.

Prior studies have demonstrated that notch signaling inhibits cardiomyocyte differentiation [114,147]. This suggests that some of the effects of pitx2c overexpression in these experiments may be mediated by inhibition of notch signaling. Furthermore, null mutation in ES cells of RBP-J, which facilitates transcription of notch downstream targets such as hes-1, increases the number of beating clusters [114]. These observations *in toto* suggest that inhibition of notch signaling in ES cells might be essential for the generation of cardiomyocytes. Pitx2c has not previously been described to be an inhibitor of Notch signaling. However, notch/HES signaling is known to be antagonistic to wnt/β-catenin signaling. It has been shown that ablation of notch signaling in the epidermis by targeted disruption of the Notch 1 gene elevated β-catenin signaling, and that reintroducing a dominant active form of notch restored β-catenin to normal

levels in these animals [148]. Furthermore, presenilin, the gamma-secretase responsible for intramembrane proteolysis of notch following notch-ligand binding, has been shown to be a negative regulator of β-catenin signaling. Since pitx2 is a direct downstream target of β-catenin [40], the effects of inhibition of notch signaling on cardiomyocyte lineage commitment could thus possibly be explained, in part, by relief of notch repression of β-catenin regulated genes.

Several cell types such as hematopoietic stem cells [149], fibroblasts and primary myocardial cell cultures [150] have been used for transplantation to improve recovery of cardiac function in infarcted mice. However, the benefit of such interventions seems to rely more on neoangiogenesis and hence increased perfusion to the damaged ventricular wall rather than direct substitution of infarcted tissue with transplanted cardiomyocytes. In this study, we demonstrate that ES cells with a preferential cardiac differentiation program engraft and differentiate into cardiomyocytes *in-vivo.* We did not observe the formation of teratomas, neoangiogenesis or evidence of electromechanical coupling of the transplanted cells with the host myocardium. Some of the transplanted cells were observed to be in a laminar arrangement in isolated places in the middle of the scar. Based on measurements of contractility, we believe that the transplanted cells might have actively participated in normal contractions, but some of the beneficial effects might be explained by altered elastic properties of the infarcted tissue after engraftment. Notably, not all of the engrafted cells appeared to adopt the cardiomyocyte phenotype, suggesting that both mechanisms could be responsible for the beneficial effects. Furthermore, as against the common observation that most transplanted cells are lost within the first few days after transplant, we observed that transplanted cells remained engrafted in the myocardium even after one month of transplantation. It is also possible that improvement in cardiac function is

secondary to some unknown beneficial paracrine factors arising from pitx2c activation that may also contribute to the continued survival of the transplanted cells, remodeling of the scar tissue and improved contractility of the residual myocardium which synergistically help in the recovery of the infarcted mice. We tested the possibility of the involvement of paracrine factors by subjecting pitx2c overexpressing cells to hypoxia and soaking the paracrine factors onto a gel. The injection of this gel in a mouse model of myocardial infarction caused significant functional recovery as compared to mice which received a gel soaked with plain medium.

In summary, we have found that overexpression of pitx2c in ES cells reliably results in differentiation of a high proportion of the cells into cardiomyocytes. Transplantation of these cells into experimentally infarcted myocardium results in engraftment and in significant functional improvement. These findings offer a reliable technique for generating large numbers of cardiomyocytes for regenerative therapies.

Materials and methods:

Cell Culture and Differentiation: R1 ES cell line was obtained Dr. Rex Chisholm (Northwestern University). ES cells were cultured without feeder cells on gelatin coated tissue culture flasks in media described previously [151] with LIF (ESGRO, Chemicon). Cells were routinely observed for SSEA-1 and Oct4 staining by immunofluorescence and were never kept in continued cell culture for more than 15 passages. For EB inductions, dissociated ES cells were counted by trypan blue exclusion and seeded at 2-5x 10^5 cells/ml in 10 cm petri dishes (Falcon) in induction media to enable them to grow in suspension without attaching to the bottom of the dish. Induction media was identical to the cell culture media except that LIF and β-mercaptoethanol were not added. Cells were grown in suspension as EBs for a total of 8 days; media was replenished every two days. On day 8 of the induction, EBs were dissociated by incubation with 0.25% trypsin-EDTA (Invitrogen) for 5 minutes and were plated onto laminin or fibronectin coated glass coverslips (with equivalent results) in induction media. One 10 cm petri dish was plated into 12 wells of a 24 well plate after trypsinization. Spontaneously contracting cell clusters were observed 2 days afterwards and were observed for up to 4 weeks.

Cloning and RT-PCR: Pitx2c was cloned from day 4 embryoid bodies by RT-PCR analysis. Reverse transcription was performed using the Thermoscript RT-PCR kit (Invitrogen) and PCR (30 cycles) was performed. Primers used for both the cloning and the detection of pitx2c was as follows: forward primer (BamH1 site), 5'-ACG GAT CCA TGA ACT GCA TGA AAG GCC CGC TG-3'; reverse primer (XbaI site), 5'-TTT CTA GAT CAC ACC GGC CGG TCG ACT GC-3'. Template for cloning pitx2c cDNA was RA treated EBs. All PCR cloning was done

using the Thermoscript PCR, high fidelity kit so as to minimize the number of random mutations.

Quantification: Quantification was done by induction of 5 million cells with pitx2c and an equivalent number of ES cells with empty vector. EBs were cultured for 8 days in suspension and plated on fibronectin on the 8th day. The plated EBs were cultured for two days and fixed with 4% paraformaldehyde for immunostaining. EBs with more than 50% cardiac troponin T (Santa Cruz Biotech CA) positive staining were counted. The same protocol was applied for two different pitx2c expressing ES cell clones to control for the site of integration of the transgene. Three hundred EBs were counted for each clone and statistical analysis for significant difference was determined by Bonferroni/Dunn Test.

Real time quantitative PCR: Total cellular RNA was extracted using Trizol (Invitrogen, Carlsbad, CA) reagent according to the protocol provided by the company. Trace amounts of genomic DNA were removed by using Turbo DNA-free kit (Ambion). 18S was used as an internal control and all reactions were performed on an ABI 7700 Prism system. Fold change of mRNA levels were calculated using the delta delta Ct method after verifying the linear range of amplification. All time points were run in duplicate and three such independent studies were done. ES cells transfected with empty vector were used as controls for comparison of fold changes of cardiac specific mRNA levels. PCR products were verified by melting temperature dissociation curves and by DNA sequencing.

Generation of Stable Cell Lines: The effects of pitx2c on cardiac lineage commitment by ES cells were examined by transfecting the cells with full length pitx2c. Since ES cells undergo several rounds of mitosis during the process of differentiation *in vitro*, stably transfected cell lines were established to avoid possible effects due to dilution of transiently transfected cells and/or preferential proliferation of nontransfected cells. The genes of interest were placed in the pcDNA3.1 expression plasmid (Invitrogen) under the control of a CMV promoter. Constitutively active promoters are widely used to study *in vitro* differentiation of ES cells [152,153]. Cells were transfected by electroporation. Selection media containing 250μg/ml geneticin (Life Technologies) was added 2-3 days post electroporation. Colonies of geneticin resistant ES cells were picked and expanded. Genomic DNA was extracted by proteinase K digestion and analyzed for insertion of plasmid DNA by PCR. PCR positive cells were expanded in selection media while differentiation experiments were performed in media without geneticin. Stable cell lines were routinely tested by PCR of genomic DNA to ensure continued integration of the construct. None of the cell lines were observed to alter expression of SSEA-1 and Oct 4 or alter undifferentiated morphology. All experiments were repeated in independently derived clones to control for possible positional effects.

Transfection of ES cells: Plasmid DNA was linearized and purified in sterile conditions. 25 μg of the plasmid was electroporated using a BioRad GenePulser II machine. The voltage of the pulse was set at 250 Volts and the capacitance at 330 μF. Cells were incubated in ice for 20 minutes immediately after administering the pulse. Cells were transferred to gelatin coated tissue culture dishes with ES medium and selection was started after 48 hrs.

Intracellular Ca^{2+} Imaging: Intracellular [Ca^{2+}] was measured as the fluorescence intensity of the Ca^{2+} indicator Fluo-4AM. Fluorescence was measured on the stage of a laser scanning confocal microscope (Zeiss LSM 510). Beating cultures were incubated at 37°C for 30 minutes in DMEM containing 40μM Fluo-4AM. The coverslip was then placed in an experimental chamber on the microscope stage and perfused with DMEM (32°C). Spontaneously beating EBs were exposed to light from a 25mW argon laser (488nm) and emitted light (>505nm) was collected. A scan line was drawn across an individual EB and fluorescence light intensity was measured from the line at a scan rate of 2msec in order to build a 2-D image (time vs distance) of fluorescence intensity across the various cells in the EB of interest. Fluorescence intensity is presented as a ratio compared to intensity at rest immediately before activation of Ca^{2+} transients (F/F0). Platinum field stimulating electrodes were placed 1 mm from either side of the EB in order to apply square pulse stimulation (2Hz, pulse duration 5msec, 1.2x threshold) across the EB. Both spontaneous and stimulated fluorescence transients were recorded from each EB whenever possible.

Action Potential Measurements: Beating EBs were placed in an experimental chamber on the stage of an inverted microscope (Nikon Diaphot). The chamber was constantly perfused with bubbled (95% O_2: 5% CO_2) DMEM maintained at 36°C. Recordings were made using high resistance microelectrodes (25-50megohms) pulled from borosilicate capillary tubing (1B100F, World Precision Instruments) on a horizontal puller (P-87, Sutter Instruments). Microelectrodes were filled with 1M KCl and fitted into a holder attached to the headstage of an Axoclamp-2 amplifier (Axon Instruments). Data acquisition and analysis and stimulation protocols were performed using pClamp6 software using a TL-125 DMA interface (Axon Instruments). Cell

stimulation (2Hz) was accomplished by delivering 2msec pulses (1.2x threshold) through the pipette in bridge mode.

Immunocytochemistry: Cells plated on cover slips were fixed in 4% paraformaldehyde and permeabilized with 0.2% Triton X-100. Primary antibodies used were the following: anti-cardiac specific troponin-T (Santa Cruz), anti-Nkx 2.5 (Santa Cruz), anti-GATA4 (Santa Cruz), anti-Oct4 (BD Pharmingen) and anti-SSEA-1 (Developmental Studies Hybridoma Bank). Appropriate secondary antibodies were purchased from Southern Biotechnology Associates. Cover slips were mounted onto glass slides (Fisher) with Antifade Kit (Molecular Probes).

Cell Transplantation: Pitx2c overexpressing ES cells and R1 ES cells were differentiated into embryoid bodies by withdrawal of LIF in the medium. Both the cell types were differentiated using exactly the same protocol. Eight day old embryoid bodies were trypsinized with 0.25% Trypsin-EDTA (Gibco) and washed with PBS and approximately $1x10^6$ cells were directly injected into the wall of the infarcted left ventricle. The study group consisted of twelve mice divided into four groups (n=3 for each group): Infarcted mice receiving differentiated pitx2c expressing cells, infarcted mice receiving differentiated untransfected R1 ES cells, infarcted mice and healthy mice. Myocardial infarction was induced in 129SvJ female mice anaesthetized by ketamine after which the chest was opened and the left anterior descending coronary artery was transiently ligated 2-3 mm from the origin of the LAD for 30 minutes and the circulation was restored thereafter.

Figure 28: Ventilator used for intubating anesthetized mice.

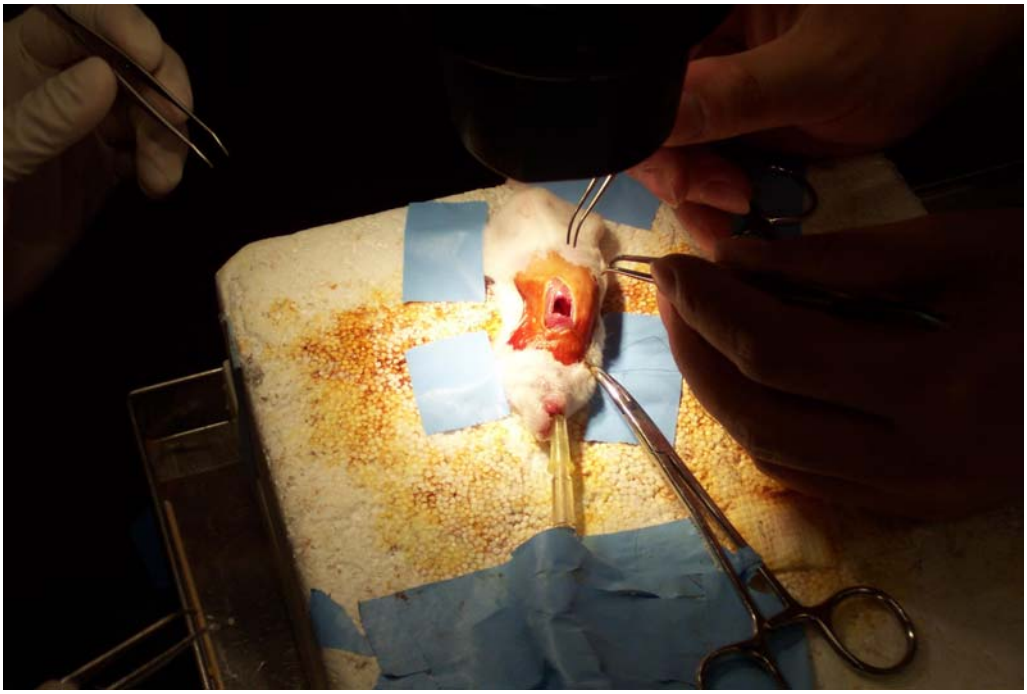

Figure 29: Open chest incision showing thoracic cavity.

Infarcted mice were monitored for one week and the cell transplantations were performed on the eighth day of post-infarction by direct injection into the myocardial scar tissue in the left ventricle. Mice were evaluated for functional improvement after one month of cell transplantation. Blood pressure, contractility (dP/dt) and relaxation (-dP/dt) were measured with SPR-839 microcatheter using a Millar Pressure-Volume system. Pressure-Volume curves were analyzed on PVAN software provided by Millar. Mice were sacrificed after functional studies and infarct size was inspected after fixing the heart. Triphenyltetrazolium staining of the infarcted hearts revealed approximately 44% ischemic area (IA) in the area at risk (AAR) (IA/AAR = 44.6 $\pm$ 4.6) and approximately 43% area at risk in the left ventricle (AAR/LV = 43 $\pm$ 3.2%). FISH analysis and immunoflourescence measurements were performed to verify the presence of differentiated transplanted pitx2 overexpressing cells with cardiac phenotype. All procedures were approved and were done according to Institutional guidelines.

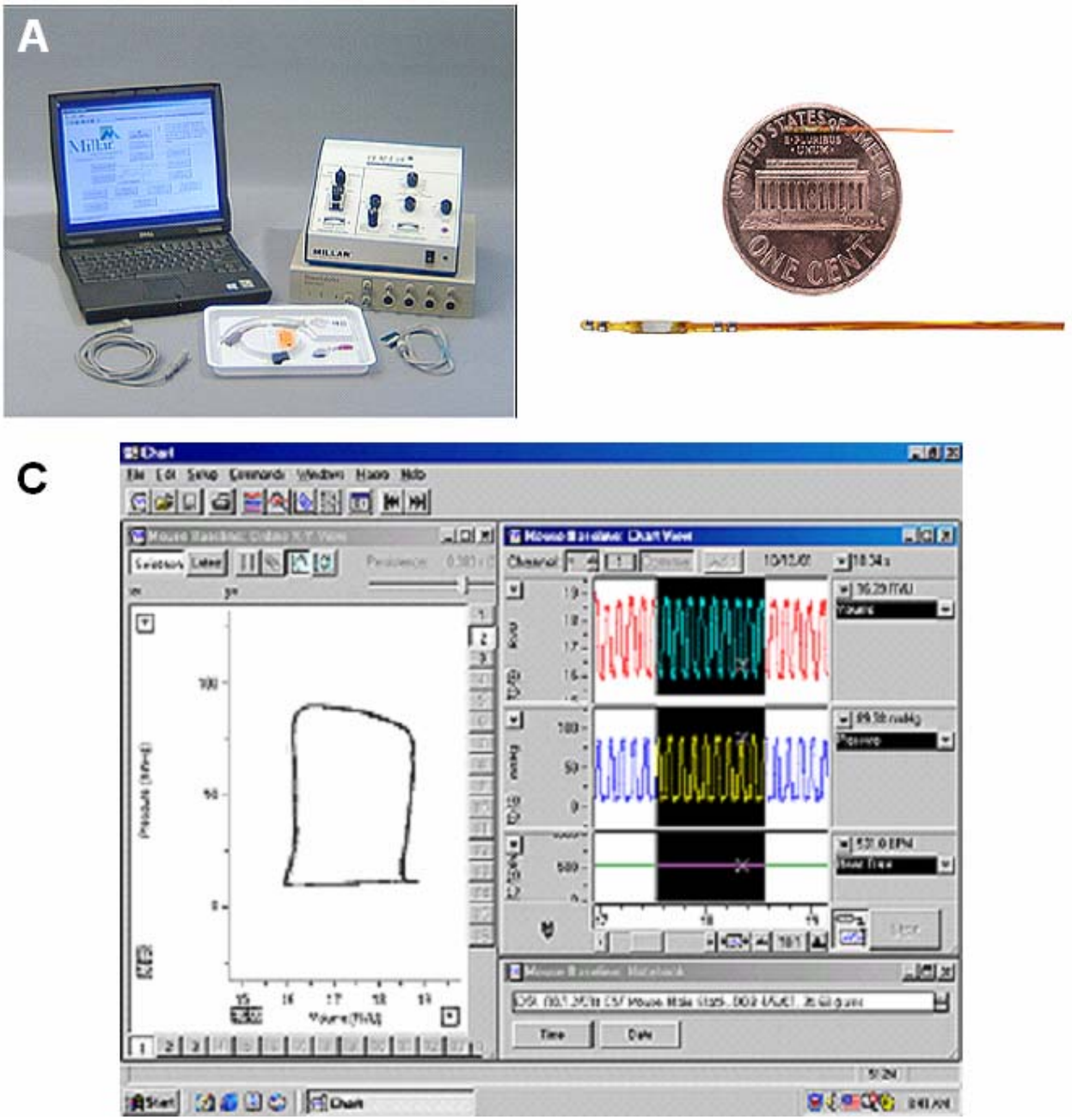

Figure 30: Instrumentation used for functional measurements of infarcted mice.

Panel A: Amplifier and interface.

Panel B: Transducers at catheter tip.

Panel C: Pressure-Volume loop seen in software.

FISH analysis: Four micron thick sections of the fixed cardiac tissue were stained for the Y chromosome using a FITC labeled probe (Open Biosystems) using the protocol provided by the company. These sections were immediately adjacent to those used for immunostaining for Troponin-T (Figure 5F and E are adjacent sections at different magnifications).

Statistical Analysis: Statistical tests were conducted with post-hoc ANOVA tests. A p value less than 0.05 was considered to be statistically different and all values are reported as means with positive and negative standard deviations except electrophysiological measurements which are reported as means $\pm$ standard error of means.

Conditioned Media Loading of a Peptide Amphiphile Scaffold: Heparin Binding Peptide Amphiphile (HBPA: $C_{16}H_{31}O$-AAAAGGGLRKKLGKA-NH_2) was synthesized as previously described [133] and purified using reversed phase high performance liquid chromatography. A solution of HBPA (3%w/v) was triggered to self-assemble by mixing with an equal volume of a solution of heparin sodium salt derived from porcine intestinal mucosa (Sigma-Aldrich, 2%w/v). The resulting gel was allowed to cure for 30 minutes at room temperature. Hypoxic-conditioned $Pitx_2$ media was prepared by culturing $Pitx_2$ cells at 5% O_2 for 24 hours, at which point the media was removed, centrifuged, and stored at -80°C for future use. The cured HBPA gel was soaked in 1mL of this media at 4°C for 24 hours. The gels were used as a treatment in a mouse myocardial infarction model (CD1 mice from Charles River). Following tracheal intubation, the heart was exposed and the left anterior descending artery was ligated for 30 minutes. After this time, 15µl of gel was injected, and the tissue was reperfused. Animals were phenotyped for functional performance at 30 days using a pressure-volume catheter inserted into the left

ventricle through the right carotid artery. Control measurements were done on healthy animals, animals receiving an infarction without treatment, and animals receiving HBPA gels soaked in unconditioned media (serum-free DMEM).

Protocol used for cell transplantation in Chagas disease model: This work was done primarily by Dr. David Engman's lab members. At 21 days post exposure to trypanosomal antigens, mice were injected with dissociated 8 day old EBs. Mice were sacrificed after one month and hearts were examined for cardiomyopathy.

