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GENERATION OF RECOMBINANT MOUSE EMBRYONIC STEM CELL LINES AND
THEIR APPLICATION FOR IN VIVO BIOLUMINESCENCE IMAGING IN THE HEART

by

RAMANA KUMAR KAMMILI
M.S. University of Abertay, 2002

A thesis submitted in partial fulfillment of the requirements
for the degree of Masters of Science
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at the University of Central Florida
Orlando, Florida

Summer Term
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Major Professor: Steven N Ebert
ABSTRACT
Cardiovascular disease is the major cause of death in the United States, with 80 million people suffering from some form of heart disease each year. One major limitation is the inability of the heart to repair the damaged tissue. Stem cell therapy holds enormous promise to repair and regenerate the damaged myocardium, but there are many technical difficulties that must first be overcome. One such difficulty is the present lack of ability to track and assess transplanted stem cells over time in vivo. The central hypothesis of this thesis is that in vivo bioluminescence imaging is a safe and useful method for monitoring transplanted stem cells in mouse hearts. To evaluate this hypothesis, two aims were performed. In aim 1, stable recombinant mES cell lines expressing the firefly luciferase (fLUC) reporter gene under the control of constitutive and cardiac-specific promoters were generated and characterized in vitro. In aim 2, these fLUC-expressing recombinant cell lines were evaluated following transplantation into neonatal mouse hearts. The major findings are: (1) Novel stable recombinant mES reporter cell lines were developed for in vivo bioluminescence imaging; (2) One of these cell lines was created using the glyceraldehyde 3-phosphodehydrogenase (GAPDH) promoter fused to the fLUC reporter and it showed similar levels of fLUC expression in undifferentiated (pluripotent) compared to cardiac-differentiated mES cells; (3) Another cell line was produced using the cardiac-specific sodium-calcium exchanger 1 (NCX1) promoter fused to the fLUC reporter and this cell line showed markedly increased fLUC expression following induction of cardiac differentiation in culture when compared to the pluripotent cells. (4) Transplantation of the recombinant fLUC-expressing cells into neonatal mouse hearts produced bioluminescent signals that persisted for at least 24 days, the maximum timepoint analyzed in this study; (5) Transplantation of 100,000 or more mES cells to the heart consistently produced teratoma and tumor formations, regardless of which recombinant clone was used or whether the mES cells were injected as pluripotent or cardiac-
differentiated cells, (6) Transplantation of between 10,000 and 50,000 cardiac-differentiated NCX1-fLUC mES cells (containing mixed population of other cells) per heart resulted in measurable bioluminescent image signals in vivo with low incidence of tumor formation, and (7) Some of the transplanted NCX1-fLUC mES cells were identified in ventricular muscle tissue in postmortem histological sections where it was found that they had developed cardiomyocyte characteristics. In summary, I developed stable recombinant mES cell lines suitable for non-invasive bioluminescence imaging to study the survival and proliferation of the cells in vivo. These results demonstrate that bioluminescence imaging in the neonatal mouse heart model is an effective strategy for non-invasive monitoring of transplanted stem cells over time in vivo, and minimizes animal usage through elimination of the need for animal sacrifice at multiple timepoints.
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<th>Description</th>
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<tbody>
<tr>
<td>DMEM</td>
<td>Dulbecco’s modification of Eagles medium</td>
</tr>
<tr>
<td>EBs</td>
<td>Embryoid bodies</td>
</tr>
<tr>
<td>ES</td>
<td>Embryonic stem cells</td>
</tr>
<tr>
<td>GAPDH-LUC</td>
<td>Luciferase driven by glyceraldehyde phosphate dehydrogenase promoter</td>
</tr>
<tr>
<td>HH</td>
<td>Hepes hanks solution</td>
</tr>
<tr>
<td>HBSS</td>
<td>Hanks balanced salt solution.</td>
</tr>
<tr>
<td>hES</td>
<td>Human embryonic stem cells</td>
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<tr>
<td>mES</td>
<td>Murine embryonic stem cells</td>
</tr>
<tr>
<td>MEF</td>
<td>Murine embryonic fibroblasts</td>
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<tr>
<td>MHC-LUC</td>
<td>Luciferase driven by α-myosin heavy chain gene promoter</td>
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<tr>
<td>NCX1-LUC</td>
<td>Luciferase driven by cardiac sodium calcium exchanger-1 promoter</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>ROI</td>
<td>Region of interest</td>
</tr>
<tr>
<td>YFP</td>
<td>Yellow fluorescent protein</td>
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CHAPTER 1
INTRODUCTION

Cardiovascular disease is the primary cause of death in the United States and in developing countries. Nearly 80 million people suffer from some form of serious heart diseases each year and 800,000 people die of myocardial infarction each year (AHA, 2008). Myocardial infarction, cardiomyopathy, hypertension, and valvular heart diseases are the most common heart diseases where myocardial cell death occurs. A common factor among these heart diseases is remodeling which occurs as a compensatory response to pathophysiological condition. For example, myocyte cell death occurs through necrosis and apoptosis, and initiates a cascade of events leading to scar tissue formation. There is also strong evidence that stem cells can regenerate in the heart (Beltrani, et al. 2004) and cardiac tissue cannot enter the normal cell cycle as the heart is terminally differentiated. Thus, cell replacement is currently investigated as a therapeutic option.