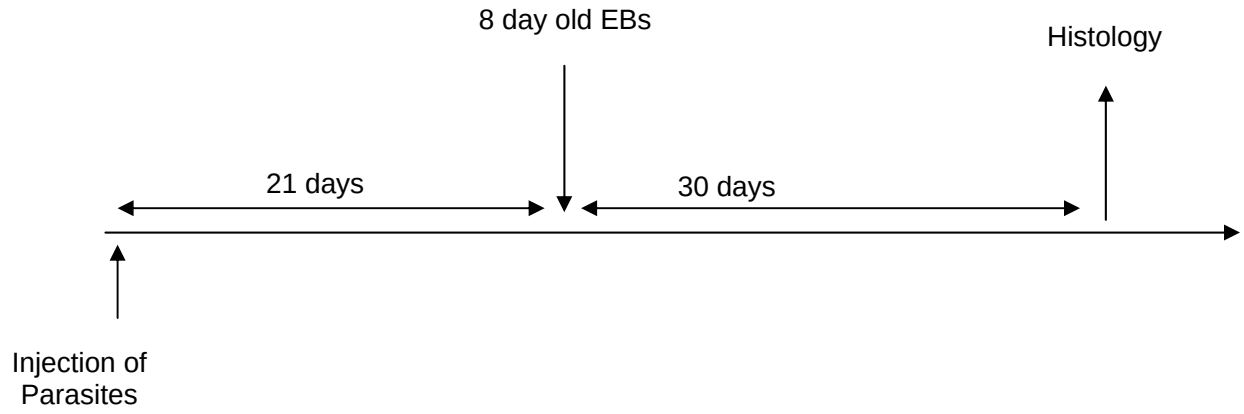

Figure 31: Protocol for cell transplantation in Chagas disease model of mice.

CHAPTER 3

Study of Lineage Changes in Cardiogenesis from ES cells

Rationale:

Embryonic stem cells have the potential to differentiate into all three lineages and they hold the promise of a therapeutic supply of differentiated cells that can be used for cell replacement therapy. However, the molecular mechanisms and pathways by which these cells differentiate into different lineages are not completely known. ES cells with multiple lineage stage-specific promoters driving markers would be an important tool to understand such molecular pathways. It would also be directly relevant to therapeutically since it would be possible to isolate pure populations of such cells to test their therapeutic efficiency. Lineage analysis has been classically done with the insertion of a specific promoter driving a fluorophore or an antibiotic resistance gene. This method requires the use of flow cytometry or antibiotic selection after transfection of the plasmid carrying the promoter-fluorophore combination. It is currently not possible to engineer multiple promoters on the same plasmid due to promoter interference with each other. Introducing multiple constructs with a single promoter-fluorophore element in each construct would require the use of multiple antibiotics which tend to differentiate ES cells. Conventional methods of producing ES cell lines with multiple promoter-fluorophore elements in the genome would require creating different transgenic animals each with a genomic insertion of a promoter-fluorophore element and cross breeding them to produce animals with multiple transgenes in them. These animals can be subsequently used for deriving ES cells to study lineage commitment in-vitro. This method is expensive, time consuming and requires a uniform genetic background for all the transgenic animals used. Another method would be to do a serial transfection of each promoter-fluorophore element with one antibiotic selection at a time but this method increases the passage number of the cells. ES cells at high passage number are known to

accumulate genetic mutations and differentiate poorly. In this study, we show two different methods by which multiple plasmids can be stably integrated into the genome. The prime applicability of this method is to insert multiple stage specific promoters-fluorophore elements into the genome at different loci and it also removes the possibility of these elements interfering with each other. It is possible that the site of insertion and copy number can influence the activity of these promoter-fluorophore elements but we show in this study that by comparing multiple ES cell clones with each other, clones which poorly represent the differentiation process can be eliminated easily. The most obvious advantage of this technique is the low passage number which is critical in the creation of transgenic mice with multiple stage specific and lineage specific promoter-fluorophore elements.

Early Markers: These enhancers/promoters mark all cardiac progenitors. They are driven at a stage where specification is taking place but differentiation has not started. The candidate genes whose enhancers are suitable are: Nkx2.5, Mef2c and Isl 1 promoters.

Late Markers: These enhancers/promoters mark lineage-specific cardiac progenitors. They are driven at a stage where specification has occurred and differentiation has started. The candidate genes whose promoters are suitable are: α-MHC, MinK, MLC2a and MLC2v. The enhancer elements of Nkx2.5, ∝-MHC and MinK have been tested in the H9C2 cell line with a retinoic acid induction-differentiation protocol

A combination of these elements could be potentially useful to create an ES cell lines that upon differentiation will express fluorophores that are helpful in FACS purifying the differentiated mixture of cells into a pure population.

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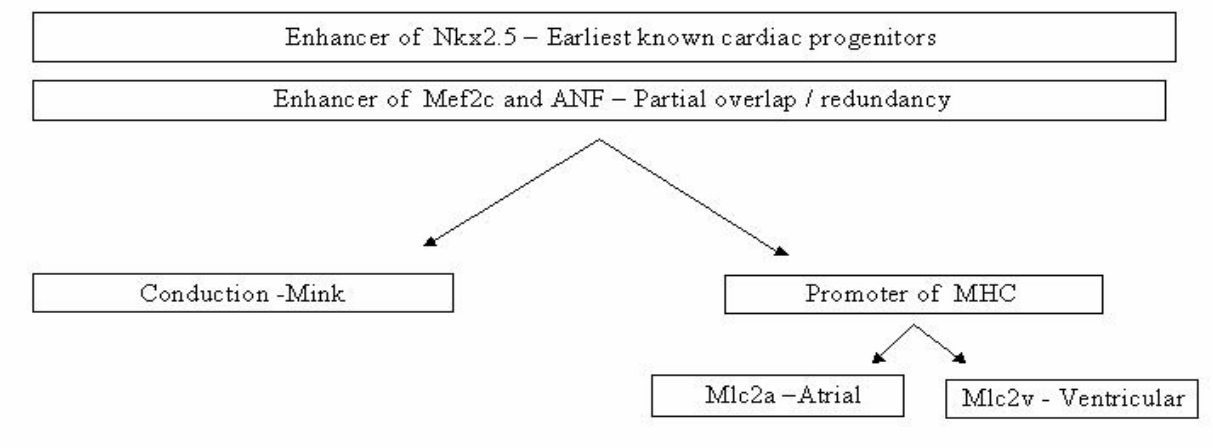


Figure 32: Schematic of promoter combination for different stages of cardiomyocytes.

To study the cardiac lineage commitment of embryonic stem cells, we have developed a novel way of inserting multiple cardiac-specific promoters using three separate constructs interdependent on each other for antibiotic resistance. We demonstrate that the promoters are active at different stages of differentiation and the stage of differentiation can be visualized by the different fluorophores driven by these promoters. This technique can be broadly applied to different types of stem cells and can be used to study the effect of morphogens on the lineage commitment of these cells.

Various strategies to insert multiple promoters into the genome:

Stable ES cell lines are made by selecting electroporated cells with an antibiotic. This does not ensure that the gene of interest also has integrated into the genome and gives a high number of false positive resistance colonies. Internal Ribosome Entry Site (IRES) is viral sequence was characterized in encephalomyocarditis virus and allows the insertion of a second reading frame after the first in the same mRNA molecule [154]. Using an IRES cassette ensures that the gene of interest and the promoter also are integrated. However, this would require that the promoter has to be constitutionally active to ensure antibiotic resistance.

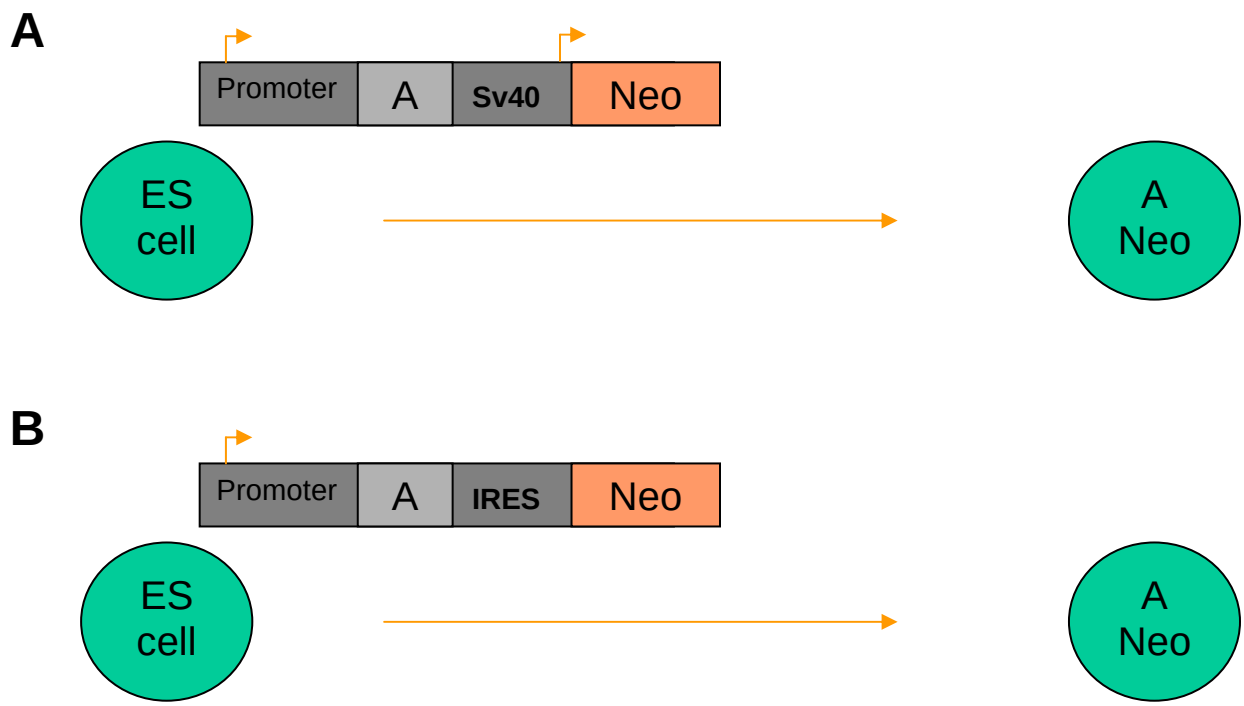


Figure 33: Schematic of Bicistronic and IRES constructs.

Panel A: The antibiotic resistance cassette has its own promoter.

Panel B: Antibiotic expression is dependent on promoter activity via IRES cassette.

The expression of the second protein after the IRES segment has been shown to be significantly lower than the first protein [155]. The expression of the antibiotic after two IRES segments has also been demonstrated [156]. Introducing multiple plasmids with the same antibiotic resistance would result in far more false positive colonies than a single plasmid. The integration of all genes can be confirmed by PCR; however, one or more of them can be silenced since the cells do not require more than one active antibiotic cassette to maintain antibiotic resistance. It is known that different promoters have different efficiency of expression in transgenic ES cells [152]

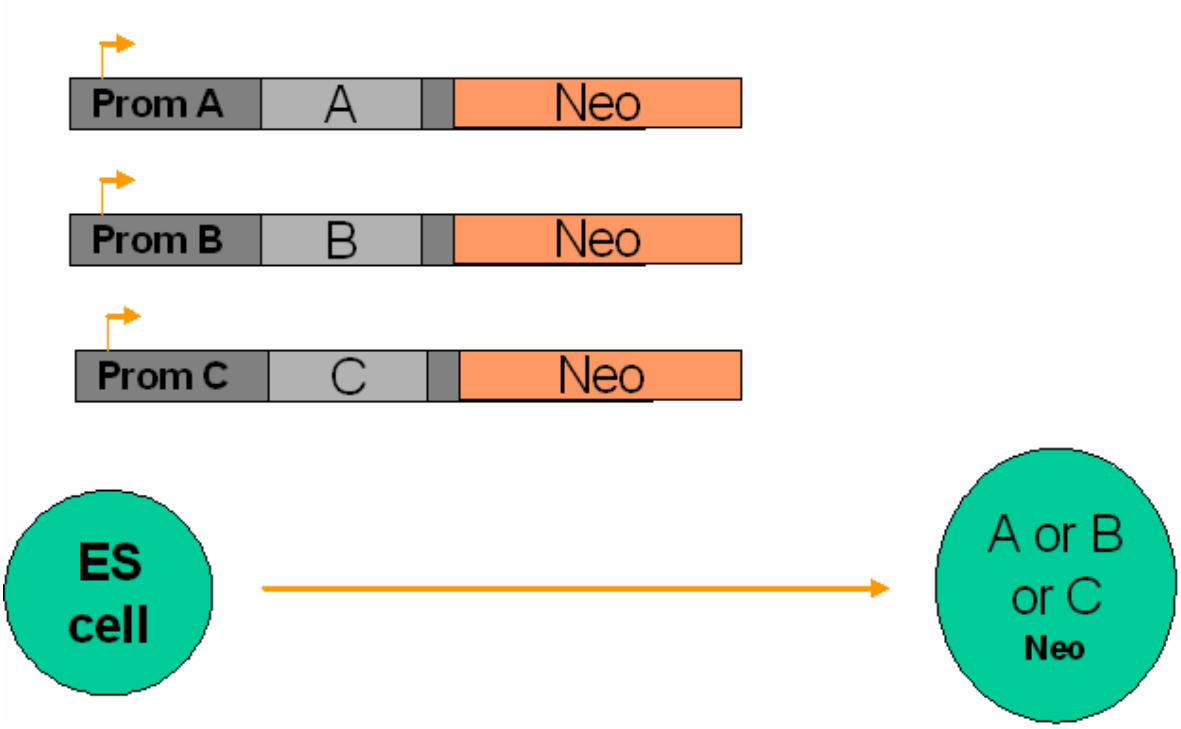


Figure 34: Introduction of multiple plasmids with the same antibiotic resistance. The efficiency of this process is extremely low.

The second strategy would be to use multiple plasmids carrying different antibiotic resistance genes. This increases the number of false positive colonies and also increases the rate of differentiation in these colonies. This approach is applicable for constitutive promoters but not stage-specific promoters.

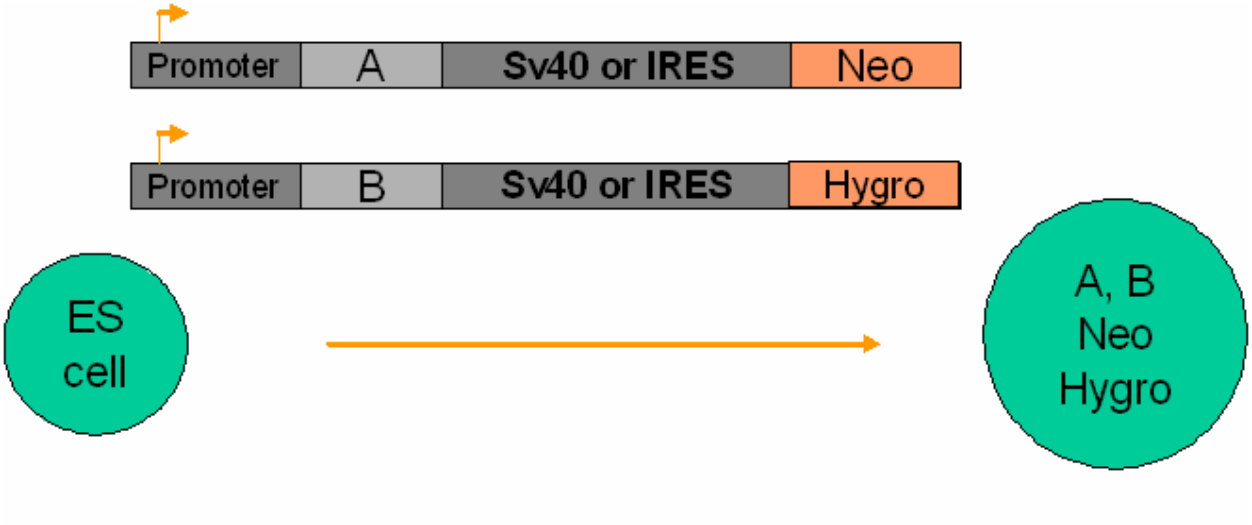


Figure 35: Schematic of multiple plasmids utilizing different antibiotic resistance genes.

The third approach would be to introduce the plasmids in serially. However, the disadvantages are that the stable cell lines will increase in passage number over multiple serial selections. It is also possible that the earlier inserted plasmids may get silenced. The increased passage number affects the ability of ES cells to maintain pluripotency. As a result differentiating such cells will not necessarily reflect the true pattern of differentiation. It has also been shown that ES cells at high passage number have karyotype defects and increased tendency to transform into cancer cells [157-160].

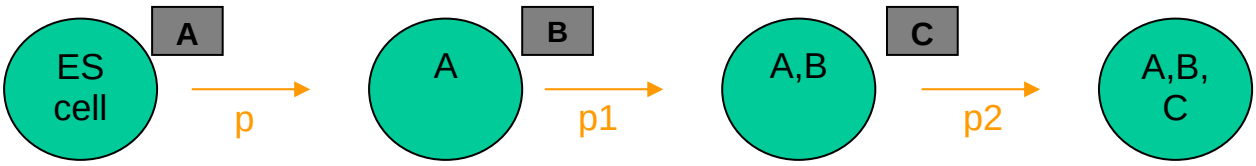


Figure 36: Serial transfection of plasmids increases the passage number.

Tissue and stage specific promoters have a temporal pattern of activation. Classically the promoters were characterized by knocking in a marker like LacZ and genetically modifying different segments of he promoter to study their effects on its activity. Such studies have been applied extensively to purify specific types of cells from differentiating ES cell cultures. However, the usage of more than one such promoter on the same plasmid can potentially interfere with each other activity since the distal elements of promoters can have multiple effects on proximal elements [56].

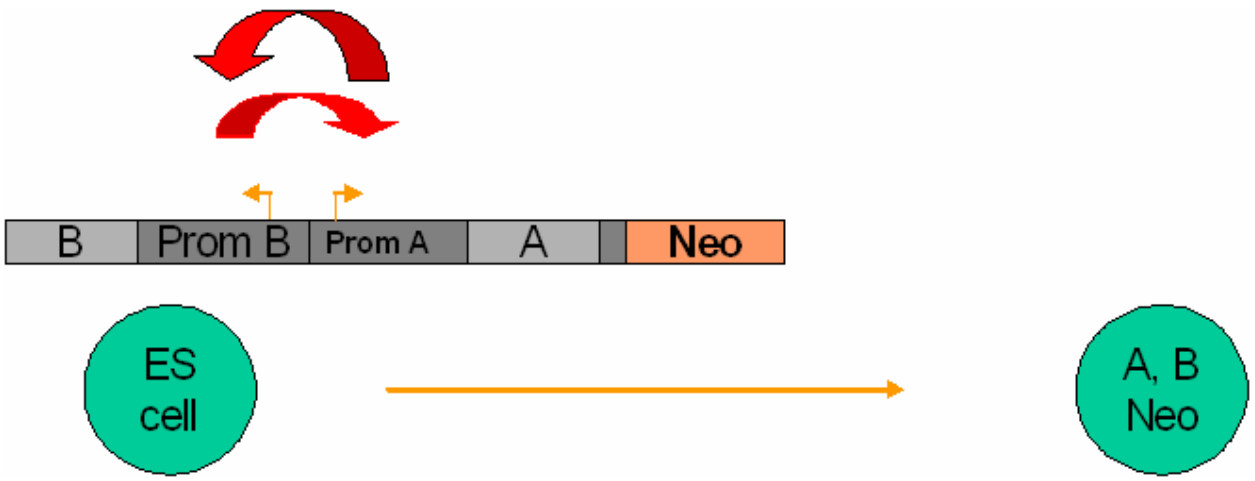


Figure 37: Mutual interference of adjacent tissue specific promoters.

One possible way to insert to insert multiple plasmids into a cell and ensure that all of them are integrated is to make them interdependent on each other for antibiotic resistance. This would entail that each plasmid contributes to a part of the neomycin molecule either at the RNA level or the protein level. This would allow the usage of tissue specific promoters flanked by antibiotic resistance cassettes.

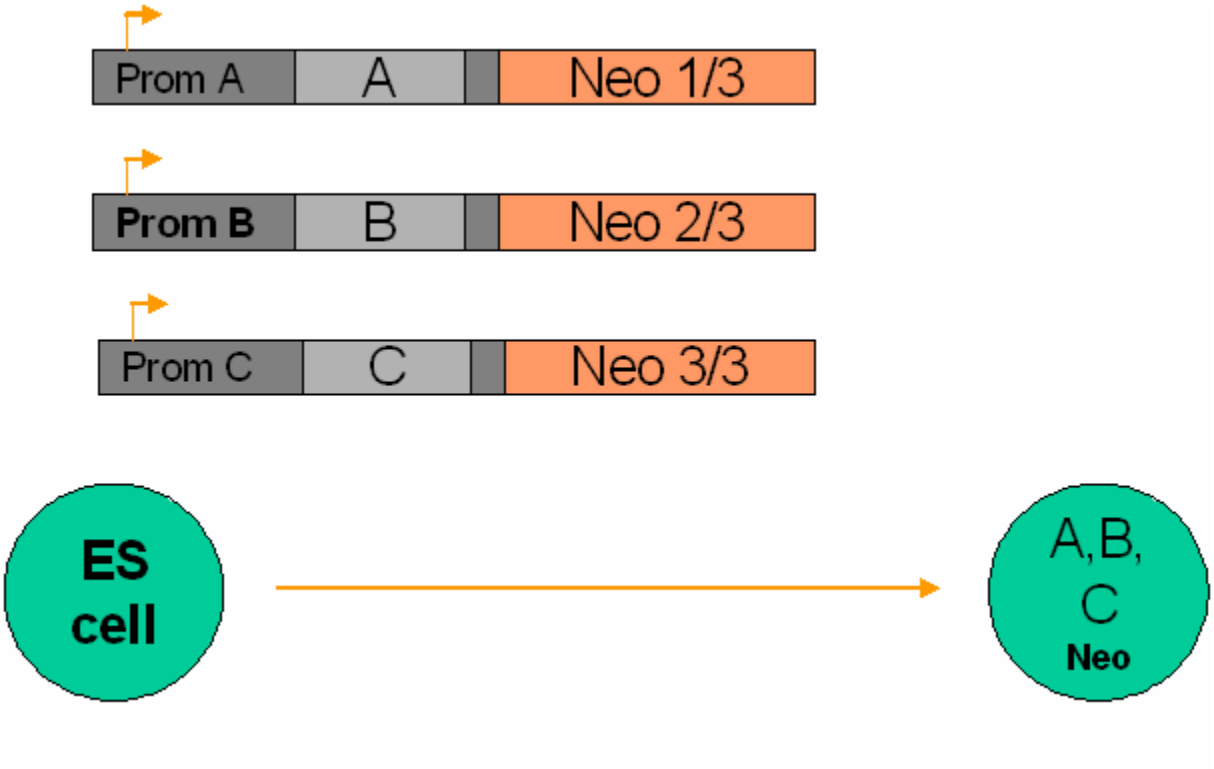


Figure 38: Contribution of multiple plasmids towards antibiotic resistance.