**Stem cell treatments – current status and future potential**

Stem cells have the potential to regenerate cardiac tissue (Sengers, et al. 2008). A variety of stem cells from several sources and endogenous resident cardiac stem cells have been transplanted into infarcted hearts (Murry et al, 2007 and Klug, et al, 1996). The ability of cells to repair or regenerate myocardial tissue is an intense area of investigation and sometimes controversial. Novel markers are identified and characterized from embryonic and adult tissues to identify specific stem or progenitor cell populations (Kattaman, et al. 2007 & Ebert, et al. 1996). Most studies use autologous bone marrow stem cells, but the engraftment and further functional improvements are marginal (Kolsov, et al. 2006). Consequently, new strategies are needed to address this important clinical problem.
Several markers for cardiac stem cells have been identified such as Isl-1, flk-1, Pnmt, and c-kit, (Ebert, et al. 1996, Kattman, et al. 2006, Morreti, et al. 2006). Embryonic stem cells can give rise not only to myocytes, but also to fibroblasts, endothelial, and smooth muscle cell types (Singla, et al. 2005). Although there is substantial evidence that cell transplantation results in cardiac tissue repair and improvement in cardiac function such as ejection fraction,(Min, et al. 2003 and Caspi, et al. 2007) there is no proper correlation among the cell type, cell delivery mechanism, and the number of cells for functional improvement with minimum safety concerns. Specific issues about cell viability, proliferation, and differentiation potential of the transplanted cells are not clearly understood. In addition, little is known about the proliferation, and differentiation status of the transplanted embryonic stem cell type, making the long term effects of cellular therapies uncertain.

**Cell sources for cardiac repair:**

Several potential therapeutic cell types are human umbilical cord blood cells, bone marrow cells, satellite cells, endothelial progenitor cells, fetal and neonatal cardiomyocytes, embryonic and adult stem cells, including resident cardiac stem cells. Most cell types have been shown to improve cardiac function marginally in terms of increasing in ejection fraction (Min, et al. 2003, Lough, et al. 2006, Murry, et al. 2007). Possible mechanisms leading to cardiac improvement are the recruiting of stem cells into the ischemic tissue, cardiomyocyte differentiation, or integration into the myocardium, and paracrine factors (Murry, et al. 2005, Behfar, et al. 2002). Myoblast transplantation into ischemic myocardium can differentiate into myotubes *in vivo* (Laflamme and Murry 2005) and improve ventricular function (Menasche, et al. 2007) in animal experiments. Cell therapy efficiency depends on cell type, transplantation time, and method of transplantation. Each cell type has its own advantages and disadvantages. Bone marrow cells have no ethical
issues and can be easily expanded. Bone marrow-derived hematopoietic cells have been reported to differentiate into cardiomyocytes when transplanted into infarcted hearts of mice (Leri, et al. 2005). Mesenchymal stem cells (MSC) are less immunogenic than any other cell type (Amado, et al. 2005) and there was evidence that transplantation of mesenchymal stem cells into hearts improved cardiac function in pigs and rats (Shake, et al. 2002 and Ma, et al. 2005). The morphology of the mesenchymal stem cells injected into pig hearts looks like fibroblasts even though they express muscle markers and there was no engraftment and reduced ventricular dilation indicating attenuation of modeling (Martin, et al. 2005). Fetal cells integrated well and formed gap junctions with host myocardium and LV functional improvement in rats (Skobel, et al. 2004). However, the disadvantage is the difficulty in acquiring fetal cells and requires immune suppression during transplantation and the possibility of secondary infections as a result of immune suppression.

**Cardiomyocytes from pluripotent ES cells:**

The first functional organ to develop in vertebrates is the heart. In mice, the primitive heart begins to beat irregularly at embryonic day E7.5-8.0 and starts to beat regularly by embryonic day E8.5-9.0 (Kaufman, et al. 1989) Mouse ES cells were isolated from the embryonic blastocyst stage. Of several sources of transplanted cells, embryonic stem cells (ES) are potentially the most suitable source for cardiomyocyte multiplication in large scale because of their pluripotency. It was shown that human embryonic stem cells (hES) possess immune privilege properties (Li Baroja, et al. 2004). ES cells have great proliferative capacity and express markers such as Oct4, Nanog and Sox2 (Pesce, et al. 1999 and Chamberes, et al. 2003) and Ronin (Dejosej, et al. 2008) and these markers decline as the cells are committed. ES cells can be used as a source for
cardiomyocyte generation (Bohelar, et al. 2002) but the percentage of cardiomyocytes is so small (1-2% of total) such that, preselection may be necessary to enrich cardiomyocytes prior to transplantation. ES spontaneously differentiate in culture, but can be inhibited from differentiation by the addition of Leukemia Inhibitory Factor (LIF) (Bader, et al. 2000) in mES cells and basic Fibroblast Growth Factor (bFGF) in human ES cells. Withdrawal of these growth factors result in cell aggregation. These aggregates will enlarge with time and form embryoid bodies. Embryoid body development partly recapitulates embryogenesis and results in the formation of all three germ layers: ectoderm, mesoderm, and endoderm. Embryoid bodies have thus been used as a reproducible model to study cardiac development (Wobus, et al. 2005). Embryoid bodies have sparse and discontinuous myofibrils which can become continuous upon maturation. The morphology and sarcomere length of ES-derived cardiomyocytes is similar to neonatal cardiomyocytes (Westfall, et al. 1997). The myofibrillar architecture and length of sarcomeres are similar to neonatal cardiomyocytes. Cardiomyocytes change with time and develop into pacemaker, atria, ventricular nodal, purkinge and His cells (Hescheler, et al. 1997 and Maltsev, et al. 1993)). The ionic currents and action potentials generated by cardiomyocytes derived from ES cells simulate cardiac development stage. Earlier cardiac developmental stages have calcium and transient potassium current in contrast to late cardiac developmental stage where sodium, calcium, and all the 5 different types of potassium currents, and I_f currents are present (Maltsev, et al. 1994). There are several lines of evidence that morphology (Westfall, et al. 1997) and pharmacological properties (Normstorm, et al. 2006) of stem cell-derived cardiomyocytes were similar to those described by adult cardiomyocytes (Piper, et al. 1982). It was shown that stem cell-derived cardiomyocytes express cardiac genes and transcription factors in developmentally regulated fashion as in normal cardiac development (Zaffran, et al. 2002).
The mRNA of both GATA4 and NKX2.5 appeared before Atrial Natriuretic Factor (ANF), myosin light and heavy chains, sodium calcium exchanger, and phospholamban. Sarcomeric proteins such as actin and troponin are also developmentally regulated (Metzer, et al. 1997). From this, it is clear that ES-derived cardiomyocytes have similar morphology, (Dai, et al. 2007) action potentials, and ionic currents as adult cardiomyocytes. The major disadvantage with these cells is tumor formation (Wakitani, et al 2003) and ethical concerns.

Singla reported pluripotent ES cell transplantation into infarcted heart resembles in multiple cell types including cardiomyocytes, vascular smooth muscles, and endothelial cells and improvement in cardiac function without evidence of tumor formation (Singla, et al. 2005). Undifferentiated ES cells injected into the rat hearts have shown an improvement in cardiac function such as ejection fraction, contractility, (Murry, et al. 2007, Befar, et al. 2002) and up-regulation of cardiac specific genes (Min, et al.). Nelson, et al. showed transplantation of ES cells containing β-galactosidase gene fused with αMHC promoter into the infarcted heart resulted in restricted engraftment and tumor formation. Wang, et al. reported ES cells tagged with GFP injected into the tail veins migrated and homed into the heart. Improvement in cardiac outcome is only transient (Meyer, et al. 2000) and is dependent on the ability of the cell to commit to cardiac phenotype and engraftment.

Transplantation of undifferentiated ES cells resulted in teratoma formation and immune rejection (Murry, et al. 2007, Befar, et al.). Kolossov (2006) and Murry (2007) showed formation of teratomas when pluripotent ES cells were injected into heart. Transplantation of cardiac-induced cells resulted in the improvement of cardiac function and increased contractility (Yang, et al. 2006). These cells also showed expression of VEGF and played an important role in inducing angiogenesis as measured by an increase in capillary density. Field and Murry group showed
genetically selected cells for cardiomyocytes did not form teratomas (Klug, et al. 1996). Cardiac-induced cells, when delivered with a prosurvival cocktail of growth factors, had a better chance of survival and improvement in cardiac function than those delivered without cocktail (Laflamme, et al. 2007).

Even though several studies have shown cardiac function improvements by hemodynamic and echocardiography assessments, by transplantation of various cell types, most basic mechanisms addressing the survival, proliferation, and viability of the cell type, an important issue for predicting the outcome of cellular therapies in long run are less studied. Thus there is need for developing technologies to accurately understand basic mechanisms in real time.

**Molecular imaging**

Molecular imaging is the visualization and quantification of biological processes in living organisms. Existing imaging modalities such as Magnetic Resonance Imaging (MRI) and Positron Emission Tomography Imaging (PEI) label the cells using iron particles and radioactive materials respectively, and study the cell fate in transplantation experiments. The major disadvantages with these modalities are: 1) Cell viability cannot be assessed in vivo; 2) The presence of iron particle or radioactive material labeled in the transplanted cell is potentially harmful; 3) How transplanted cells labeled with particles interfere with cell engraftment and function is uncertain; 4) The proliferation and differentiation potential of the cells, a critical issue for functional outcomes, cannot not be adequately monitored in vivo, and 5) Higher costs with MRI and toxicity issues associated with PET. Most methods for visualizing cells are based on postmortem analysis depending on histological assessment, which precludes longitudinal monitoring of cells for long-term studies. Since cardiac function, repair, and regeneration are dependent on cell retention at the injected site, imaging techniques for precise identification of
cells need to be developed. Advances in bioluminescent imaging techniques will allow for non-invasive repetitive long-term monitoring with minimal animal usage. If a fluorescent or luminescent reporter gene is driven by a cardiac specific promoter, then the differentiation potential and tissue-specific expression of the cells can be assessed (Kolossov, et al. 1998).