Trans-splicing has been demonstrated in trypanosomes [161,162] and in mammals [163-165]. It involves the annealing of 5' and 3' ends of different RNA molecules such that the spliceosomal machinery jumps from the first RNA molecule onto the second RNA molecule and gives rise to an intergenic transcript [166,167]. This techniques has been used to correct aberrant transcripts

in cystic fibrosis [168] and sickle cell disease [169] and is an emerging technology in gene therapy [170]. Shielding of splice donor and acceptor sites by annealing small RNA has been demonstrated to contribute to trans-splicing [171,172]. It is also possible to divide antibiotic resistance into three parts by using the following strategy:

1. Two introns with stop codons are introduced into the reading frame of the antibiotic.
2. Two small RNA molecules are designed such that each RNA molecule uniquely binds to the splice donor and splice acceptor sites of the parent mRNA molecule.
3. Each plasmid expresses one component of this system and antibiotic resistance is possible only if all of them are active and present. This method was implemented and was found to be extremely inefficient.

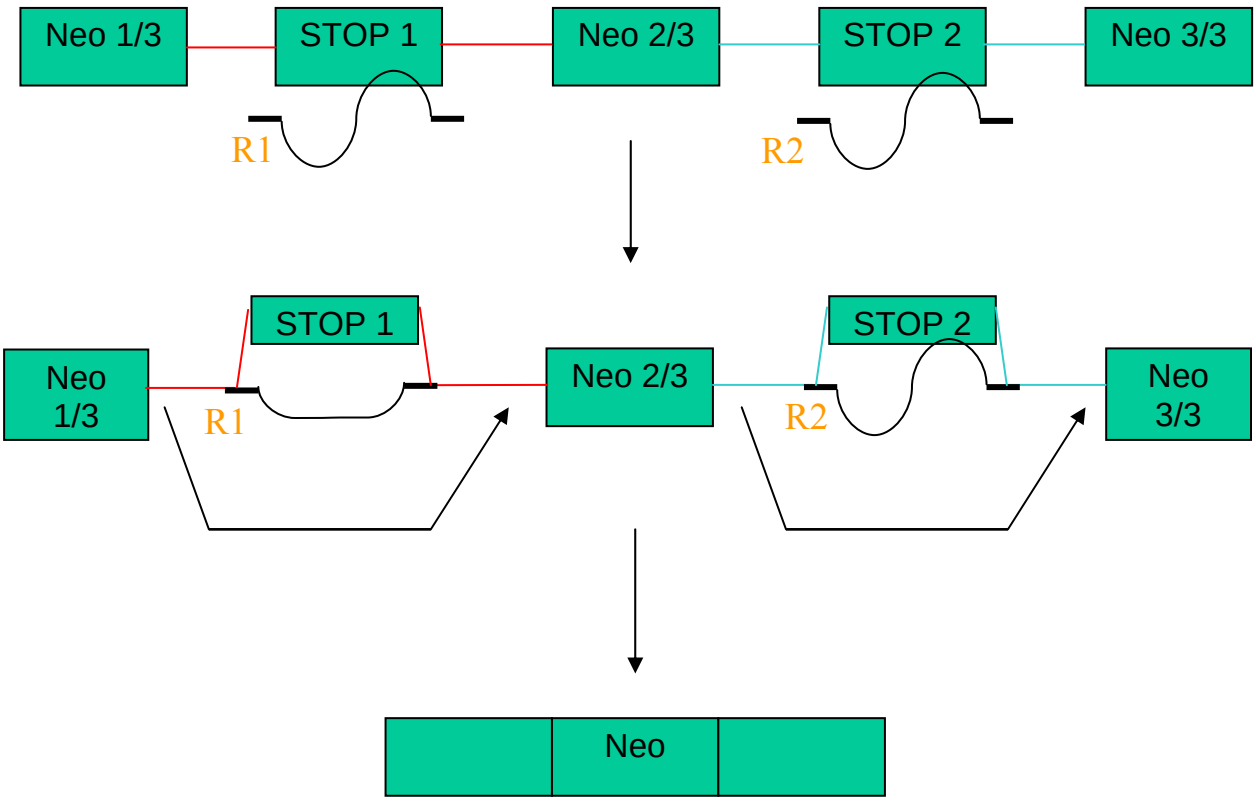


Figure 39: Exon shuffling strategy for antibiotic resistance.

It is possible to reconstruct a functional protein by complementing its parts at the RNA level or the protein level. Protein complementation has been demonstrated for GFP [173], beta lactamase [174], beta galactosidase [175] and luciferase [176] and engineered neomycin phosphotransferase [177]. There are no known instances where three or more fragments have been used to complement a complete protein. The option of using multiple fragments is available if trans-splicing can be utilized.

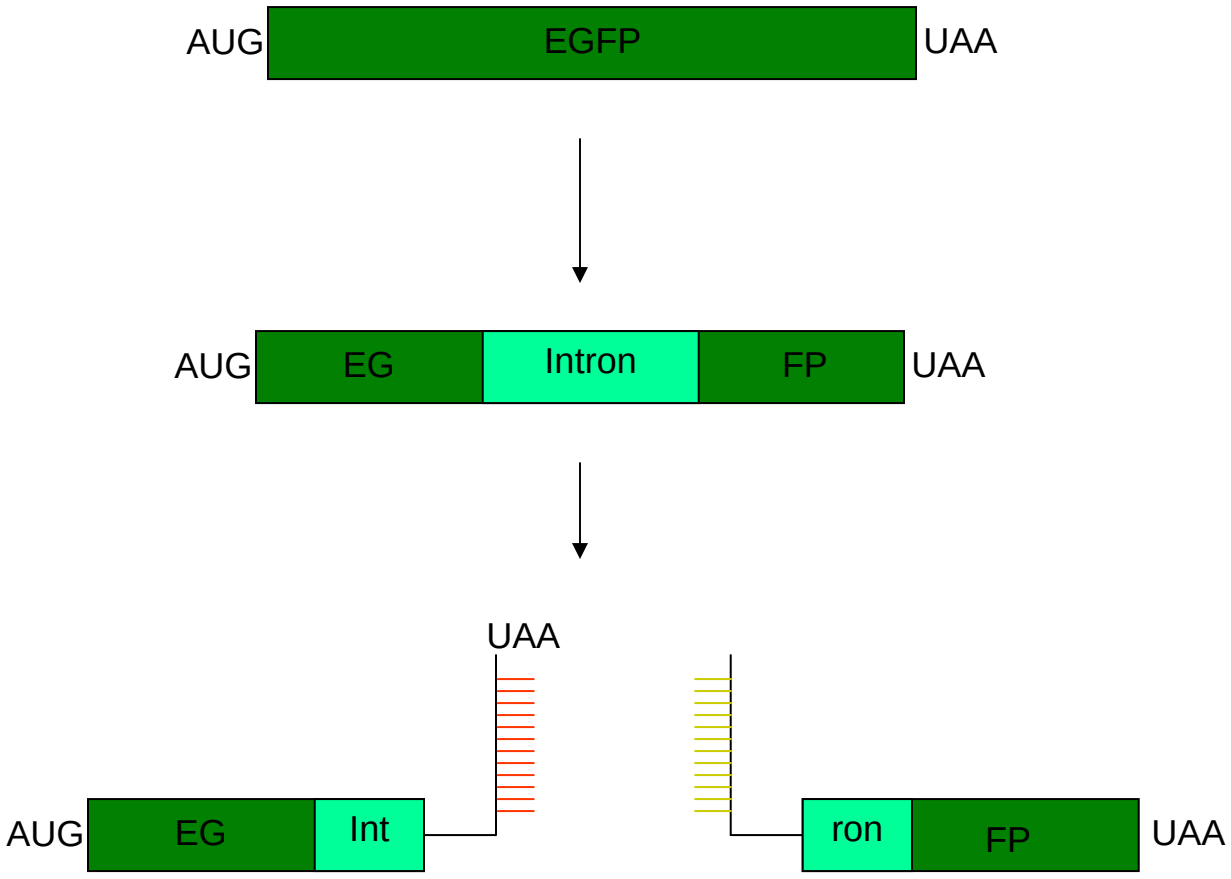


Figure 40: Design of self annealing fragment for trans-splicing of GFP.

The design of trans-splicing RNA fragments involves an annealer sequence in the 3;' end of the first molecule and a complementary annealer sequence at the 5' end of the second molecule. It would be useful not to have a start codon in the second fragment. This will rule out the possibility of protein fragment complementation. A similar approach for luciferase molecule has been successfully demonstrated. The sequence of the annealer should be unique and long enough to complement the second molecule for functional trans-splicing to occur.

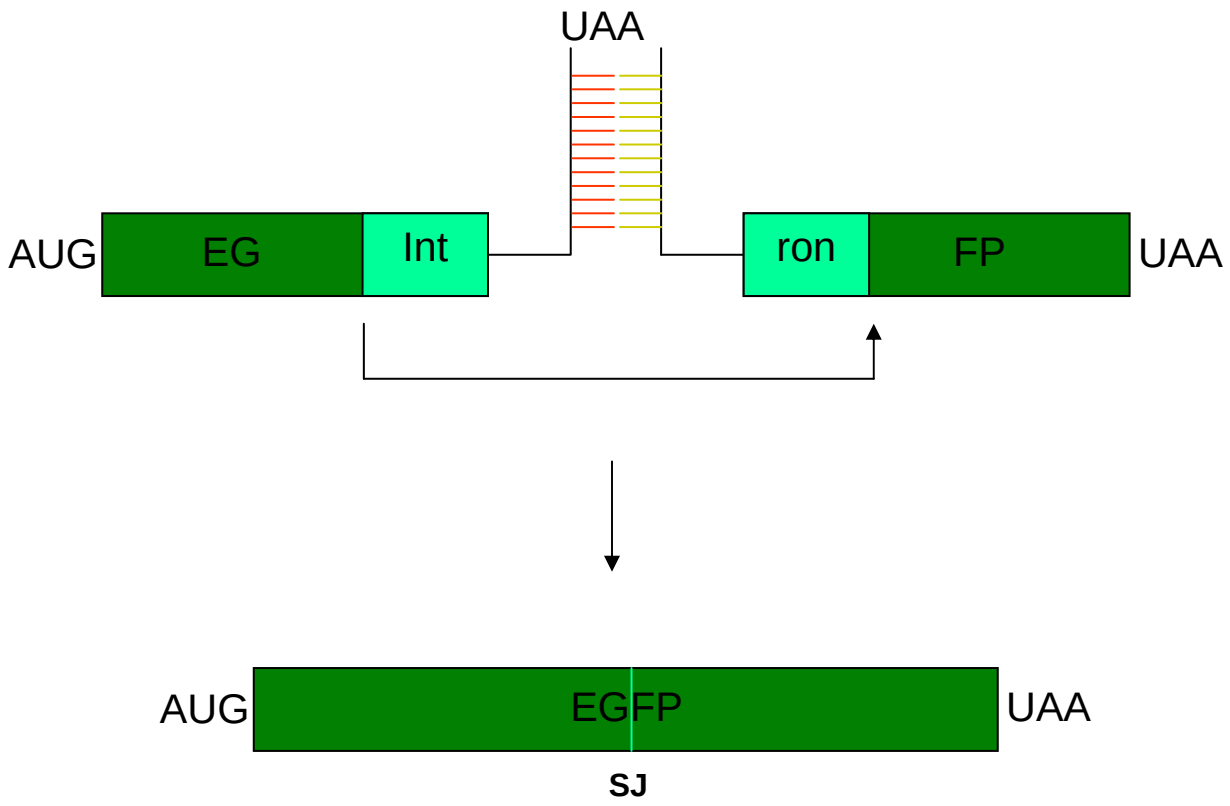


Figure 41: Annealing of complementary fragments leads to trans-splicing into a functional GFP transcript (SJ = Splice Junction after trans-splicing).

The introduction of a third and/or fourth fragment as a bridge in the annealing process enables the expression of a functional transcript to be dependent on multiple plasmids. The efficiency of this process is likely to be very minimal since it requires the random interaction of multiple fragments multiple times to produce a reasonable amount of marker molecule or antibiotic resistance.

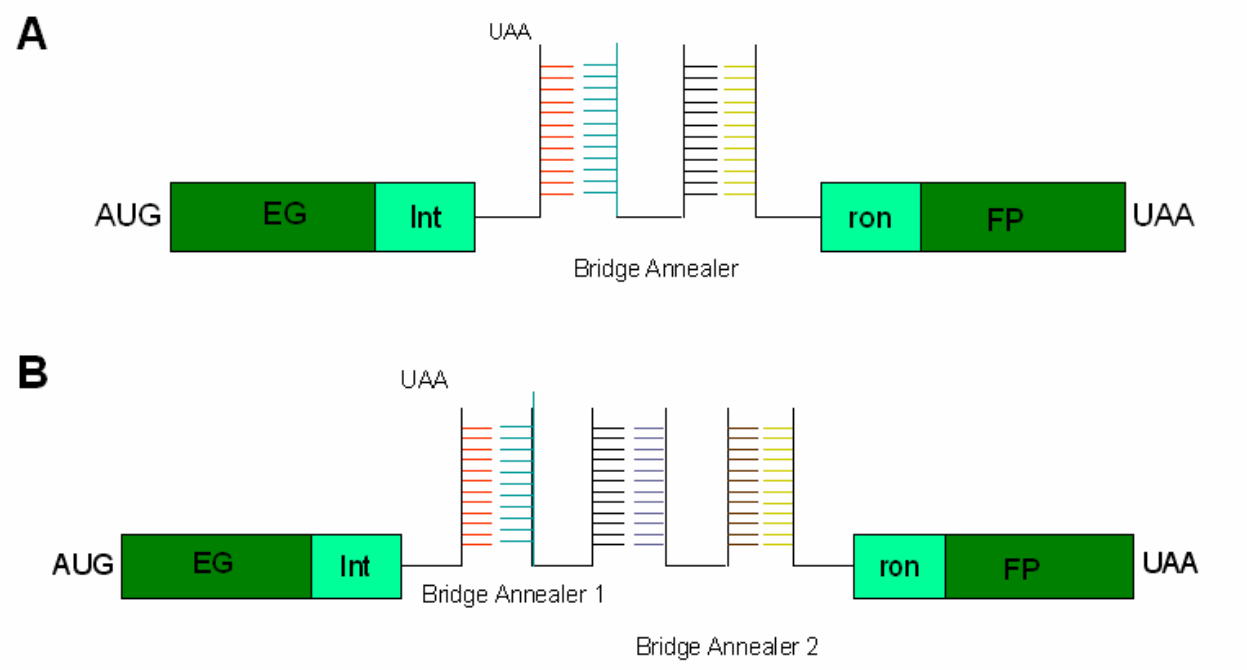


Figure 42: Trans-splicing using multiple fragments.

Panel A: A bridge annealer can bring all three fragments together for trans-splicing.

Panel B: Two bridge annealers can bring four fragments together for trans-splicing.

An alternative to the low efficiency of multiple bridge fragments is to engineer some exonic sequence into the bridge fragments. This approach closely resembles exon shuffling but is involves the three fragments each with some amount of exonic sequence.

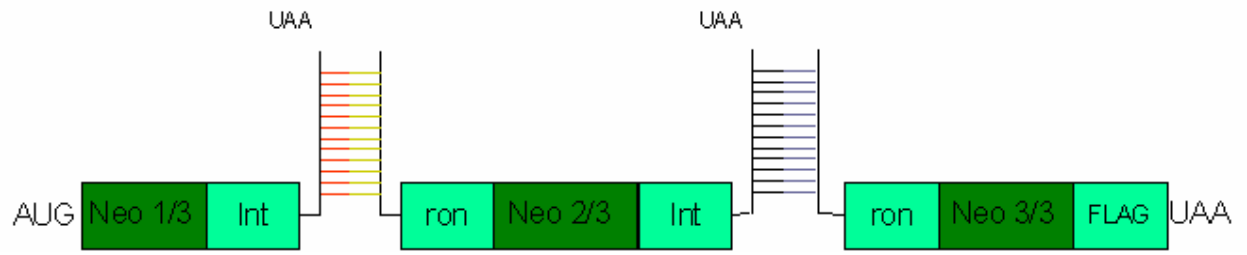


Figure 43: Trans-splicing involving thee exonic segments.

The approach that was finally implemented in this thesis work was chosen for its efficiency. It involves the heterodimerization of two proteins which bind to a response element and drive the expression of an antibiotic resistance gene. A similar system is used in the yeast-two hybrid studies. This interaction can be easily implemented such that three different plasmids each express one component of the system.

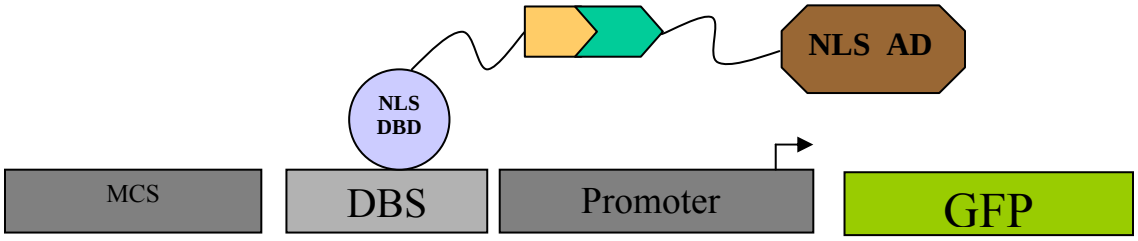


Figure 44: Schematic of a three element system to drive marker expression / antibiotic resistance. MCS: Multiple cloning site for Tissue-specific promoter-Fluorophore. DBS: Binding site for the DNA Binding Domain (DBD) of the first component.

The efficiency of the three element system is based on the complimetarity and affinity of the binding proteins. Initial tests with p53 and Sv40 T antigen should excellent expression of the reporter gene. This proves that the system is feasible but p53 and Sv40 T antigen cannot be used per se since Sv40 T antigen is known to transform cells [178]. The alpha and omega fragments of beta galactosidase have been shown to complement in mammalian cells [179] but the resultant molecule could possibly interfere with transcriptional apparatus. Gal 11 and LGF2 are interacting partners commonly used as positive controls in bacterial-2 hybrid systems[180] and their utility has not been demonstrated in protein fragment complementation assays. A three element system was designed taking advantage of the ability of a modified ecdysone receptor (VgEcR) to bind to a modified retinoid X receptor (RXR) [181,182].

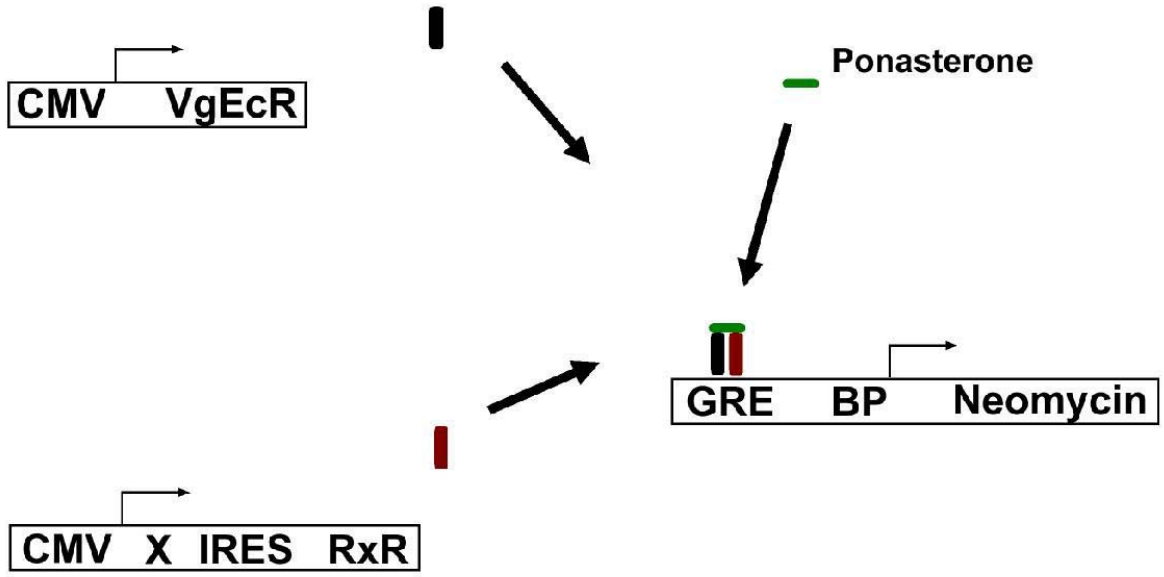


Figure 45: Three element system utilizing VgEcR and RXR heterodimerization.

X denotes any gene of interest that can also be overexpressed in this system.

GRE denoted Glucocorticoid Response Element.

A three element system can also be implemented in a 'cascade' design. In this system the first plasmid would make a transcription factor which uniquely binds to a response element on the second plasmid. This would start the transcription of a second transcription factor which uniquely binds to a response element on the third plasmid. The second transcription factor would lead to the transcription of a marker or an antibiotic resistance gene. This system could not be verified due to problems with LexA binding to its cognate site.

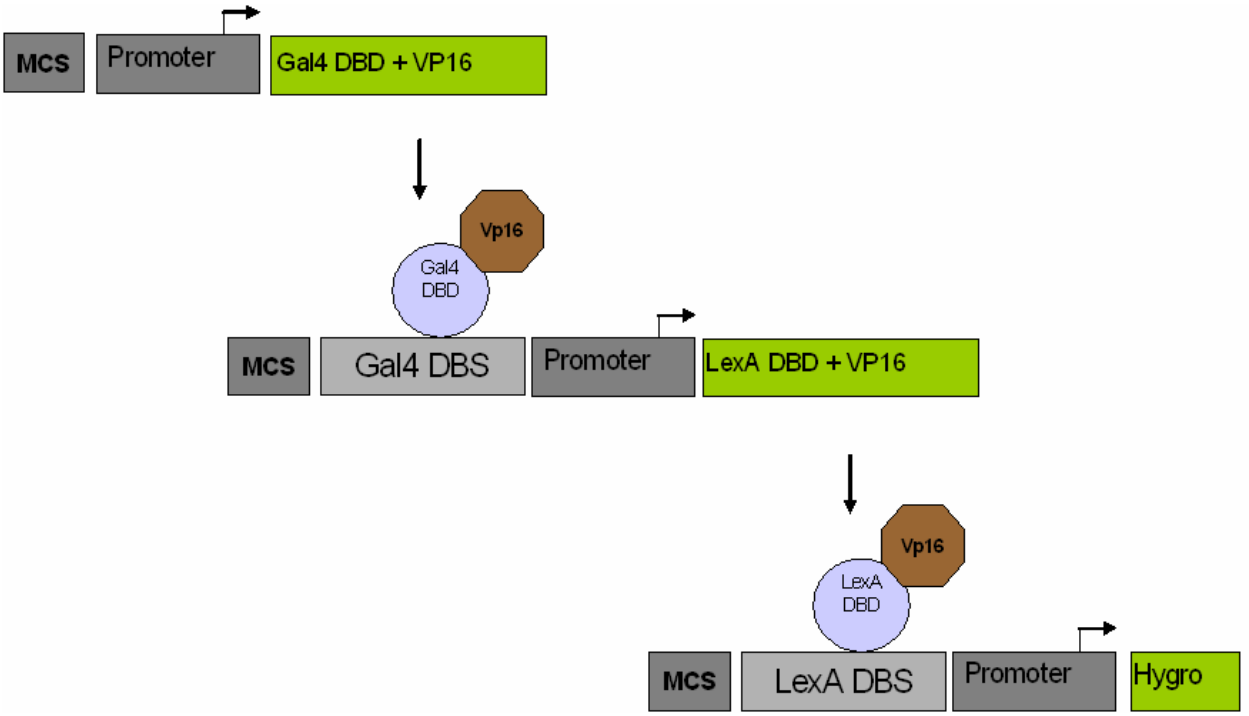


Figure 46: Three element system utilizing a cascade of transcription factors. This system has two unique DNA binding domains: Gal4 and Lex A but uses the same type of activation domain. DBS denotes DNA Binding Site.

The three element system was tested by cloning in three different fluorophores into the three plasmids. In the first plasmid a CMV promoter driving RFP was cloned. In the second plasmid CFP was cloned between the CMV promoter and RXR. In the third plasmid a CMV promoter driving YFP was cloned.

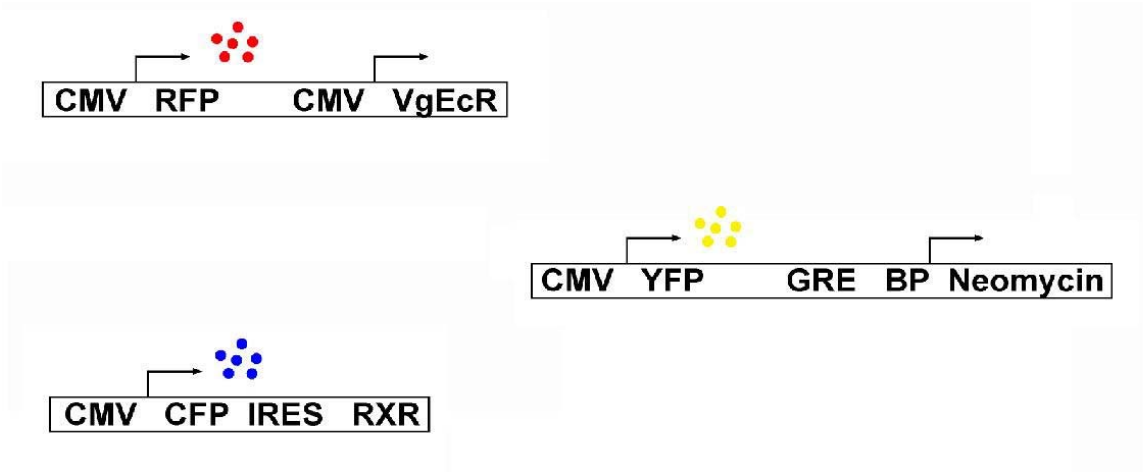


Figure 47: Schematic of the three element system expressing three different fluorophores.

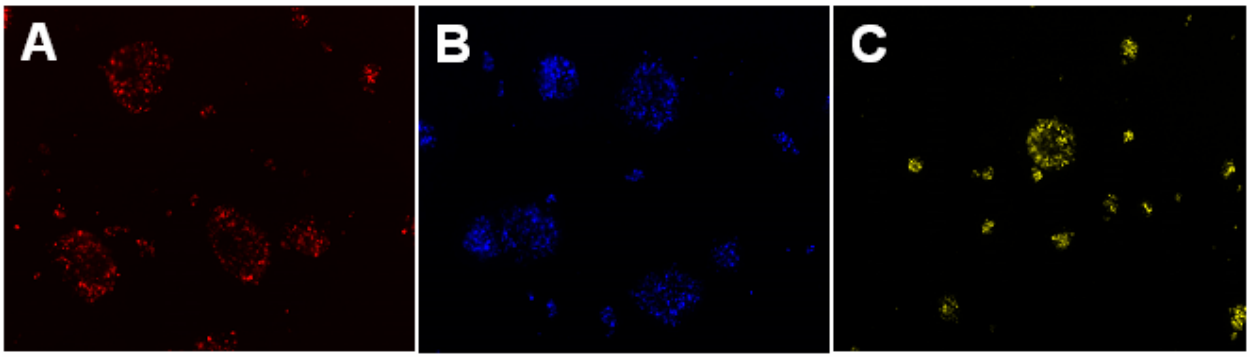


Figure 48: Testing individual plasmids of the three element system in 293 cells.

Panel A: Plasmid expressing VgEcR also expresses RFP.

Panel B: Plasmid expressing RXR also expresses CFP.

Panel C: Plasmid with GRE expresses YFP.

The constructs were tested individually in 293 cells for the activity of the promoters and were found to express all the three fluorophores. Subsequently, they were linearized and electroporated into murine ES cells and mesenchymal stem cells. Ponasterone was added to the medium on the first day of post-electroporation and neomycin selection was started 48 hours after electroporation. Triple fluorophore positive resistant colonies were formed.

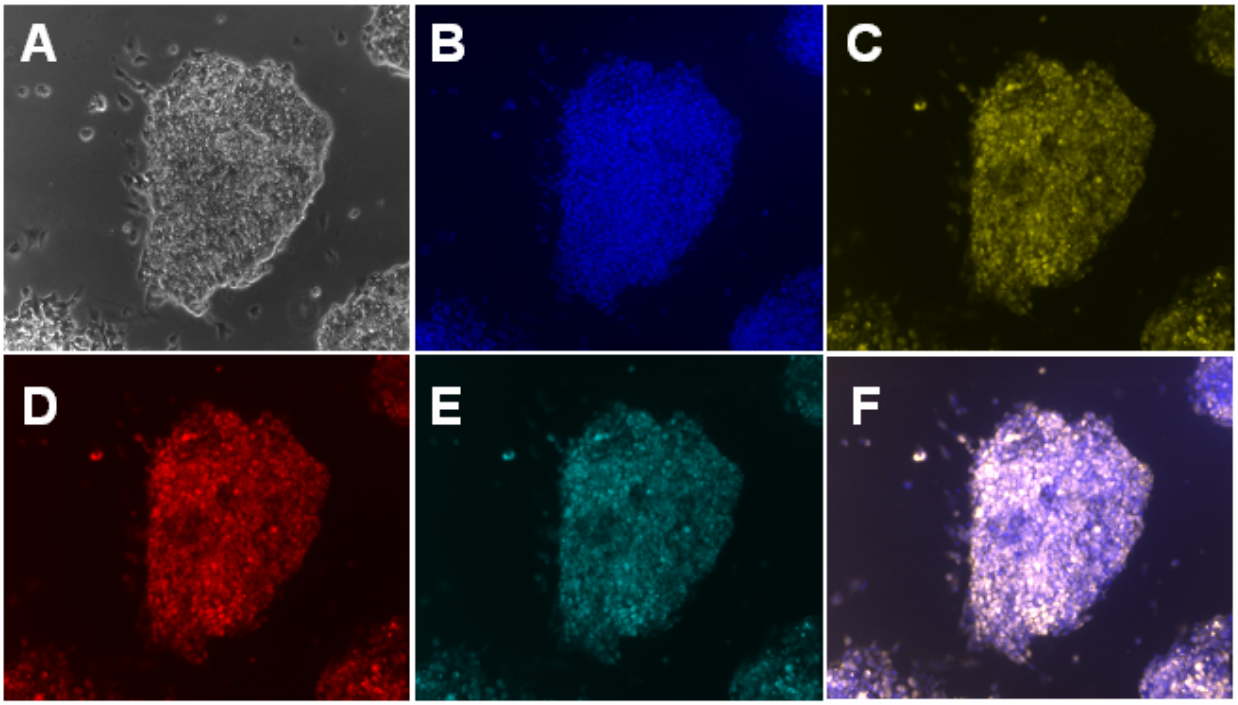


Figure 49: Testing the three element system in murine ES cells.

Panel A: Phase contrast of ES cell colony.

Panel B: DAPI staining of nuclei.

Panel C, D and E: YFP, CFP and RFP expression in ES cell colony.

Panel F: Merge of panels B, C, D and E.

Similarly, triple fluorophore resistant colonies were found in mesenchymal stem cell cultures. However, the expression of the fluorophores was much weaker compared to ES cell cultures and the number of triple fluorophores positive cells were also significantly lesser than those seen in ES cell cultures.

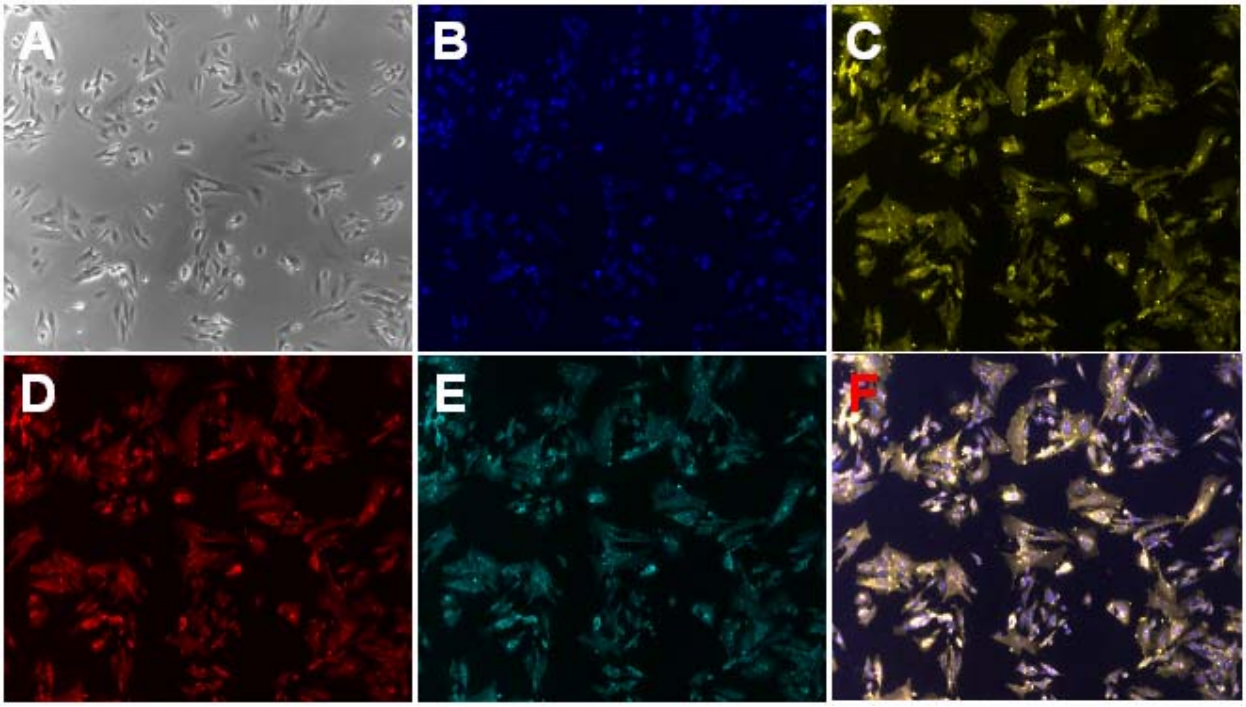


Figure 50: Testing the three element system in mesenchymal stem cells.

Panel A: Phase contrast of mesenchymal stem cells.

Panel B: DAPI staining of nuclei.

Panel C, D and E: YFP, CFP and RFP expression mesenchymal stem cells.

Panel F: Merge of panels B, C, D and E.

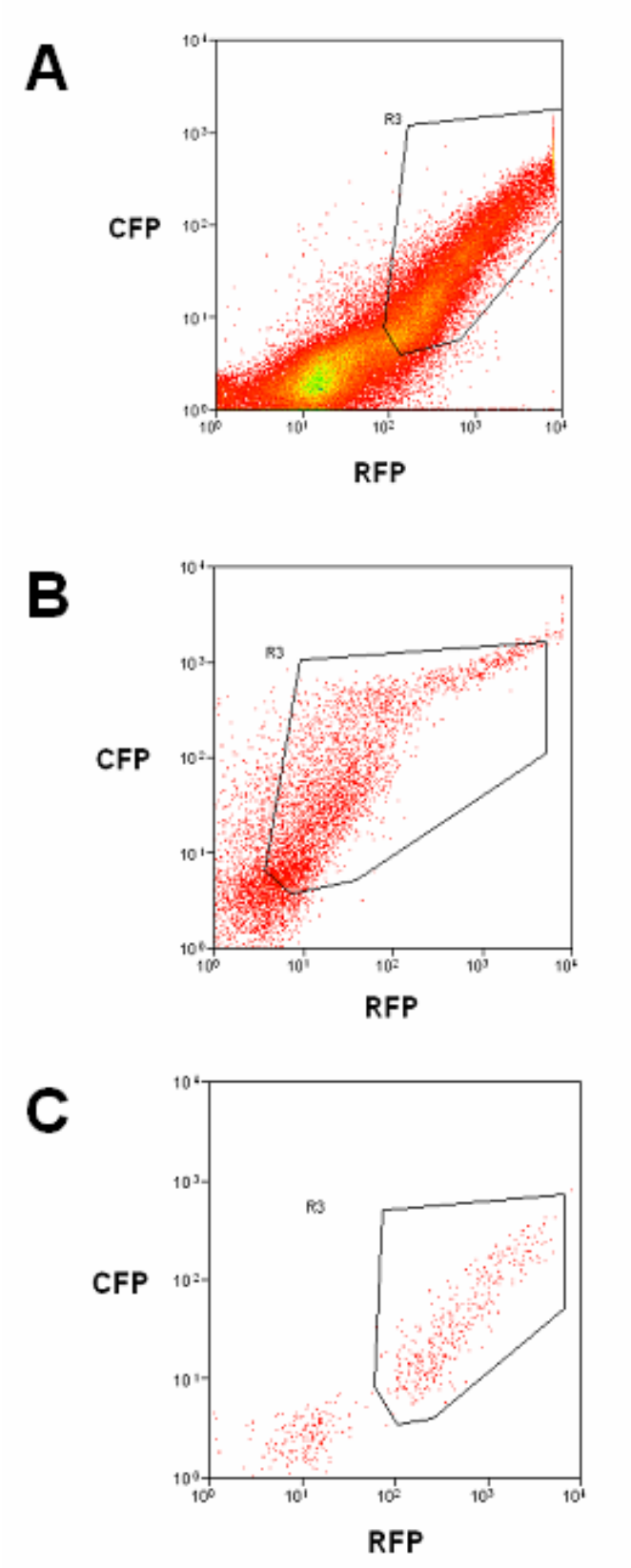
The pattern of constitutive expression of the fluorophores was dependent on the presence of antibiotic selection. The expression of fluorophores after 10 passages was tested by FACS and was found to be enriched as compared to the initial population.

Figure 51: Purity of triple fluorophore positive cells as determined by FACS.

Panel A: There are many weakly positive cells in freshly selected ES cells.

Panel B: Cells are enriched in fluorophore expression after 10 passages, which shows that the promoters do not get silenced as long as selection is applied.

Panel C: Fewer mesenchymal stem cells are positive for all the three fluorophores as compared to ES cells.


A
R3
CFP
RFP
B
R3
CFP
RFP
C
R3
CFP
RFP

The number of colonies formed with electroporation of different combinations of the three element system clearly shows minimal background. The efficiency of the system was lesser than that of using a single plasmid expressing antibiotic resistance.

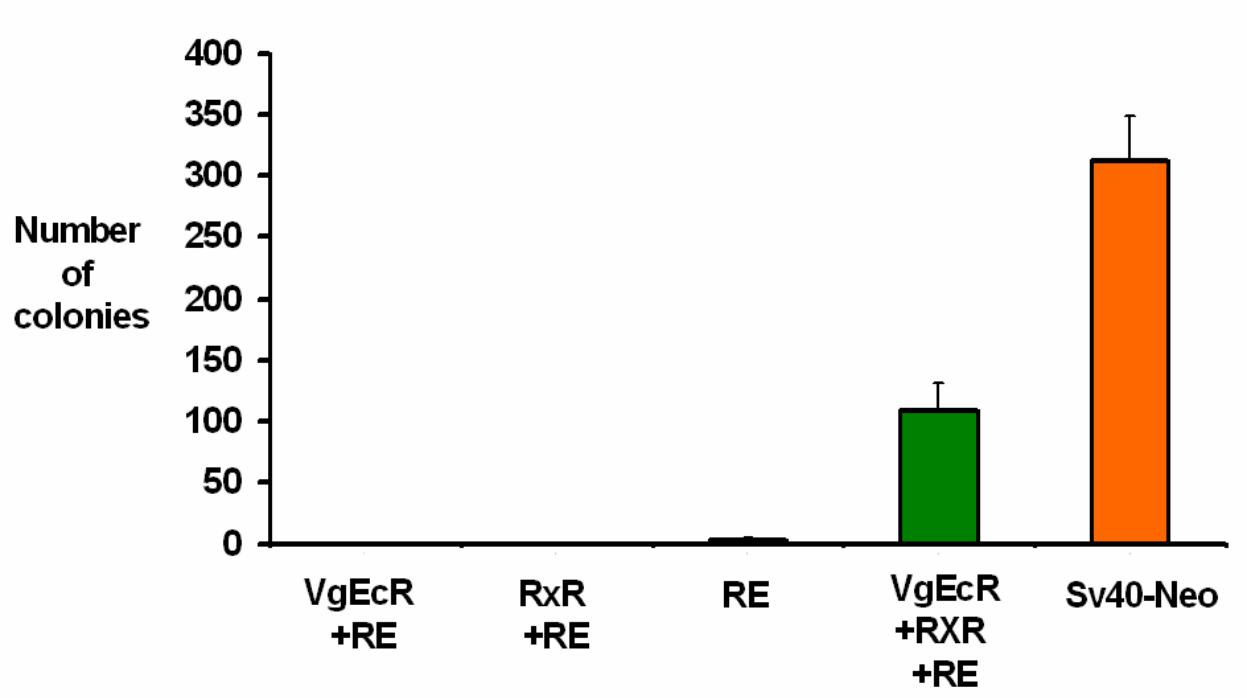


Figure 52: Number of resistant ES cell colonies formed with different combinations of the three element system.