**Promoters**

Most reporter genes are modified according to human codon usage for use in mammalian cells and are driven by viral promoters, as they are constitutively active. When adenoviral CMV- Fluc was injected into the heart, the luciferase signal was also detected in the liver (Wu Jc et al, 2002). Thus, if a reporter gene is driven by a cardiac specific promoter such as Myosin Heavy chain (αMHC), Myosin Light Chain (MLC2V), and Sodium Calcium Exchanger (NCX1), then the reporter gene will be activated only in the cardiac tissue (Wu Jc et al. 2002) with no interfering signals. This unique advantage can be exploited by using bioluminescent imaging to study survival, proliferation, and differentiation and differentiation in vivo.

**Glyceraldehyde phosphate dehydrogenase gene:**

Glyceraldehyde -3-phosphate dehydrogenase is commonly referred to as a housekeeping gene and is known to be as an enzyme involved in glycolysis. In addition to glycolytic enzyme, it has diverse functions in repairing DNA and is a microtubule-binding protein. The size of the human glyceraldehyde phosphate dehydrogenase promoter (hGAPDH is -1112 base pairs. (Shan Lu, et al. 2002)

**Alpha Myosin heavy chain protein**

α-Myosin heavy chain(MHC) is a structural protein and molecular motor of the cardiac tissue. Myosin heavy chain proteins exist in both alpha and beta isoforms. The relative amount of isoforms varies and is dependent on the developmental stage of the animal. Mutations in alpha
and beta MHC isoforms result in defects in cardiac function and heart failure. The major distinction among the isoforms is in relation to ATPase activity. Higher amounts of α-MHC results in more contractile activity where as higher amounts of β-MHC results in more force generation. The alphaMHC is expressed in atria in gestation and the beta isoform is predominantly expressed in ventricle. The beta isoform will be silenced after birth and the alpha form exists in both atria and ventricles (Buckingham, et al. 1990). Gene knock out of myosin heavy chain results in death at 11 -13 days in uteri from heart defects (Jones, et al. 1996). In αMHC +/- condition mice had lower levels of mRNA and protein which caused alterations in contractility and relaxation (Jeffrey Robbins, et al. 1996). The size of αMHC promoter used is 353 base pairs

**Sodium calcium exchanger protein**

Sodium calcium exchanger is a transmembrane protein which plays an important role in calcium homeostasis. The NCX gene family is a highly conserved gene in vertebrates. NCX1 is highly expressed in the heart. Other isoforms NCX2 and NCX3 are expressed in the brain and in skeletal muscle. NCX1 is up regulated in fetal heart and down regulates after birth (Koban, et al. 1998). Disruption of the NCX1 gene is lethal and mice die of heart failure as a result of cell death at E8.5 to E10.5 (Hee-sup Shin et al, 2000). This indicates that NCX1 is important in embryonic heart development. The over-expression of NCX leads to lower calcium handling and SERC regulation (Reed, et al. 2000). The size of NCX1 promoter is 1810 base pairs only 181 base pair are sufficient for driving cardiac specific reporter gene expression (Kenneth, et al. 2001, Mennick, et al, 2006). The promoter contains CIS regulatory elements and GATA binding and serum response factor binding sites which help in stimulation of cardiac-specific genes. Studies have shown that NCX1 is regulated by adrenergic hormones (Menick, et al. 2006).NCX1
is expressed not only expressed in cardiac tissue (Kimira, *et al.* 1996), but to a lesser extent in non cardiac tissues such as the brain and kidney. At low levels, it is also expressed in the lungs, spleen and muscle tissue (Juhaszova, *et al.*1996). NCX1’s major role is calcium regulation in the heart, kidneys, brain. A cytoplasmic loop at 5-6 domains plays a major role in NCX1 regulation.

**Bioluminescent imaging:**

Bioluminescent imaging is based on the ability to detect light in the presence enzyme and substrate. Among the various luciferase genes, firefly luciferase, Renilla luciferases are commonly used. The variation among the individual luciferase is the difference among the excitation and emission profiles. Firefly luciferase, isolated from *Photinus pyralis* (Subramani, *et al.* 1987) is a single copy gene. That gene is composed of seven exons and six introns. Of note, there was no TATA box in the 10-20 base pair, and the TATA box is located at far upstream at -141 and -116. Light emission occurring over a wide range of 480-613nm and peaks at 560nm. The efficiency of light emitted as a result of luciferase signal in biological tissue is dependent on the optical properties of tissue. Luciferase peak emission will be red-shifted if the temperature is increased from 25-37° degrees. Luciferase has several advantages in that is does not require light for excitation, thereby limiting autofluorosence(*Anversa, et al.* 2007) and fast turnover of the enzyme preventing accumulation in the cell (*Tarik, et al.* 2003). Bioluminescence imaging has several potential advantages compared to other imaging modalities. First, it is suitable for imaging in small animals with high specificity and little background noise. Second, long-term non-invasive repetitive imaging with high acquisition times compared to PET and MRI. Third, there is no use of radionuclide unlike PET and SPECT (Single Photon Emission Computed Tomography) and requires only luciferin which was shown to be non-toxic and has a high
enzyme turnover (Masood et al. 2003). It does not use light compared to Computed Tomography (CT) that requires X-rays. Therefore, it is comparatively safer technology. Fourth, it can be used to study cell survival, proliferation and migration (Swijnenburg, et al. 2008) which are not possible with MRI. Previously, our lab has shown non-invasive tracking of stem cell derived cardiomyocytes by labeling the cells with super paramagnetic microspheres and following their fate with MRI in vivo and showed these beads did not interfere with cell viability and cardiac differentiation (Ebert, et al. 2007). Now, we are moving a step head by genetically labeling the cells with luciferase genes by using a different imaging modality i.e. bioluminescent imaging because of the above advantages.