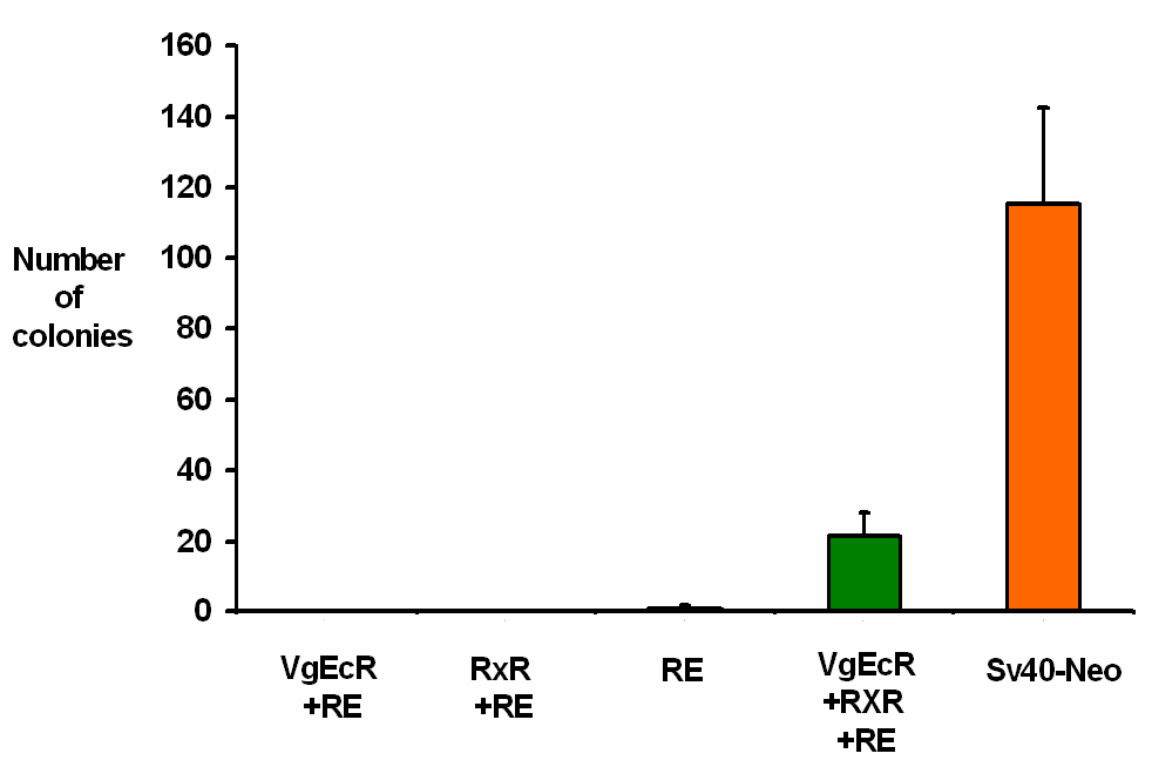


Figure 53: Number of resistant MSC colonies formed with different combinations of the three element system.

Verification of the functionality of cardiac stage-specific promoters:

The three element system was designed to insert multiple tissue specific promoters/enhancers to drive fluorophore expression to study lineage changes in stem cells. Four promoters which represented four different stages of the cardiac differentiation process were cloned into the three element system. The first plasmid carries the cardiac-specific enhancer adjacent to a basal promoter which drives CFP expression when activated. The second plasmid carries the Mef 2c promoter driving RFP when activated. The third plasmid carries the alpha MHC promoter during

GFP and the MLC2v promoter driving YFP when respectively activated. Together this composite promoters system will mark the earliest cardiac progenitors, differentiated cardiac progenitors, ventricular progenitors and terminally differentiated ventricular cardiomyocytes in the ES cells during differentiation.

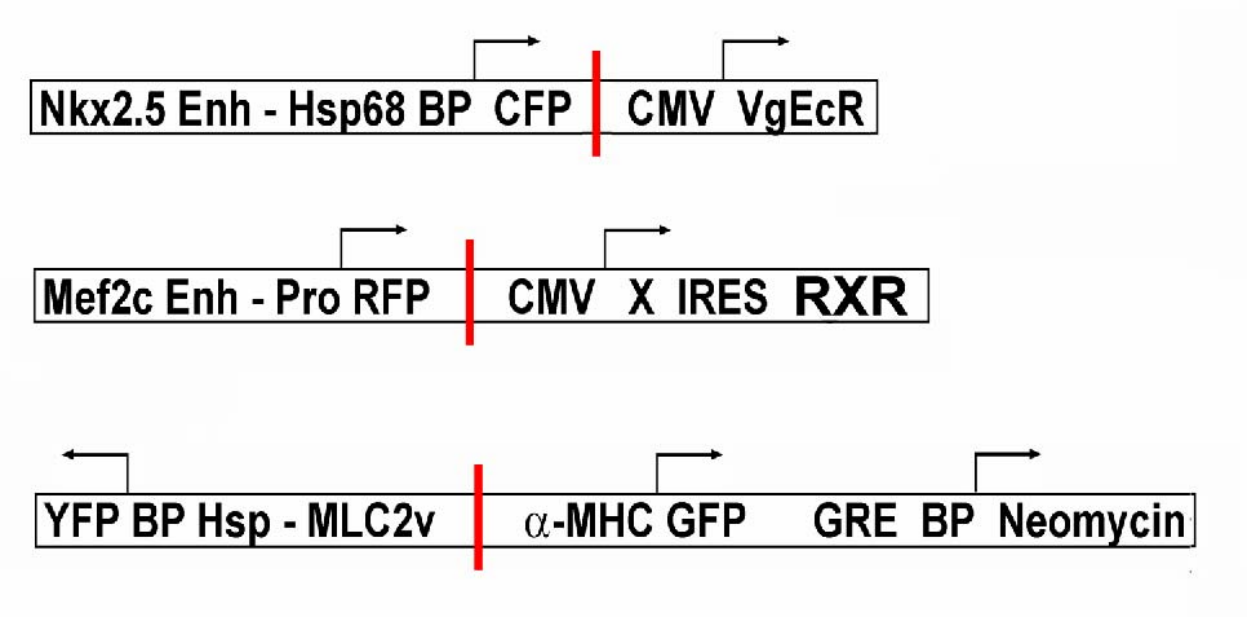


Figure 54: Schematic of four different cardiac stage-specific promoters in three plasmids. Red line indicates the site of linearization before transfection.

The functionality of the promoters was tested individually in murine neonatal cardiomyocytes. There is minimal leakage of the promoters in 293 cells when compared to their activity neonatal cardiomyocytes.

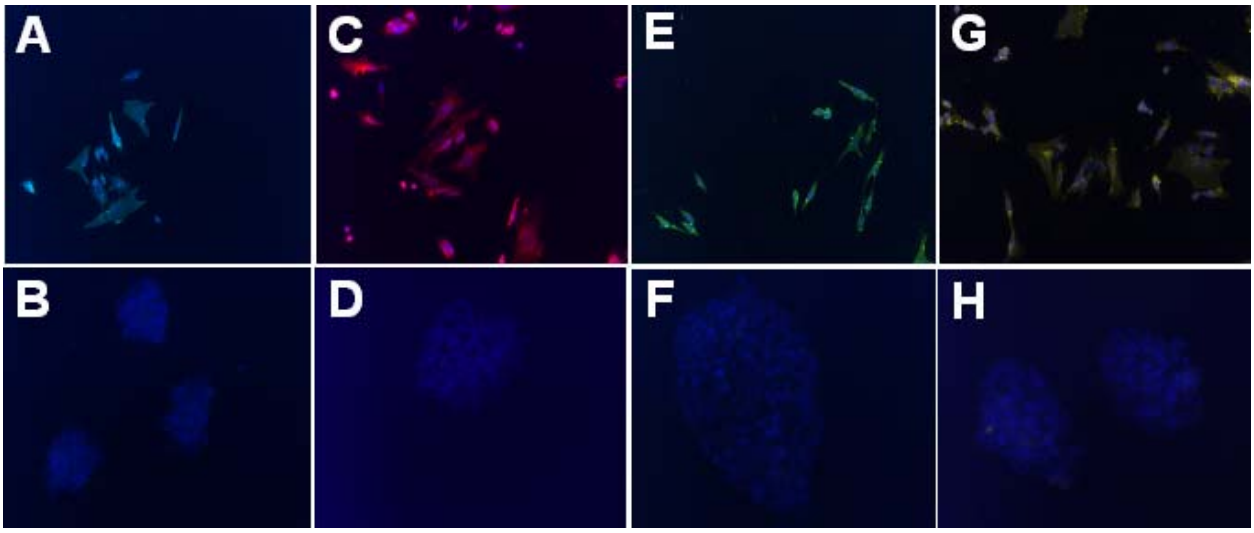


Figure 55: Activity of cardiac stage-specific promoters in murine neonatal cardiomyocytes and 293 cells.

All panels are stained with DAPI to visualize nuclei. Panels A, C, E and G are murine neonatal cardiomyocytes. Panels B, D, F and H are 293 cells.

Panel A and B: Nkx 2.5 cardiac specific enhancer driving CFP.

Panel C and D: Mef2c promoter driving RFP.

Panel E and F: Alpha-MHC promoter driving GFP.

Panel G and H: MLC2v promoter driving YFP.

These systems of four promoters on three plasmids which are interdependent on each other for neomycin resistance were electroporated and resistant colonies were picked and expanded. PCR screening for the insertion of the full length plasmids into the genome was performed and the

positive colonies were further expanded. These conies were differentiated to check for the functionality of all the promoters.

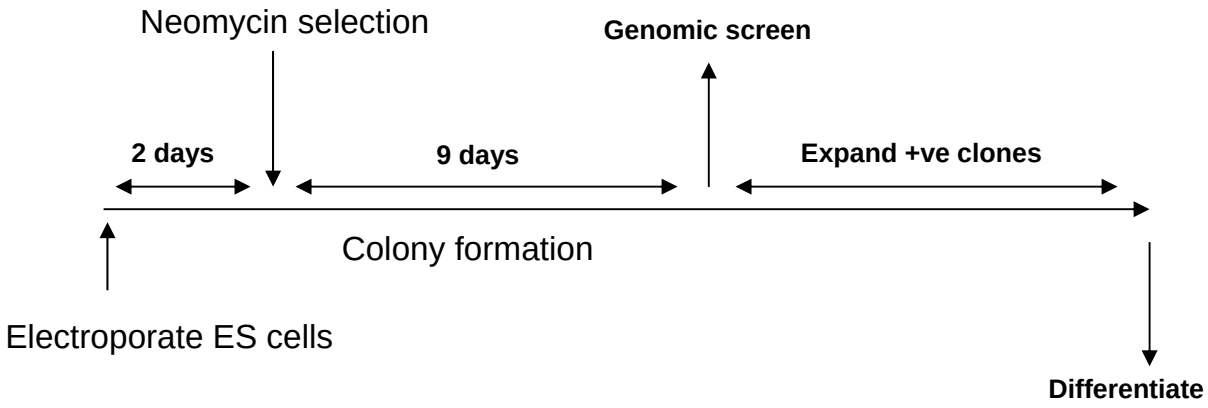


Figure 56: Protocol used for the selection and testing of ES cells positive for the integration of the four cardiac stage-specific promoters.

Differentiation was induced by withdrawal of LIF and suspension culture 10 days. CFP positive cells were seen as distinct loci on the third day of differentiation. RFP positive cells appeared on the fifth day of differentiation and entirely co-localized with CFP positive cells. No migration of these cells was observed at this stage also. GFP positive cells were observed on the seventh day of differentiation and it coincided with a marked decrease in the CFP and RFP fluorescence. GFP positive cells thereafter exhibited spontaneous contractions.

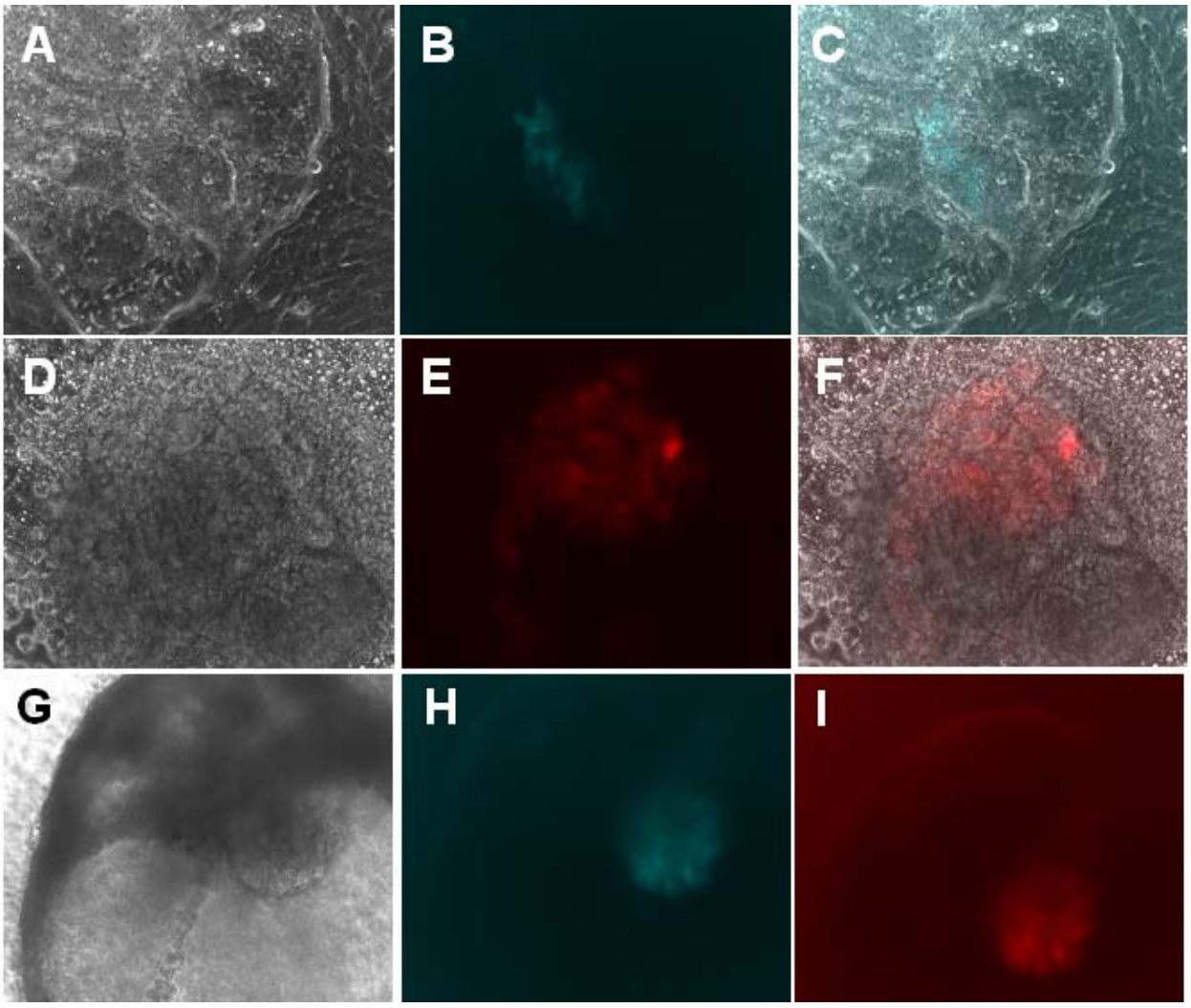


Figure 57: CFP and RFP positive foci in differentiating embryoid bodies.

Panels A, D and G: Phase contrast pictures of embryoid bodies.

Panel B: CFP positive cells seen in the middle of the embryoid body.

Panel E: RFP positive cells seen in the middle of the embryoid body.

Panels C and F: Merges pictures of fluorescent and phase contrast pictures.

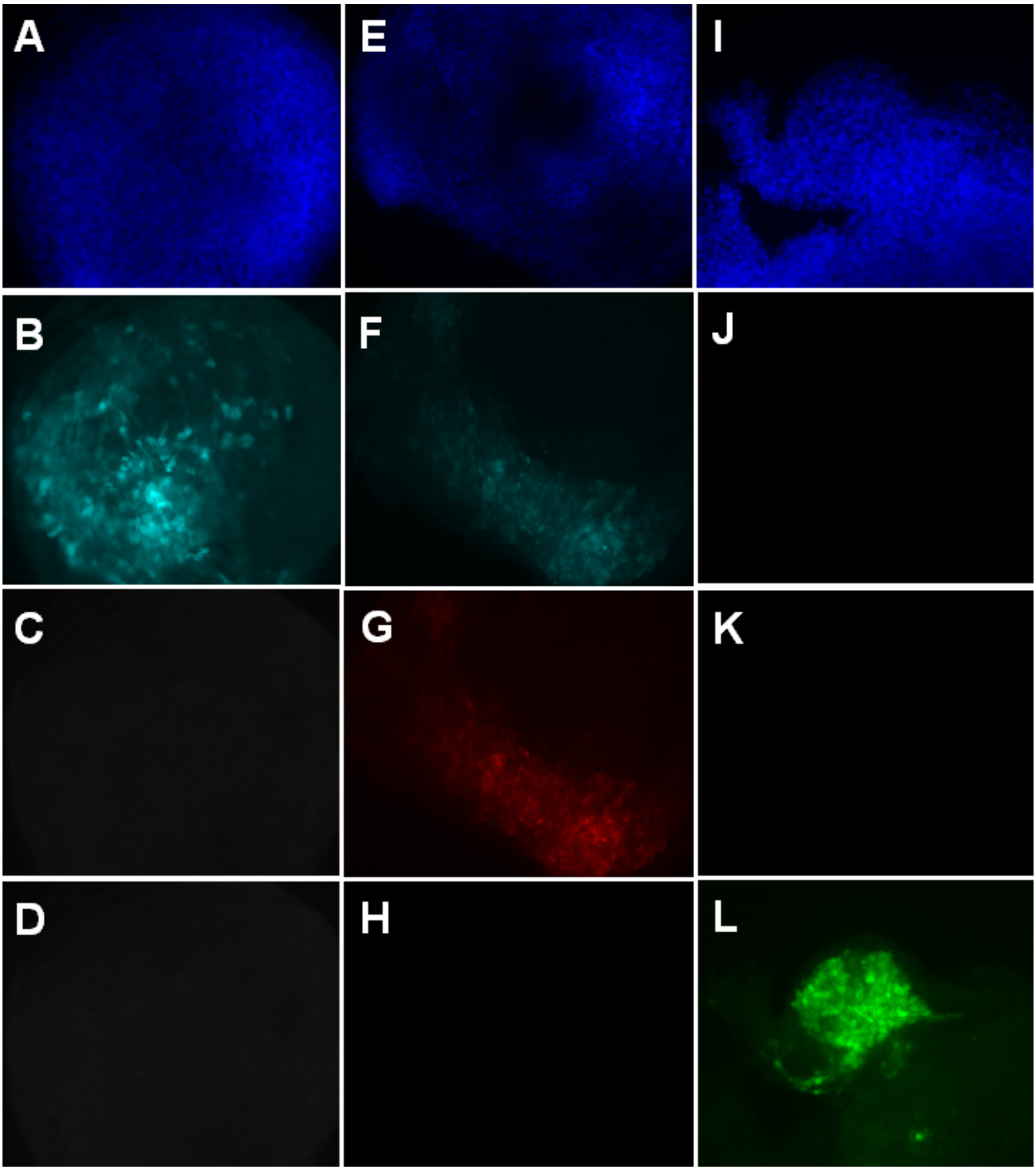
Panels H and I show the co-localization of CFP and RFP positive cells.

Figure 58: Fluorophore changes are distinct in the later stages of differentiation.

Panels A, B, C and D are the DAPI, Cyan, Red and Green Channels on an embryoid body at day 3.

Panels E, F, G and H are the DAPI, Cyan, Red and Green Channels on an embryoid body at day 6. The cyan and red cells co-localize.

Panels I, J, K and L are the DAPI, Cyan, Red and Green Channels on an embryoid body at day 9. The green cells do not have any cyan and red fluorescence in them.


A
E
I
B
F
J
C
G
K
D
H
L

The cardiac nature of the GFP positive cells was confirmed by immunostaining them with cardiac troponin T. These cells lose cyan and red fluorescence which also reflects their maturation along the cardiac lineage.