For these unique reasons, the purpose of this thesis is to explore the potential of a bioluminescent imaging strategy by testing the following hypothesis: **Bioluminescent imaging is safe and useful non-invasive method for monitoring transplanted stem cell in mouse heart. Two specific aims were performed to test this hypothesis;**

Aim 1: Development of recombinant ES cell lines expressing fLUC reporter genes under the control of constitutive and cardiac-specific promoters for in vivo bioluminescent imaging.

Aim 2: Transplantation of pluripotent and cardiac-differentiated ES cells into neonatal hearts and monitoring cells by in vivo bioluminescent imaging
CHAPTER 2
MATERIALS AND METHODS

Cell cultures

YFP ES cell line

7AC5/YFP ES cell line (Hadjantonakis, et al. 2002) is R1 cell line obtained from ATCC (Scrc-1033) and maintained in Dulbecco(DMEM medium) (Gibco cat no.11995)) supplemented with 15% fetal bovine serum( Hyclone, cat no.SH30070.03 with lot no: ASD29137), 1.0% l-alanyl glutamine, (ATCC cat no: 30-21115 lot no: 3000521), 1.0%non essential amino acids GIBCO cat no:11140), 1.2%Pencillium streptomycin, (GIBCO cat no 15140), leukemia inhibiting factor, (Chemi-Con, ESGRO batch no:07111424501), and 2 mercapto-ethanol (cat no.21985-023 lot no.409045). The YFP cells were grown on mitomycin-treated (MEF’s) isolated in our lab. YFP ES cells were allowed to grow for at least 3 passages on mouse embryonic fibroblasts and then were transferred to gelatin coated plates. The cells were grown for 3 more passages before the ES cells were used for electroporation for the development of stable cell lines. After electroporation the cells were maintained on feeder layers for initial selection procedure and started screening by withdrawing the feeder layers.

Mouse embryonic fibroblasts

The MEF’s were isolated from 11 days post coitus from pregnant mice. The fetus was isolated from uterus and removed all the forelimbs, hind limbs and tail. The rest of the body was minced, 0.05%trypssin was added which allowed the cells to be dissociated. After 15min, 10 ml of MEF cell culture media was added and plated on to 100mm cell culture plates and transferred to incubator. The culture medium composition, DMEM (Dulbecco medium) (Gibco cat no.11995), was supplemented with 10% fetal bovine serum (Hyclone, cat no.SH30070.03 with lot no.
ASD29137), and 1% penicillium streptomycin. The next day the medium was aspirated and washed 3 times with 1x PBS and added 10 ml of culture media on to the plates. Cells were confluent in 24 hours. Fibroblasts grow faster in the earlier passages and slower during the later passages. After 10-12 passages the cells growth was slow and the cells were discarded. Freshly thawed MEF’s cells were grown from frozen vials for further expansion.

Electroporation

Preparation of plasmids for use in electroporation

NCX1 plasmid was generous gift from Dr. Donald Mennick and the size of the plasmid is 7.2kb. α-MHC plasmid was generous gift from Dr. French Brent and the size of the α-MHC plasmid is 5.7kb plasmid. Gapdh plasmid was generous gift from Dr. Shan Lu and the size of the GAPDH plasmid is 5.2 kb. MC1neo plasmid was from our lab and the size of the MC1neo plasmid is 3.9kb plasmid. Plasmids were multiplied in E.coli strain and extracted large scale using qiaegen kits for further use in electroporation procedure. Purified plasmids were cut with unique restriction enzymes in the ampicillin gene region so that sequence of interest is not disrupted, and for linearization of the plasmids for use in electroporation. The NCX plasmid was cut with PvuI. α-MHC plasmid was cut at Bam H1 and MC1neo plasmid was cut with unique restriction sites at AlwNI and linearised for better integration into the chromosome during electroporation procedure.