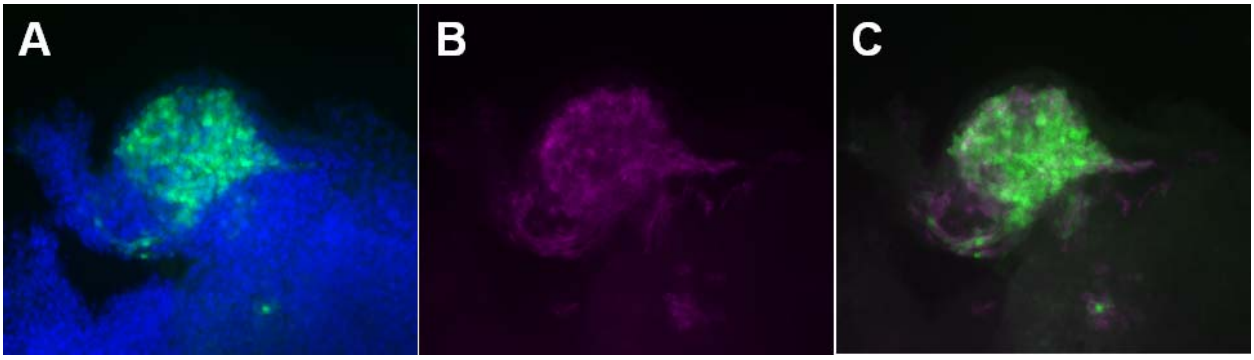


Figure 59: Cardiac Troponin T staining of GFP positive cells in the embryoid body.

Panel A shows a merge picture of DAPI and FITC of the same embryoid body depicted in panels I-L in the previous figure.

Panel B shows troponin T staining.

Panel C shows a merge of troponin T staining and FITC positive cells.

The fidelity of the cardiac stage-specific promoters was verified by visualizing the respective RNA transcripts of the four genes. Semi-quantitative PCR of the transcripts was performed on RNA collected from CFP, RFP and GFP positive cells. Their levels were compared to total embryoid body RNA collected on days 3, 6 and 9. The level of native RNA of these four genes closely reflects the activity of the four promoters. This further validates the usage of this system of cardiac stage-specific plasmid system for lineage analysis in differentiating ES cell cultures.

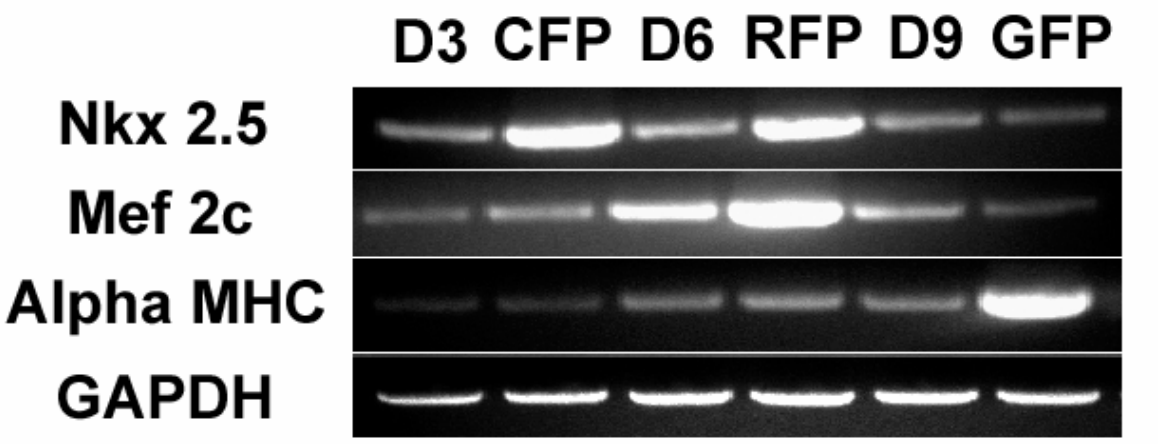


Figure 60: Semi-quantitative RT-PCR comparing the transcripts of the four genes in embryoid bodies and CFP, RFP and GFP positive cell populations.

Utility of multi-promoter-fluorophore system in studying lineage changes during cardiogenesis from ES cells:

This combination of promoters was used to study the effect of retinoic acid on ES cell differentiation into cardiomyocytes. Retinoids are expressed in a gradient in the heart tube during development suggesting that RA signaling may be important for cardiogenesis [183-185]. Although such a well-organized gradient is not likely to be present in an embryoid body, RA signaling might still enhance commitment of ES cells to specific chamber specific cell types. In mES cells, RA treatment at a concentration of 100nM increases levels of alpha MHC in late EBs but not in the earlier stages stages. It is possible that the cardiac progenitors (GFP positive cells) are moving away from ventricular lineage (YFP positive cells).

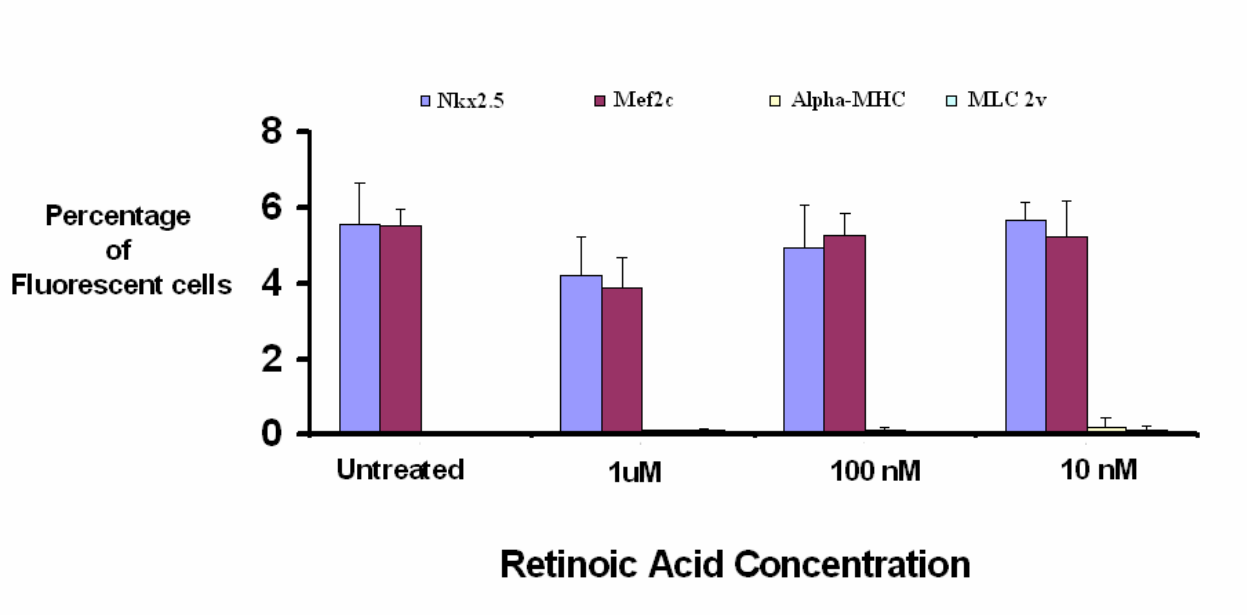


Figure 61: Pattern of differentiation after retinoic acid treatment on Day 5.

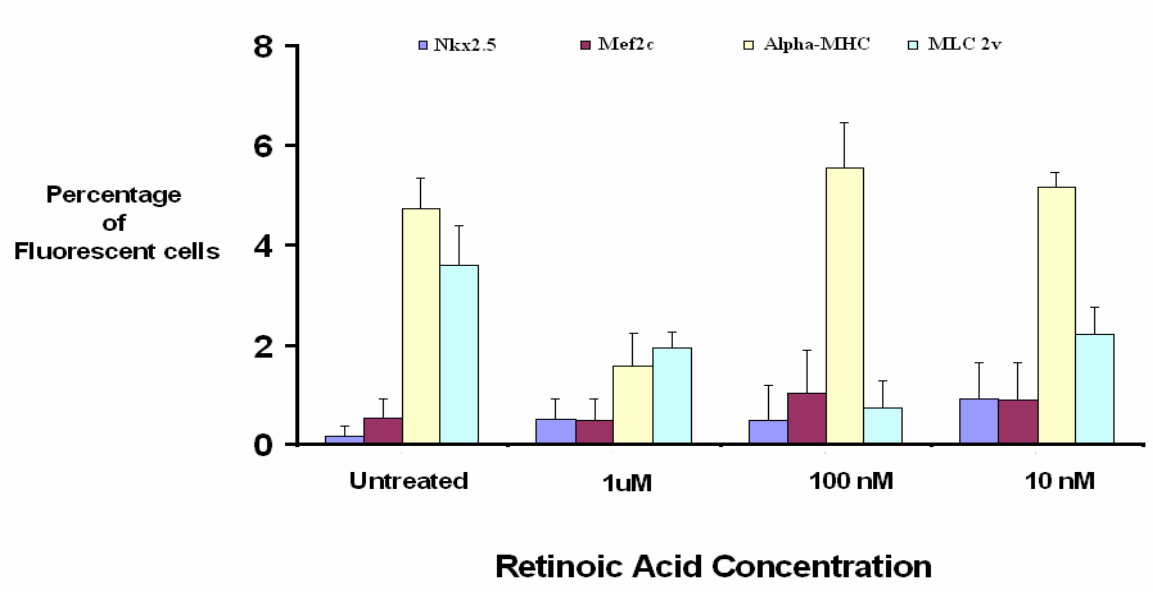


Figure 62: Pattern of differentiation after retinoic acid treatment on Day 9.

It is possible to introduce more promoters using a second system with a different antibiotic. This system utilized both the 'transcriptional cascade' concept and trans-splicing. This system is more

efficient since there is only one step of transcriptional cascade involved and trans-splicing is most efficient when there are only two fragments involved. Together, this combination represents a second 'three element system' whereby potentially more tissue-specific promoters driving markers can be inserted using dual antibiotic selection.

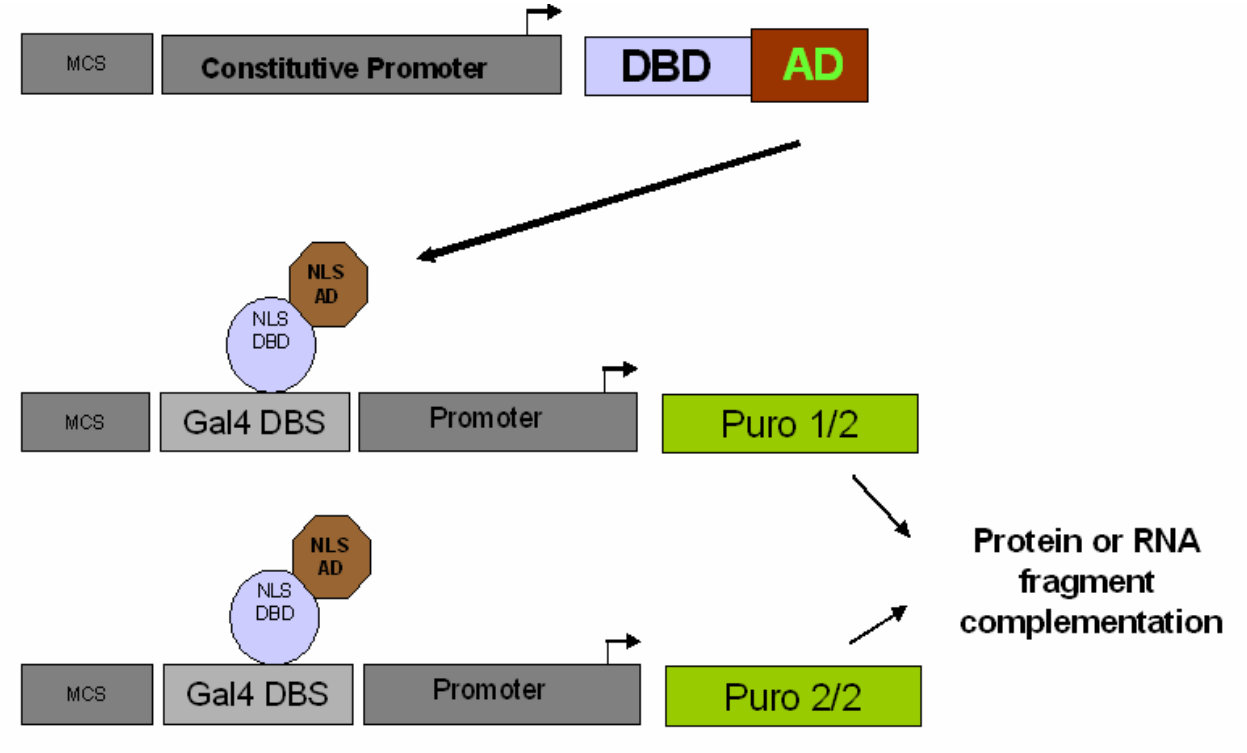


Figure 63: Three element system using transcriptional cascade and trans-splicing.

DBD denotes DNA Binding Domain.

AD denotes Activation Domain.

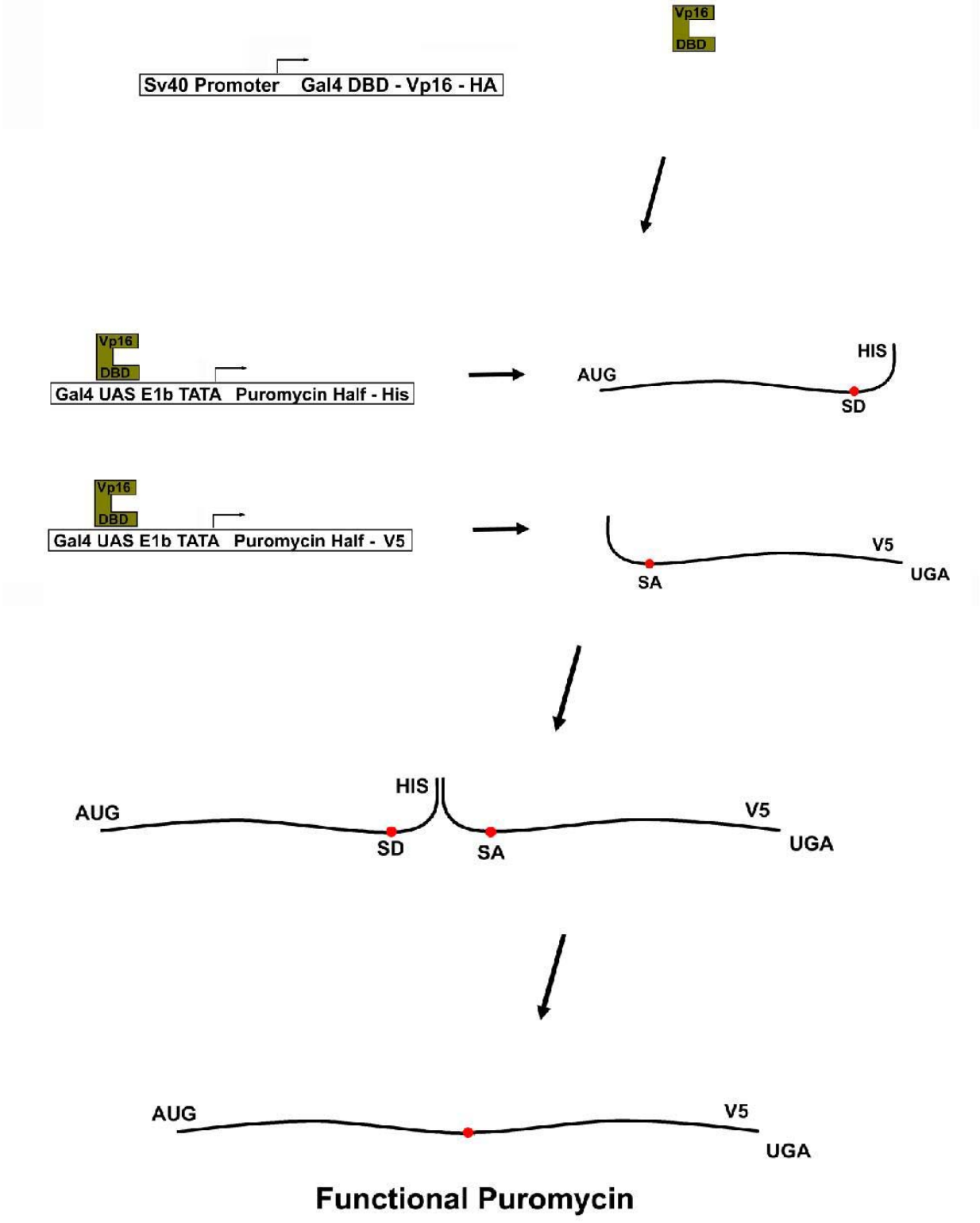


Figure 64: Three element trans-splicing system for puromycin resistance.

The alternate system designed here incorporated a hemagglutinin epitope at the end of the reading frame of a Gal4 DBD–Vp16 chimeric transcription factor. This allows for monitoring the activity of the first plasmid. The second plasmid carries a His tag at the end of the annealer sequence. The level of this protein reflects the amount of untrans-spliced product. The third plasmid does not have start codon in the reading frame and carries a V5 tag at the end of the puromycin reading frame. The level of V5 reflects the amount of puromycin produced. The efficiency of this method was inferior to that of the three element sytem utilizing protein heterodimerization.

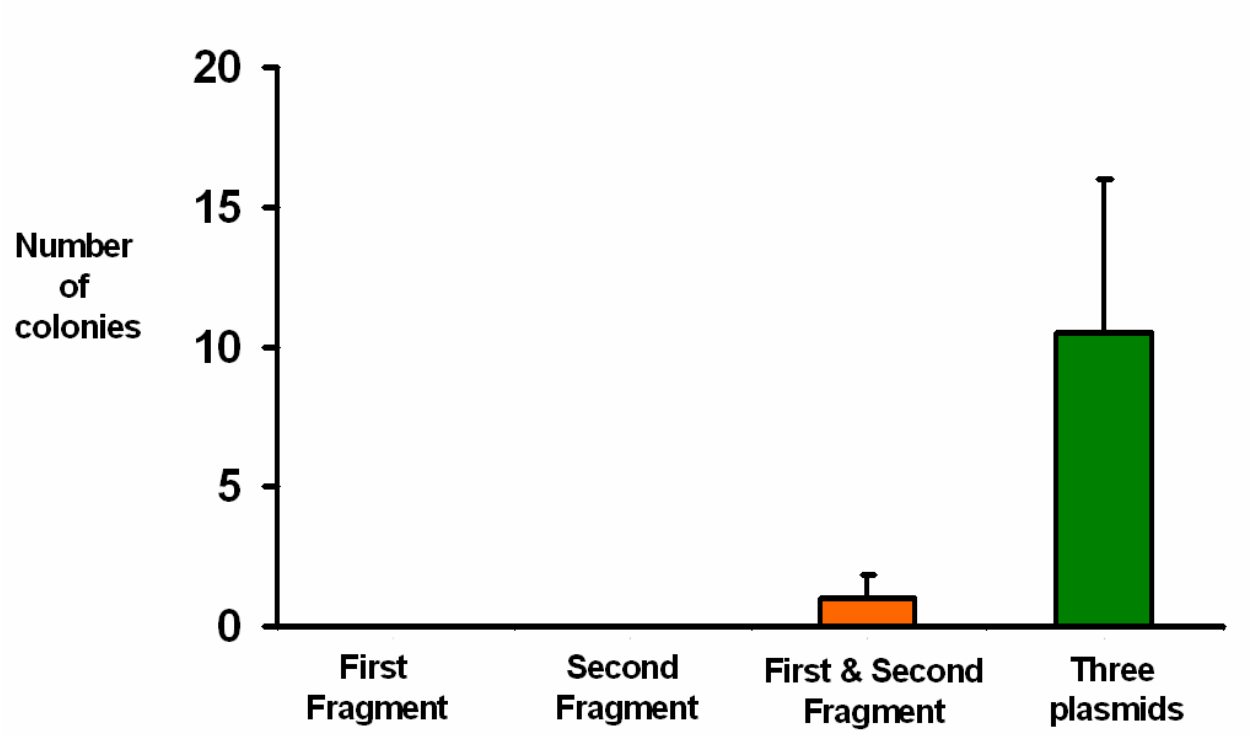


Figure 65: Number of resistant ES cell colonies formed using the trans-splicing system.