Development of Recombinant ES cell lines using electroporation

Current methods employed for the development of stable cell lines are electroporation and viral mediated transfection using adenovirus or lentivirus. We used electroporation for the development of stable cell lines.
ES cells grown for electroporation should be subconfluent with clearly defined ridges and these
should not begin to differentiate (Patricia et al, 2004). Recombinant YFP R1 ES cells were
grown on mitotically inactivated fibroblasts. Plasmids were amplified in *E.coli* and extracted
with standard quiagen kits. Approximately 50 µg plasmid DNA was taken. These plasmids were
linearised by cutting within the ampicillin gene so that the promoter of interest and luciferase
were not disturbed during the linearised procedure. The DNA used for electroporation was clean
without salts which would otherwise interfere with electroporation procedure. Four plates of ES
cells at 50-70% confluency and clearly defined ridges were washed with 1x PBS and trypsinised
with 1ml of 0.05% Trypsin / EDTA (GIBCO cat no. 25300) for each plate and incubated for
5min. Cells would be dissociated into single cells suspension. This is the most critical step in
electroporation. I added 4 ml YFP ES cell media to stop the reaction and the cells were pooled
into a 15ml tube and spun in the centrifuge to remove serum that may interfere with
electroporation. I then added 0.4ml Hanks (1x) medium and linearised plasmids at
3:2(LUC:MC1) concentrations to a cuvette and electroporated in biorad gene pulser at 0.4KV,
25µfd for 0.5 sec. The concentration of the GAPDH and MC1 NEO plasmids used during
electroporation were at 25µg/µl. The concentration of the αMHC and MC1 NEO plasmids used
during electroporation were at 60µg/µl. The concentration of the NCX1 and MC1 NEO plasmids
used during electroporation were at 50µg/µl and 30 µg/µl. Electroporated cells were allowed to
recover for 5 minutes and cells were transferred to the plates containing mitomycin inactivated
fibroblasts.

Cells were allowed to grow in normal culture media for 24 hours and then selected with
antibiotic (G418) Geneticin, (GIBCO cat no. 118110031 lot no.1394394 (350 µg/ml media).
Colonies appeared at 7 days. The day prior to picking colonies, 24 well plates were preseeded
with mouse embryonic fibroblasts that were inactivated with mitomycin to stop cell division. Each colony was checked under microscope for clear defined ridges. Each colony was picked and trypsinised in a 24 well plate. The trypsinised cells were transferred to 24well plate containing mitomycin-treated fibroblasts. After careful expansion for 2 batches one batch was frozen for backup and the other batch was maintained for subsequent screening. For 5 successive passages cells were maintained on mouse embryonic fibroblasts (feeder layers). After 5 passages recombinant ES clonal cells were grown without feeder layers and then screened for luciferase signal using standard Promega luciferase kit by (Glo-Lysis buffer 1x Cat No: E266A Lot no:24815901) lysing the cells and assay them with luciferase kit (Steady –Glo luciferase Assay System(E2520). Luciferase signal was quantified both in undifferentiated and cardiac-induced cells.

**Cardiac differentiation of recombinant LUC ES cells**

Cardiac differentiation was induced in ES cells by hanging drop and rotary shaker methods. For selection and screening of luciferase we followed typical hanging drop Protocol (Maltsev, 1994). For in vivo experiments, we needed the cardiac differentiated cells in larger quantities, so we used rotary shaker methodology (Richard, L.Carpendo, et al. 2007). Rotary shaker protocol is very feasible for getting large numbers of cardiac-induced cells. Cardiac-differentiated cells contained a mixed population of other cells in addition to cardiomyocytes.

**Hanging-drop protocol**

Approximately 400 cells/20µl volumes were plated as hanging drops and maintained for 2 days in hanging drops to aggregate the cells followed by 5 days in suspension phase. After 7 days (2 days in hanging drops+ 5 days in suspension) the embryoid bodies were plated on gelatin coated plates(Chemicon cat no. ES-006-B and Lot no. 80108-1) and allowed for cardiac differentiation.
The size of the embryoid body is greater than 100µ. Cells start beating from 7+1 days and beating areas will increase overtime. Even though the beating areas increased over time, less than 10% of EBs were differentiated into cardiomyocytes. For screening recombinant ES cell clones for luciferase expression in cardiac-differentiated state, 6 embryoid bodies from each clone were plated on 48 well plates. The cells started differentiating from 7+1 days and increased in beating activity over time. Once the cardiac differentiation was confirmed by checking beating areas under microscope, the cells in that well were lysed with lysis buffer (Glo-Lysis buffer 1x Cat No. E266A Lot no.24815901 for lysing the cells and assay them with luciferase kit (Steady –Glo luciferase Assay System (E2520). Luciferase signal was quantified both in undifferentiated and cardiac-induced cell and in vitro bioluminescent imaging with Xenogen IVIS system.

Rotary culture:
Rotary cultures create microgravity environment and allow the cells to aggregate and form embryoid bodies (Richard, et al. 2007). ES cells were trypsinized and seeded at a density of 400,000 cells/ml in 100mm culture plate and then placed on rotary shaker and maintained in 37°C and 5% CO2. The rotary shaker system was rotated at 20rpm. Cells were strained with 100µ sieve after 2 days. Embryoid bodies were washed twice 1x PBS (GIBCO cat no.10010) and transferred to 100 mm bacterial plates. They were allowed to stay in suspension for 5 more days by changing the EBs with differentiation media every day. One day after plating (7+1 days) we typically observed beating areas. The composition of the differentiation media is Dulbecco medium (DMEM) (GIBCO cat no. 11995) supplemented with 15% fetal bovine serum (Hyclone, cat no.SH30070.03 with lot no. ASD29137), 1.2% L-glutamine, (GIBCO cat no. 25030)1% essential amino acids (GIBCO cat no. 11140), and 1% Pencillium streptomycin (GIBCO cat no.15140).
Luciferase Assays.