Conclusions:

We demonstrate two different methods, where in each method; antibiotic resistance is dependent on the presence of all the three constructs. The first method uses protein dimerization and the second uses RNA trans-splicing. Gene expression systems expressing multiple genes with different degrees of control have been proposed earlier [183], but we have adopted this system for inserting promoters instead of expressing different genes with external regulators like tetracycline. The first method utilizes the dimerization properties of the ecdysone receptor (EcR) and the retinoic acid receptor (RXR). This complex binds to a modified response element and drives transcription in the presence of ponasterone. This mechanism has been exploited in commercially available inducible gene expression control systems. We modified this system by separating the expression of EcR, RXR and the response element driving neomycin resistance into three different constructs and tested their interdependency with neomycin selection. We cloned three markers (RFP, CFP and YFP) into separate constructs and checked for the expression of all the three fluorophores. This is a direct functional evidence of the insertion all the three constructs in the genome. This system was validated by electroporation of these constructs into murine ES cells and mesenchymal stem cells (MSCs). The identity of the MSCs was verified by differentiating them into osteogenic, chondrogenic and adipogenic lineages. All colonies that formed after selection were positive for all the three fluorophores. Constructs were also electroporated individually and in various combinations to investigate for false positive colonies due to leakage of the response element. The response element alone formed very few colonies as compared to all the three constructs. These colonies were passaged to rule out episomal expression and for silencing of the promoters. All colonies continued to be positive for

the three fluorophores after more than 10 passages. However, MSCs had a significantly fewer number of resistant colonies as compared to ES cells. We attribute this to the lesser propensity of foreign DNA fragments to integrate into their genome. The second method uses three constructs in which the first construct constitutively expresses a transcription factor that binds to response elements on the other two constructs. They in turn express the N-terminal and C-terminal halves of the antibiotic resistance molecule (puromycin N-acetyl transferase) which trans-splice to form a complete transcript. The protein products of this system were tagged with HA tag, V5 and His tag. Detection of these tags is evidence that antibiotic resistance is due to trans-splicing of the constituents parts. We further tested the utility of this system by cloning four promoters specific to cardiac lineage into three constructs that utilize protein dimerization. Nkx2.5 is a transcription factor which marks the earliest known cardiac progenitors in heart development [54]. We inserted a 2.5 kb cardiac specific enhancer of Nkx2.5 with a basal promoter driving cyan fluorescent protein (CFP) in the first construct constitutively expressing VgEcR. Mef2c is transcription factor which is expressed at a later stage in the anterior hear field [53]. We cloned the 4 kb Mef2c enhancer with its endogenous promoter before RFP and inserted it into the second construct constitutively expressing RXR. Alpha Myosin heavy chain (α-MHC) is a structural protein which is expressed in progenitor cardiomyocytes [61]. We have cloned the 5 kb α-MHC promoter driving GFP into the third construct which has the response element driving neomycin resistance. Myosin light chain ventricular isoform (MLC 2v) is a structural protein which is expressed in mature ventricular cardiomyocytes. This promoter has been used in conjunction with a CMV basal promoter to select pure populations of ventricular cardiomyocytes [184]. We cloned this element of 3 kb driving YFP into the third construct such that after linearization the promoters would be separated by a 6 kb region of the plasmid. We checked the

functionality of these constructs in neonatal mouse cardiomyocytes. We next electroporated ES cells with these constructs to create reporter lines that will glow with different fluorophores at different stages of differentiation. All resistant clones were screened by for the complete insertion of the constructs by PCR and approximately 35% of all the colonies had complete integration of all the three constructs. Clones which were verified for genomic integration of these constructs were investigated for concatenated insertion by PCR and we found that none of the clones had concatenated insertions. We next investigated the functionality of these clones by differentiating them and found that 2 out of 34 clones were weak expressers. The fidelity of expression was also verified by comparing the mRNA levels of the four genes being investigated in these clones with respect to wild type differentiated cells and found them to correlate with each other. It is interesting to note that the cardiac differentiation in ES cells was synchronous in all embryoid bodies (EBs). We did not find any EBs which had delayed or earlier cardiac differentiation in them. It was also observed that the usually one or two foci of cardiac differentiation would be present and they would differentiate at an equal pace. Furthermore, all EBs which had focus of cardiac differentiation would give rise to predominantly one cardiac cell subtype (ventricular or non-ventricular). One of the challenges in the cell replacement therapy is to find the most suitable state of differentiation between stem cells an differentiated cells where the cells are committed to the lineage but still have the proliferative abilities of progenitor cells. This system enables the isolation of pure populations of cells at different stages of commitment and hence will provide a basis for the measurement of their proliferative potential. This system is also useful for testing the effect of morphogens and small molecules on cardiogenesis. Retinoic acid is known to alter cardiogenesis and we verified this effect in our system by measuring the changes in fluorophores expression and correlated them with changes in cardiac specific

transcripts compared to untreated controls. We also quantified the relative changes in cell number by FACS analysis. It is potentially possible to use the second system utilizing RNA trans-splicing to introduce at least three more promoters (one on each construct) into the genome at the cost of only one added antibiotic resistance.

To summarize, in this study we demonstrate two novel strategies to insert multiples promoters into the genome of ES cells. This method could be very useful in studying the lineage commitment of ES cells in varied settings and to purify pure populations of stage-specific cells for cell replacement therapy. These twin systems could also be used for the over expression of six genes in a manner which doe not require genomic PCR verification.

Materials and methods:

Cell culture: All initial tests with trans-splicing of the GFP molecule were done in 2093 cells grown in basal medium (DMEM + 10% FBS + antibiotics). Mouse ES cells were cultured on gelatin coated dishes in a medium with DMEM, 20% FBS, LIF, 2-mercaptoethanol, non-essential amino acids, L-glutamate and antibiotics. Mesenchymal stem cells were isolated from mouse femur and grown in tissue culture dishes in isolation medium consisting of RPMI, 9% FBS, 9% equine serum, L-glutamate and antibiotics. Adherent cells were continuously cultured for two weeks in isolation medium and monitored for cell proliferation. The medium was changed to expansion medium consisting of IMDM, 9% FBS, 9% equine serum, L-glutamate and antibiotics. Differentiation of ES cells was achieved by withdrawal of LIF. Retinoic acid

was added to the differentiation medium at varying concentrations to investigate changes in pattern of differentiation.

Transfection of ES cells: Plasmid DNA was linearized and purified in sterile conditions. 25 μg of the plasmid was electroporated using a BioRad GenePulser II machine. The voltage of the pulse was set at 250 Volts and the capacitance at 330 μF. Cells were incubated in ice for 20 minutes immediately after administering the pulse. Cells were transferred to gelatin coated tissue culture dishes with ES medium and selection was started after 48 hrs. Ponasterone was added in the first 12 hours to enable the production of neomycin phosphotransferase and subsequently included in all media preparations involving the three element system.

Plasmid construction: RXR and VgEcR were cloned from pERV3 (Stratagene). The glucocorticoid response element was cloned from pEGSH. RFP was cloned from mRFP vector. CFP and YFP were cloned from pECFP, pEYFP respectively. Neomycin was cloned from pcDNA 3.1. Puromycin was cloned from EC-Puro Exchange cassette (Stratagene). p53, Sv40 T antigen, Gal 11 and LGF2 were cloned for the positive controls of the bacterio-match vectors and mammalian-two hybrid vectors (Stratagene). The cardiac specific enhancer of Nkx 2.5 was a kind gift of Dr. Eric Olson. Mef2c promoter was kindly provided by Dr. Brian Black. Alpha – MHC promoter was gifted by Dr. Gulick and the MLC2v promoter was cloned from genomic DNA. The annealer fragment is a repeat of the sequence: GTA and is 30 nucleotides in length. The artificial intron used is 80 nucleotides long and has a canonical CAGG sequence.

RT-PCR: Total cellular RNA was extracted using Trizol (Invitrogen, Carlsbad, CA) reagent according to the protocol provided by the company. Trace amounts of genomic DNA were removed by using Turbo free DNAase kit (Ambion). cDNA was made using oligo dT and RT reaction was done with Thermoscript kit (Invitrogen). GAPDH was used as an internal control and. PCR products were run on an agarose gel.

Image acquisition: All images were acquired on Zeus Axiovert fluorescence microscope.

Flow cytometry: All samples were suspended as single cells and sorted at the FACS facility on a Dako Cytomation MoFlo machine. 293 cells transiently transfected with constructs expressing single fluorophores were used as controls to set the gates for fluorescence intensity.

Immunostaining: Embryoid bodies were plated on fibronectin coated coverslips and fixed with 4% paraformaldehyde. Free floating embryoid bodies also were centrifuged and fixed. Coverslips were washed in PBS twice and incubated with PBS-tween for five minutes. Goat serum was added to the solution to a total concentration of 5%. Primary antibodies for cardiac troponin T (Santa Cruz) and alpha-MHC (MF20-Developmental Hybridoma Bank) were added to the solution at 1: 500 dilution and incubated overnight. The next day, after three washes with PBS, secondary antibody conjugated to Alexafluor 647 was added for 1 hr. The coverslips were then washed thrice with PBS and DAPI was added for 10 minutes. After a set of three final washes with PBS, the coverslips were mounted with Fluormount G.

CHAPTER 4

Studies on Cardiogenesis in Mesenchymal Stem Cells

Rationale:

Mesenchymal stem cells are functionally defined by their ability to give rise to osteogenic, chondrogenic and adipogenic lineages [185]. Mesenchymal stem cells from the bone marrow are isolated by their ability to adhere to cell culture dishes and can be expanded over a million fold which makes them good candidates for cell replacement therapy. The homogeneity of this population has been questioned but it is generally agreed that these cells are positive for CD 29, CD 105, CD 166 and are negative for CD 34 and CD 45 [186]. Bone marrow derived mesenchymal stem cells acquire cardiac markers when treated with 5 Azacytidine and form stable intracardiac grafts when injected into healthy mice [187]. These cells can also be propagated as clones and are known to change their signature action potentials from sinus node-like to more ventricular-like action potentials. However, in spite of their electrophysiological properties and cardiac-specific mRNA, these cells exhibit a different phenotype from cardiomyocytes and form unusually long, tube-like, spontaneously contracting structures some of which may reach 3000 μm [188]. These cells are positive for Nkx 2.5, Gata 4, Mef2c and negative for TEF 1. The chief disadvantage of these cells being the prolonged time required for differentiation and the lack of in-vivo studies to demonstrate their applicability for cell transplantation. Activation of notch signaling by jagged 1 has been shown to differentiate mesenchymal stem cells into cardiomyocytes [189] Untreated mesenchymal stem cells have been shown to home to infarcted myocardium when injected intravenously [190]. The subsequent differentiation of the engrafted cells into cardiomyocytes, smooth muscle cells and endothelial cells has been controversial. Intracardiac injection of human mesenchymal stem cells into nude mice shows cells positive for cardiac Troponin T, beta myosin heavy chain and alpha actinin

when examined two months after injection [191]. The engrafted cells exhibited sarcomeric organization but numerically fewer in the infarcted myocardium. A similar study using bone marrow stromal cell injected into the infarcted myocardium of the left ventricle detected cells positive for cardiac Troponin I, intercalated discs and gap junctions. The phenotype in this study was attributed to cell fusion [192]. Extracts from rat cardiomyocytes when transiently exposed to human adipose derived stem cells cause them to acquire cardiac markers like cardiac Troponin I, alpha sarcomeric actin, desmin and connexin 43 [193]. Formation of binucleated, striated and spontaneously contracting cell clusters responsive to chronotropic drugs points towards reprogramming of the genome, however other mechanism like epigenetic modifications could be involved. Low frequency of formation of cardiomyocytes and the use of cellular extracts precludes these cells from clinical use. Cytokines and growth factors in the medium can also influence the differentiation of bone marrow derived mesenchymal stem ells into cardiomyocytes. A mixture containing transforming growth factor beta 1 (TGF-β1), bone morphogenetic protein 2 (BMP 2), insulin-like growth factor 1 (IGF 1), fibroblast growth factor 4 (FGF 4), leukemia inhibitory factor (LIF), alpha thrombin, vascular endothelial growth factor A (VEGF A), tumor necrosis factor alpha (TNF α) and retinoic acid has been used to differentiate human bone marrow derived mesenchymal stem cells into cardiopoietic population positive for Nkx 2.5, Mef 2c and alpha actinin [194]. These cells do not form spontaneously contracting clusters and have not yet been shown to possess calcium transients. Cells derived from the stromal vascular fraction of adipose tissue when cultured in medium supplemented with insulin, transferrin, IL 3, IL 6 and SCF acquire contractile phenotype after 14 days in culture. These cells are positive for Nkx 2.5, Mef 2c, Gata 4, atrial natriuretic peptide, connexin 43, beta myosin heavy chain, atrial and ventricular isoforms of myosin light chain. These cells are

multinucleated, have organized sarcomeric apparatus and are responsive to chronotropic drugs [195]. The frequency of differentiation (0.02 – 0.07 %) is low in this method and in-vivo properties of these cells have not been determined. Co-culture of mesenchymal stem cells with cardiomyocytes has been shown to influence their differentiation into cardiomyocytes [196-199]. Although very few cells acquire cardiac phenotype [200], none of them mature to form spontaneously contracting clusters. There is also a further unresolved question if co-culture with neonatal or adult cardiomyocytes is more efficient in inducing cardiac differentiation. A very small number of cells also acquire cardiac phenotype when co-cultured with fixed cardiomyocytes [201]

Results:

Mesenchymal stem cells are functionally defined by their ability to produce adipogenic, chondrogenic and osteogenic lineages. These cells when grown in adipogenic medium for two weeks acquire abundant lipid droplets in their cytoplasm.

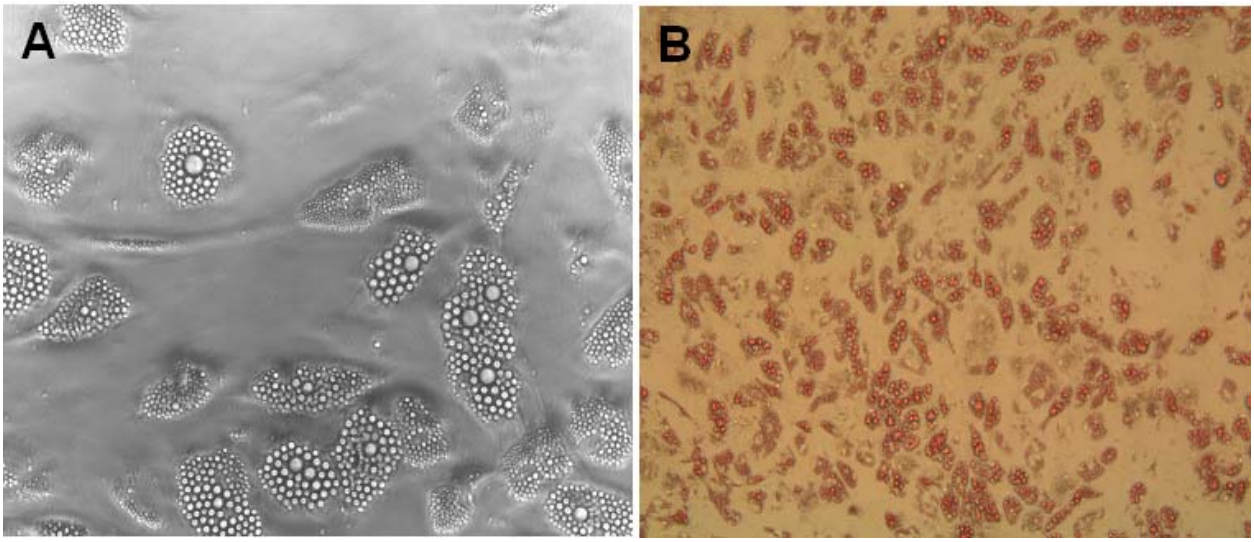


Figure 66: Phase contrast picture (A) and oil red staining (B) of differentiated adipocytes.

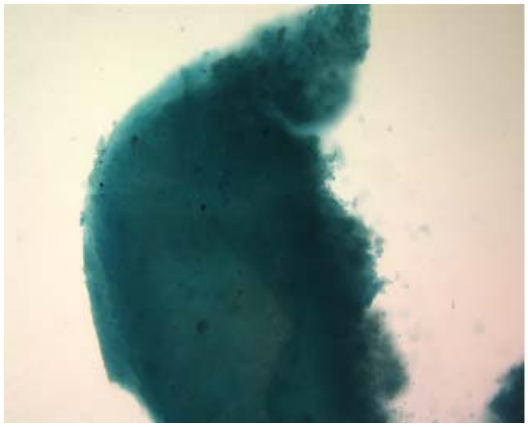


Figure 67: Alician Blue of cartilage pellet produced by culturing mesenchymal stem cells in chondrogenic medium.

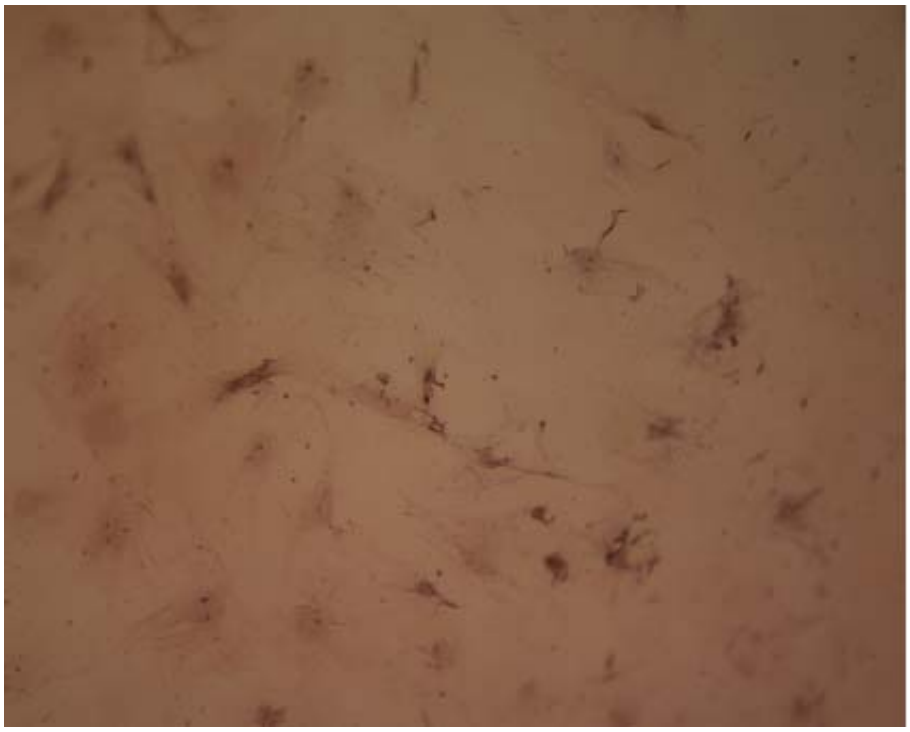


Figure 68: Calcium staining of osteoblasts by alizarin red treatment.

Effect of membrane proteins and media supplements on cardiogenesis from mesenchymal stem cells:

Cell cultures were done at three different concentrations (1250, 2500 and 5000 per 2 cm^2 per well) for all the following conditions:

Substrate variations:

1. Fibronectin.
2. Gelatin

3. Laminin
4. Matrigel.
5. None (untreated cover slip).

Substrate variations with membrane extracts:

Membranes from different cells were added in these combinations to test for effect of membrane epitopes on the cardiac differentiation of mesenchymal stem cells:

1. Matrigel + Neonatal mouse cardiomyocyte membrane
2. Matrigel + H9C2 cell membranes.
3. Matrigel + Adult mouse cardiomyocyte membranes.
4. Matrigel + 293 cell membranes.
5. Fibronectin + Neonatal mouse cardiomyocyte membrane
6. Fibronectin + H9C2 cell membranes.
7. Fibronectin + Adult mouse cardiomyocyte membranes.
8. Fibronectin + 293 cell membranes.
9. Methylcellulose + Neonatal mouse cardiomyocyte membrane
10. Methylcellulose + H9C2 cell membranes.
11. Methylcellulose + Adult mouse cardiomyocyte membranes.
12. Methylcellulose + 293 cell membranes.

Media variations:

1. Basal medium + 5' Azacytidine (9μmol/L for 24 Hrs).
2. Basal medium + Trichostatin (1μM for 24 Hrs).

3. Basal medium + Dimethyl Sulphoxide (DMSO for 24 Hrs).
4. Enriched medium (medium supplemented with Insulin, transferrin, Ascorbate etc – complete composition of medium is in Materials and Methods section).
5. Methocult medium (methylcellulose medium supplemented with SCF, IL-3. IL-6 etc - complete composition of medium is in Materials and Methods section).

Cells were cultured in duplicate in all the above 66 conditions (66) for 45 days and observed for spontaneously contracting cell clusters. They were subsequently fixed and stained for cardiac troponin T.

The morphology of the cells during this time period did not significantly change. None of the above conditions resulted in the formation of any spontaneously contracting cell clusters. No cardiac troponin T positive cells were detected.

Conclusions:

Enriched medium and methocult medium has been shown to induce cardiogenesis in adipose derived mesenchymal stem cells of rodents [195,202]. We did not observe any such changes in human adipose derived cells. It is possible that the mechanisms which are responsible for such effects in rodents are completely different in humans. Co-culture with neonatal cardiomyocytes has been extensively used as a culture technique to induce cardiomyocyte differentiation in a variety of cells. There are also reports of a similar effect when co-culture is done with fixed cardiomyocytes. We attempted to reproduce this phenomenon by co-culture with the membrane

extracts of various cells. We have not observed any signs of cardiomyocyte formation. It is possible that the membrane extracts added to the medium had degraded rapidly in the first 24 hrs of culture. This loss of epitope exposure to the mesenchymal stem cells could have been offset by fixing the membrane extracts with paraformaldehyde or by frequently replenishing the culture medium with fresh membrane extracts. Efforts to identify any such membrane components which have a cardiogenic effect would be of great benefit to cell-based cardiac therapy.

Materials and Methods:

Cell culture: Adipose derived human mesenchymal stem cells were purchased from ZenBio Inc. The cells were at a passage 3 during acquisition. Three vials representing cells extracted from three different patients were used in the experiments to rule out variability due to age and BMI. They were expanded into passage 5 by culturing in a maintenance medium consisting of DMEM/F-12, fetal bovine serum, hepes and antibiotics (penicillin, streptomycin and amphotericin B). No spontaneous differentiation was observed and cells retained their morphology. 293 cells were grown in a basal medium with DMEM, 10% fetal bovine serum and antibiotics. Adult mouse hearts were extracted immediately after sacrifice and stored in liquid nitrogen.