Steady-Glo luciferase assay System (Promega cat no. E2520) is the luciferase assay used for *in vitro* experiments. Luciferase signal was measured both *in vitro* and *in vivo*. This assay is designed for high-throughput screening and produces stable luminescence with half-life greater than 5 hours. Bioluminescent imaging was performed using with standard In Vivo Imaging System (IVIS) (Caliper). It contains white lights to identify the position of the plate or animal, a dark chamber, CCD camera to detect bioluminescent signals and a computer software and hardware for data analysis.

**IVIS System**

![IVIS System Diagram](image)

**Figure 1: In vivo imaging system (calipers)**

**In vitro assay**

For *in vitro* assays, the culture medium was slowly removed from the culture plates, and then a volume of 100µl lysis buffer (Glo-Lysis buffer 1x Cat No. E266A Lot no. 24815901) was added and put in the incubator to allow the cell to lyse thoroughly. After 10 minutes, an equal volume of Steady–Glo luciferase assay reagent (Promega cat no. E2520) was added and the
luminescence was measured immediately using IVIS imaging system and subsequent Scintillation counting.

**In vivo assay**

For in vivo imaging there is no cell lysis step. The animals were anesthetized with 2% isofluorane and D-luciferin (Caliper cat no. XR1001 Batch no. 124357945) was used at a concentration of 150 mg/kg body weight. D-luciferin was weighed and dissolved in 1X PBS as per the requirement. The animals were injected with D-luciferin solution by intraperitoneal injection AT 20µl volume and then allowed to stay for at least 5 minutes to circulate the substrate and then imaged in IVIS system for 1 minute and 5 minutes. The region of interest (ROI) was quantified. The ROI was taken at an approximate distance and calculated in photon/sec/cm²/sec, and background noise was subtracted to quantitate the exact luciferase signal. Animals were euthanized at various points under full anesthesia for immunohistochemical staining.

**Neonatal mice cell injections**

Neonatal mice models have certain advantages over adult mice because they do not require open heart surgery and recovery. Their immune system is premature and the environment will be more conducive for cell survival (Christensen, *et al.* 2000). Prior to cellular transplantation in neonatal mice, cells were trypsinzed (0.05%Trypsin EDTA GIBCO cat no.25300 and lot no.389921) for pluripotent cells and 0.25% Trypsin EDTA (GIBCO cat no.25200 and lot no.361343) for cardiac differentiated cells and put in an incubator for 5 minutes. Cells dissociated well when trypsin was added. They were checked under microscope. Some culture media or DMEM was added to neutralize the activity of Trypsin-EDTA. Cells were transferred into 15 ml tube and spun for 5 min. The supernatant was removed carefully and then the cells were resuspended in Hanks balanced salt solution (HBSS). The cells were counted and diluted
with HBSS such that the injection volume should be 20µl for each pup at the required concentration of cells. One intramyocardial injection at the heart was given and the pups were swabbed with alcohol before and after injection procedure to sterilize the site of injection and pups were dabbed with peanut oil and then returned to their litter. We gave one concentration of cells to each litter as it was difficult to mark the pups. In each litter, we maintained two controls by injecting with HBBS solution with no cells by docking the tails. ES cells were used from SV129 to minimize immune rejection.

**Tissue preparation for immunostaining**

The animals were euthanized at specific time points and the hearts were removed and fixed in 4% para-formaldehyde in 0.1m phosphate buffer saline, pH 7.3 for 1-2 hours on ice. Animals less than 3 weeks were fixed in 2% para-formaldehyde overnight on ice at 4ºC and then transferred to 30% sucrose solution. Animals older than 4 weeks were fixed in 4% para-formaldehyde by perfusion for 5 minutes. Hearts were transferred to 4% para-formaldehyde for an additional 2 hours on ice and transferred to 30% sucrose solution in PBS pH 7.3 and then sectioned using cryostat to correlate the bioluminescent data with immunohistochemistry. Hearts were cryosectioned at 8µm on to Super Frost Plus microscope slides (Fischer Scientific). The sections were frozen and stored at -80 for subsequent use for immunostaining.

**Immunofluorescence histochemistry**

To perform immunostaining the slides were first thawed to room temperature and the sections were ringed with PAP pen for forming well for proper incubation. The tissues were rehydrated with 1X PBS for 1 hour. Next the slides were replaced with PBS and incubated with blocking buffer for 1 more additional hour (0.3% Triton X-100, 5% powdered non fat dry milk, and 0.02% sodium azide in PBS) at room temperature. The blocking solution was removed slowly, and then
replaced with fresh blocking solution containing primary antibody α-actinin at 1:100 dilutions. The primary antibody incubation step was proceeded for one hour at room temperature followed by overnight incubation at 4°C in a humidified plate. Following this incubation period, the next day primary antibodies were removed and sections were washed 3 times with PBS 15 minutes or longer each wash. Next, the subsequent steps were done at room temperature. The PBS was replaced with Texas Red fluorescent-tagged secondary anti-mouse antibodies (1:100 dilutions) were incubated for 2 hours at room in dark. After 2 hours the slides were removed and washed three times with PBS (1x) 15 minutes each wash. Then the slides were mounted with Vecta shield mounting medium (Vector labs, Burlingame, CA). Fluorescent labeling was observed with fluorescence microscopy and pictures were taken with laser scanning confocal fluorescence microscope and processed via Adobe Photoshop.
CHAPTER 3 RESULTS

Plasmid Reporter Constructs for In Vivo Bioluminescence Imaging

A. Ubiquitous Expression  
**GAPDH promoter** -> **fLUC**  

B. Cardiac Specific Promoter  
**αMHC** -> **fLUC**

**NCX1 promoter** -> **fLUC**

Figure 2: Reporter plasmids used to generate stable recombinant mES cell lines.