Mesenchymal stem cell characterization: Mesenchymal stem cells were functionally characterized by differentiating them into adipogenic, chondrogenic and osteogenic lineages.

Adipocyte differentiation: Adipocyte differentiation was achieved by plating 75,000 cells per well in a 24 well tissue culture dish. 48 hrs after plating, medium consisting of DMEM/F-12, fetal bovine serum, hepes, biotin, pantothenate, insulin, dexamethasone, isobutylmethylxanthine and antibiotics (penicillin, streptomycin and amphotericin B) was used to differentiate the cells into adipocyte lineage. Medium was changed every 4 days and lipid droplets started appearing after one week in culture. The cells attained large collections of lipid droplets after two weeks in culture. These cells were then fixed with 4% paraformaldehyde and stored for staining. Adipocytes were stained by washing with distilled water once and propylene glycol twice for five minutes each. Oil Red was applied for seven minutes followed by 85% propylene glycol for three minutes. The stained cells were washed in distilled water and mounted with Permount.

Osteoblast differentiation: Osteoblast differentiation was achieved by plating 50,000 cells per in a 24 well tissue culture dish. 48 hrs after plating, medium consisting of DMEM/F-12, fetal bovine serum, ascorbate, dexamethasone, vitamin D3 and antibiotics (penicillin, streptomycin and amphotericin B) was used to differentiate the cells into osteoblastic lineage. Medium was changed every 4 days and cells were cultured for two weeks. These cells were then fixed with 4% paraformaldehyde and stored for staining. The coverslips were stained by washing with distilled water once and alizarin red solution for five minutes. This was followed by 20 washes in acetone and 20 washes in acetone-xylene. After a final xylene wash, the coverslips were mounted with Permount.

Chondrocyte differentiation: mesenchymal stem cells were mobilized by trypsin treatment and counted by trypan blue exclusion. One million cells were pelleted by centrifugation for 10

minutes and the supernatant medium was removed. Chondrocyte differentiation medium consisting of DMEM, fetal bovine serum, ascorbate, dexamethasone, insulin, transferrin, selenium and antibiotics (penicillin, streptomycin and amphotericin B) was added. Medium was changed carefully without disturbing the pellet very 4 days. After three weeks of culture the cell pellet hardened to form a mass of chondrocytes embedded in cartilaginous tissue. The pellet was then fixed with 4% paraformaldehyde and stored for staining. For chondrocyte staining, the pellet was washed with distilled water and treated with 3% acetic acid for three minutes. Alcian blue was added for 30 minutes. The pellet was washed twice in distilled water an mounted in Fluormount G.

Special Media composition:

Enriched medium [203] consisted of:

60% DMEM-LG/28% MCDB-201 (Sigma),

10% fetal bovine serum

1.0 mg/ml bovine insulin,

0.55 mg/ml human transferrin,

0.5 μg/ml sodium selenite,

50 mg/ml bovine serum albumin,

0.47 μg/ml linoleic acid,

10^{-4} M ascorbate phosphate,

10^{-9} M dexamethasone,

and 100 U/ml penicillin G, 100 μg/ml streptomycin sulfate.

Methocult is a proprietary medium manufactured by StemCell Technologies Inc with the following components:
IMDM, methylcellulose, fetal bovine serum, bovine serum albumin, insulin, transferrin, 2-mercaptoethanol, stem cell factor (SCF), IL-3, IL-6 and antibiotics.

Membrane extraction: Neonatal cardiomyocytes, H9C2 cells, 293 cells were lysed with a lysis solution containing 1mN EDTA, 5mM tris HCL, and O.25 M sucrose and proteinase inhibitors. Adult mouse heart tissue was frozen in liquid nitrogen and then ground to powder with mortar and pestle and the powder was added to the lysis solution. The solutions were subsequently homogenized on ice for 20 seconds. The heavy cellular components were removed by centrifugation at 1000 rpm for 10 minutes. The supernatant was spun at 40,000 rpm for 30 minutes and the pellet was resuspended in PBS. All samples were measured for protein concentration and their total volume was changed accordingly.

Immunostaining: Cells were grown on coverslips and fixed with 4% paraformaldehyde. Coverslips were washed in phosphate buffered saline (PBS) twice and incubated with PBS-tween for five minutes. Goat serum was added to the solution to a total concentration of 5%. Primary antibodies for cardiac troponin T (Santa Cruz) and alpha-MHC (MF20-Developmental Hybridoma Bank) were added to the solution at 1: 500 dilution and incubated overnight. The next day, after three washes with PBS, secondary antibody conjugated to FITC was added for 1

hr. The coverslips were then washed thrice with PBS and DAPI was added for 10 minutes. After a set of three final washes with PBS, the coverslips were mounted with Fluormount G.

Microscopy: All slides were observed under an inverted Zeus Inverted microscope. UV channel was used for DAPI staining of nuclei and FITC channel to detect staining of troponin T or alpha-MHC.

CHAPTER 5

General Discussion

Cardiac regeneration is being increasingly seen as a potentially valuable mode of therapy in heart diseases. Cardiomyocytes are considered to be terminally differentiated cells. Cardiomyocytes have been shown to switch from hyperplasia to hypertrophy to compensate for the increase in functional demands during growth and development. However, the heart as an organ has a slow turnover of cells. The source of cells which replenishes the gradual loss of aging cardiomyocytes has been an enigma until recently. It was postulated that 'long range traffic' from the bone marrow could potentially provide new cells which migrate and transdifferentiate into cardiomyocytes. The evidence for such a phenomenon is weak and controversial. The discovery of resident cardiac stem cells has provided a partial explanation. The variation in the markers of these resident cardiac stem cells and their scant and scattered distribution in the heart makes it difficult to understand how effective they are as sources of newly generated cells. Catastrophic cell losses such as in myocardial infarction do not lead to a rapid mobilization of these resident stem cells. Increased chronic functional demands also do not mobilize this compartment. It is known that the axolotl heart has excellent regeneration capacity. Further studies have shown that differentiated cardiomyocytes at the site of the injury are induced to re-enter the cell cycle and contribute to the regenerating myocardium. Mammalian cardiomyocytes do not have this capacity and it is also very difficult to immortalize functional beating cardiomyocytes. Furthermore, no defined territory of each stem cell niche has been elucidated in the mammalian heart. It is possible that these resident cardiac stem cells are vestiges of evolution or contribute exclusively to neoangiogenesis in a disease setting. These cells have an enormous expansion potential ex-vivo and it would be worthwhile to explore the clinical applications of such a potential. This expansion potential may reflect the activity of

mitogens and serum factors which are unique to culture conditions and may not be a true representation of their in-vivo potential.

Studies involving ex-vivo expansion of these resident cardiac stem cells and transplantation into disease models are yet to conclusively show that these cells are good candidates as sources for cardiac cell replacement therapy. There is one exception to this scenario. Pacemaker cells derived from resident cardiac stem cells are not required to have a high proliferative capacity. These cells could be a very valuable immunocompatable substitute for artificial pace makers. A similar paradigm involving pacemaker cells derived from embryonic stem cells has shown the proof of principle for this approach. However a major component of cardiac disease related morbidity is related to ischemic insult which requires mass replacement of cells for functional recovery. Embryonic stem cells on the other hand have a high proliferative potential and the ability to differentiate along a desired pathway. These properties are being studied and harnessed but the insight into these processes comes from studying the normal development of the heart in the embryo.

The discovery of the secondary heart field has shown that cardiac progenitors seem to take divergent pathways much earlier than previously thought. Replicating these cardiac lineages in ES cell differentiation experiments provides us with a powerful tool to produce stage-specific and chamber-specific cardiomyocytes. An even more interesting aspect of this process is to identify means to produce cardiac stem cells from differentiating ES cell cultures. Obtaining and maintaining cardiac stem cells would require precise knowledge of their differentiation pathway and factors that help maintain stemness in these cells. Recently, another approach has been used to produce ES-like cells. Expression of defined factors can cause somatic cells to be re-

programmed to ES cell like state [204]. These cells termed iPS cells have similar characteristics to those of ES cells. Cardiac differentiation of these cells has been demonstrated. However, some groups have reported transformation in these cells. This was due to the usage of c-myc as one of the factors which reprogrammed the cells. Elimination or substitution of this factor is possible and decreases the risk of transformation. These factors were delivered by retroviral vectors which inherently carry a risk of carcinogenesis due to random integration into the genome. Delivery of these factors in the protein form would be an ideal substitute to the current methods employed. The epigenetic print of the iPS cells has been shown to be similar to that of embryonic stem cells which makes them very good candidates for deriving differentiated cells. Therefore applying the principles of cardiac development observed in-vivo to ES cells could ultimately lead to refined methods of producing very specific cells for cellular cardiomyoplasty. Another promising strategy would be to derive cardiac stem cells from iPS cells. This would add the benefit of immunocompatibility to the potency of iPS cells. However the differentiation protocol for this transition from iPS cells to cardiac stem cells is yet to be investigated. Complete characterization of cells derived from resident cardiac stem cells will complement these studies. Recently, very primitive uncommitted progenitor cells have been shown to exist in the adult rodent heart. These cells are Oct 4 and SSEA 3 positive. The expression of Oct4 in the adult has been shown in primordial germ cells. There are reports of Oct4 expression in peripheral blood monocytes. The finding of these cells to be positive for SSEA 3 in the heart could point towards the most primitive and potent population of resident cardiac stem cells in the heart. These cells were shown to differentiate into cardiomyocytes, smooth muscle cells and endothelial cells. An interesting aspect of this differentiation process is that cardiac stem cell markers were found to be transiently expressed in these cells. It is possible

that the uncommitted progenitor cells in the heart transiently differentiate into cardiac stem cells and then completely differentiate into a mixture cardiac specific cell types. Cell fusion is another technique which holds great promise as a source for tissue-specific stem cells. This technique has been used to study gene occlusion. Fusing stem cells with somatic cells with polyethylene glycol (PEG) under dual antibiotic selection yields tetraploid cells. Some of these tetraploid cells are reprogrammed into stem cells. It has been shown that embryonic stem cells fused to somatic cells form tetraploid cells and maintain their pluripotency and tetraploidy under antibiotic selection for over 50 generations. Fusion of these tetraploid cells with somatic cells causes reprogramming if the tetraploid nucleus is removed by centrifugation before it fuses with diploid nucleus. This method has been successfully demonstrated in murine stem cells. To avoid the issue of tetraploidy, if it were to be possible to separate the cells after the event of cytoplasmic fusion but before the dissolution of the individual nuclear envelopes of diploid nuclei, there could be a potential scope for reprogramming. This would require that sufficient protein and RNA from the stem cell is transferred into the recipient cell. Fusing cytoplasts only to somatic cells does not reprogram them which could mean that highly unstable microRNAs are mediating this process or that reprogramming takes a longer time frame than that would be possible during a transient fusion-splitting event. If there has been no transfer of DNA, the reprogrammed donor cell would maintain its distinct MHC profile and yet attain the characteristics of tissue specific stem cells. The difficulty of the process lies in splitting the binucleated fused cells before there is dissolution of the nuclear membranes. This technique could be potentially used for the derivation of immunocompatable tissue specific stem cells from any organ system if resident stem cells of that organ are characterized both in-vitro and in-vivo. There are other sources of adult stem cells like adipose-derived mesenchymal stem cells which

are known to have high proliferative capacity in in-vitro conditions. These cells have the advantage of immunocompatibility. It has been demonstrated that these cells can differentiate into cardiomyocytes at a low frequency in rodents. The mechanisms of this transdifferentiation process are poorly understood. Co-culture with neonatal and adult cardiomyocytes has been shown to induce cardiac markers in these cells. Electrophysiological characterization of action potentials has not been done in these studies. Media supplements such as insulin, transferrin, linoleic acid and ascorbic acid have been shown to augment formation of cardiac markers in these cells. Cell culture substrates such as Matrigel and methyl cellulose have also been used unsuccessfully in these studies. A special culture medium with methylcellulose supplemented with IL-3, IL-6, insulin and transferrin has been shown to induce cardiac differentiation in rodent adipose derived mesenchymal stem cells. Electrophysiological characterization was performed on spontaneously contracting cell clusters in these studies. The most interesting aspect of these transdifferentiation experiments is the effects of fixed membrane epitopes on mesenchymal stem cells. Co-culture with fixed neonatal cardiomyocytes also induces cardiac differentiation in these cells. However, none of these studies have been performed in human adipose derived mesenchymal stem cells. It might be possible to use fixed membrane extracts as inducing agents for cardiac differentiation for these cells.

Embryonic stem cells differentiate into all the three germ layers and the understanding of the formation of the earliest mesodermal cardiac precursors is important in replicating this process in embryonic stem cells. A variety of agents have been shown to be cardiogenic in embryonic stem cells. This includes ascorbic acid, oxytocin, erythropoietin etc. Morphogens such as noggin and retinoic acid have also been shown to influence cardiac lineage commitment. Enrichment of the

differentiated mixture of cells has been achieved by different methods such as microdissection, gradient centrifugation and antibiotic selection. It is possible to attain the most homogenous population of cells by antibiotic selection. The transplantation of such a homogenous population of cells has the best outcome; however, the survival of the transplanted cells remains a major problem. Administering a pro-survival (anti-apoptotic) cocktail of cytokines along with the transplanted cells improves their survival rate in the graft. Genetically manipulating the transplanted cells to express Akt has also been used to enhance their survival rate. Engrafted cells also have to electromechanically integrate into the host myocardium to directly contribute to functional recovery. It would also require sufficient neoangiogenesis to support the metabolic requirements of the engrafted cells. Overexpression of Connexin 43 in the transplanted cells has been shown to reduce the incidence of arrhythmia due to cell transplantation in mice. Differentiated embryonic stem cells overexpressing VEGF have been shown to enhance functional improvement of post-infracted hearts. As embryonic stem cells differentiate along a specific pathway their phenotype changes and their ability to engraft, proliferate and secrete paracrine factors also changes. It would be possible to investigate these properties if there were markers which enabled the purification of live cells into specific subsets. This process not only allows studying the temporal relationship between the development of various stage-specific markers but also allows for the investigation of their proliferative potential, ability to engraft and the elucidation of their signature transcriptome. Furthermore, it is absolutely essential to be able to isolate stage-specific populations to understand the paracrine factors that they possibly secrete which helps in the remodeling of the disease heart. The lack of availability of surface markers which are specific to the stage of differentiation would require genetic manipulation such that a unique detectable marker is produced during that particular stage of differentiation. These

markers can be in the form of fluorophores or engineered membrane epitopes carrying His tag, myc tag etc.

This study addresses some of the problems that are inherent to cell transplantation work. Increasing the efficiency of the production of cardiomyocytes is certainly a requirement. Our study shows that the overexpression of pitx2c enhances the development of cardiomyocytes in the differentiated cell population. Analysis of the mRNA of various cardiac specific genes shows that there is an enrichment of cardiomyocytes as these cells differentiate. Immunocytochemistry of embryoid bodies shows a positive staining for transcription factors like Nkx2.5, GATA4 and structural proteins like cardiac troponin and myosin heavy chain. Quantification of cardiac troponin T positive embryoid bodies showed a significant increase compared to cells transfected with empty vector. A higher incidence of spontaneously contracting cell clusters was seen in pitx2c overexpressing cells. Electrophysiological studies of the action potentials of cells in the spontaneously beating clusters showed that they were similar to ventricular phenotype. Calcium transient studies involve introducing a dye in to a cell such that the dye will glow when it is bound to calcium. This acts as a surrogate measure of the calcium flux in the cytoplasm. Activation of the sarcomeric apparatus will coincide with a release of calcium from the sarcoplasmic reticulum which can be visualized by exciting the dye with a suitable wavelength light. Calcium transient studies performed on the pitx2c overexpressing cells showed the presence of calcium transients with regular rhythm. Pacing of the cells externally induced changes in the contractility rate of the beating embryoid bodies and this was reflected in the recording of the calcium transients as well. The molecular mechanism underlying his process is not clear. Pitx2c is the only isoform which is expressed in the heart and is involved in breaking

the symmetry in the early stages of development. Nodal, Lefty and Pitx2 are conserved in vertebrates and work as a functional unit. Pitx2 is a target gene of Nodal and Lefty1 prevents it from being expressed in the right side of the midline. Pitx2 is most likely working at an earlier stage when it activates Nkx2.5, GATA and Hand and at a later stage when cardiac looping occurs. Pitx2c null mice do not survive beyond birth. These mice exhibit right atrial isomerism. Pitx2c when suppressed in specific cell types gives rise to different outcomes. Myocardium specific suppression of pitx2c causes transposition of great arteries and double outlet right ventricle. Suppression of Pitx2c in cardiac neural crest causes persistent truncus arteriosus. It is possible that pitx2c may influence pharyngeal arch development. Pitx2c is known to suppress the formation of a left sided sinoatrial node. Pix2c interacts with Nkx2.5 and is involved in the formation of the pulmonary myocardium. It is possible that the cardiomyogenic effect of Pitx2c on embryonic stem cells observed in our studies is due to activation of a cascade involving Nakx2.5 and Gata4. Pitx2c binds to beta catenin and it is possible that sequestration of beta catenin by pitx2c in the early stages of differentiation could potentially explain the enhanced cardiogenesis in pitx2c overexpressing cells.

The second part of the study deals with the development of a system to study the progression of cardiac development by the expression of different fluorophores in embryonic stem cells. Classically this is achieved by knocking in a marker into the coding region of one of the alleles of the gene being studied. Such promoter-fluorophore combinations have been extensively used to study lineage commitment in various in-vitro differentiation protocols of embryonic stem cells. Mating multiple such transgenic mice into a single line where in all the promoter fluorophores combinations segregate together would be a very difficult task. An easier method

would be to engineer all the promoter-fluorophore combinations into a single construct but this strategy may fail due to interference of the promoters with each other. To separate these promoter-fluorophore elements would require the usage of multiple selection criteria which will advance the passage number to an unacceptable level. Embryonic stem cells at high passage are known to differentiate poorly and also accumulate genetic mutations. Using multiple plasmids with different antibiotic resistance genes would require administration of multiple antibiotics which tends to differentiate embryonic stem cells. Therefore it is necessary to split the antibiotic resistance amongst the multiple plasmids such that their combined presence contributes to resistance to a single antibiotic. Multiple approaches to this method are possible. Engineering the antibiotic resistance gene such that individual fragments come together and trans-splice to produce a functional molecule is a feasible strategy which has been demonstrated in this study. The usage of more than two fragments either as bridge annealers or as annealers with exonic fragments drastically decreases the efficiency of this approach. Direct protein complementation by splitting the protein has been demonstrated for marker proteins like GFP and luciferase. A similar approach for antibiotic resistance genes has not been demonstrated. In this study, a different approach involving protein heterodimerization has been demonstrated. Ecdysone receptor is a steroid receptor in insects which binds to its cognate binding protein, ultraspiracle and the complex acts starts transcription in steroid response elements in the genome. A modified system involves a modified ecdysone receptor which binds to retinoid X receptor protein and in the presence of the steroid analog- ponasterone activates transcription in modified glucocorticoid response elements. This complex does not bind to endogenous steroid receptor elements. The three components of this system were engineered into three different plasmids such that only in the presence of all the three plasmids a functional neomycin phosphotransferase molecule is

produced. Each plasmid also was engineered to carry a unique fluorophore expressing unit and this system was tested in embryonic stem cells and mesenchymal stem cells. Resistant colonies carrying all three fluorophores formed and continued to express all the three fluorophores after multiple passages in culture. This system was further modified to carry cardiac stage-specific promoters driving different fluorophores. Differentiation of these transgenic embryonic stem cells results in these stage-specific promoters being activated at different time points and the expression of the fluorophores driven by them. The fidelity of the system was further verified by mRNA analysis of cells expressing fluorophores and compared to embryoid bodies of different time points. The effects of morphogens like retinoic acid at different concentrations on the differentiation pattern of embryonic cells can be monitored by this system. It is possible to complement this system with a three-plasmid trans-splicing system by which three more stage-specific promoters can be introduced to make transgenic embryonic stem cells which carry at-least six different promoter-fluorophore elements. This would greatly augment the ability to study differentiation patterns in live cells. There are two additional interesting applications of this strategy. This strategy allows for unique overexpression of at least three different genes which does not require PCR verification of insertion in the genomic DNA. This requires that the genes of interest are cloned with IRES segments after them but before EcR and RXR genes on the two plasmids. The third plasmid would require the gene of interest to be cloned with an IRES segment before the antibiotic resistance gene. The second and more complicated application of this system is stage-specific overexpression of proteins. This system would involve cloning stage-specific-protein elements such that each promoter is activated when the cells reaches a particular stage and drives the overexpression of a protein of interest. This system would be a unique and perhaps the only way to study lineage perturbations by different proteins in a stage-

specific manner. The unique advantage of this system is that the promoter-protein elements would turn themselves down once the cell crosses a certain stage in its lineage. This would be the closest approximation to the natural activity of the stage-specific promoter as compared to an externally regulated control system like a Tet-on/off system. Pure populations of stage specific populations are not only desirable from the point of cell transplantation; they also provide a versatile tool by which paracrine factors that are secreted by them can be characterized. In conclusion, this study demonstrates potential strategies to enrich cardiomyocyte production from embryonic stem cells in-vitro and to purify the mixture of cells to a stage-specific population which can be used for biochemical characterization and cell transplantation studies.

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