(A) Ubiquitous cellular promoter–reporter constructs (GAPDH-LUC), (B) Cardiac promoter–reporter constructs, αMHC-fLUC and NCX1-fLUC. Each plasmid contained a firefly luciferase (LUC) reporter gene with an attached poly-A (pA) coding sequence. The plasmids were linearised in the vector ampicillin resistance gene and transfected via electroporation in pluripotent mouse embryonic stem (mES) cells that express YFP. To select for stable recombinant clonal cell lines, each plasmid was cotransfected with neomycin Resistance gene, (pMC1-Neo) (Casimoro, et al. 2001) and the resulting G418 clones were screened for luciferase activity.
Recombinant GAPDH-fLUC ES Cell line Screening.

We developed a noninvasive bioluminescent imaging strategy to study the proliferation and differentiation potential of transplanted cells in vivo. In this effort, we created stable recombinant cell lines that express fLUC driving luciferase under the control of Housekeeping gene Glyceraldehyde phosphate dehydrogenase(GAPDH-LUC) promoter(Shan lu, et al. 2002) through electroporation mediated transfection. The size of the promoter is approximately -1112 base pairs. This plasmid is cotransfected with Pmc1neo plasmid. Since luciferase is under the control of housekeeping gene we expected to see the luciferase signal in undifferentiated pluripotent and cardiac differentiated state. YFP ES cell line (SERC-1033) was used. This cell line has several advantages. It is derived from R1 ES cell line so that it can be germ line transmitted and these ES cells were genetically engineered with Yellow fluorescent protein so we can know the viability of the cell irrespective of the differentiation status. Geneticin (G418) was used at 350ug/ml for picking resistant colonies and the cells were maintained on feeder layers in the initial passages. Colonies appeared at day 7. The cells were grown initially on feeder layers and after several passages the feeder layers were withdrawn. After withdrawing from feeder layers recombinant GAPDH-fLUC ES cell line was screened for luciferase signal. A representative figure showing positive luciferase signal in undifferentiated state in Fig. 2. Each ES clone line was screened twice before comparing their screening states in pluripotent vs. cardiac induced state. Ninety eight colonies were picked and screened for luciferase activity in undifferentiated state. We confirmed 3 positive in the initial and secondary screening.
Figure 3: Screening for recombinant luciferase ES cell line with fLUC driven by the constitutive cellular promoter (GAPDH) using IVIS System.

Each well denotes a different clone. Cells are plated and allow them to become confluent and analyzed by luciferase assay by IVIS imaging system. Notice the expression of luciferase in 4 wells in the figure. Only one ES clone in row 2 has brighter luciferase signal and it was used for further screening.
Screening for recombinant cardiac specific $\alpha$MHC-fLUC luciferase ES cell line

We generated stable transfected mES cell lines that expressed the LUC reporter gene under control of cardiomyocyte specific promoter $\alpha$MHC. To explore the feasibility of using ES cells for studying the survival, proliferation and differentiation status of the cells following bioluminescent imaging into neonatal mouse hearts \textit{in vivo}. We transfected the same YFP containing ES cells with pMC1 plasmid and $\alpha$MHC- luciferase plasmid by electroporation in which luciferase gene is under the control of myosin heavy chain promoter and selected G418 resistant clones. Myosin heavy chain is a structural protein and exists in both $\alpha$ and $\beta$ isoforms and is found to be cardiac specific. Since luciferase gene is driven by cardiac specific promoter we expected to see luciferase only in cardiac induced state and not in undifferentiated pluripotent state. One hundred and eighty two clones were picked and screened for luciferase in pluripotent as well as cardiac induced state. Nine clones exhibited luciferase in both pluripotent and cardiac-induced state and were not studied further. Three positive clones were confirmed, namely $\alpha$-MHC-fLUC4, $\alpha$-MHC-fLUC5, $\alpha$-MHC-fLUC17 in primary screening and subsequent screening and a representative figure showing screening for luciferase signal in cardiac induced state (Fig.3.). These positive clones were further characterized to compare cardiac differentiated and undifferentiated cells on the same plate (Fig. 5). One clone $\alpha$-MHC-fLUC 4 had more LUC activity in pluripotent state and lower LUC activity in cardiac- induced state. We explored this cell line further for cardiac bioluminescent imaging by transplanting these cells in adult and neonatal mice.
Figure 4: Screening for recombinant α-MHC-fluc ES cell lines expressing reporter gene expression following differentiation by hanging drop protocol.

Six hundred clonal cells /20ul were plated in hanging drops for 2 days followed by 5 days in suspension and seeding the embryoid bodies. Cardiac induction is confirmed by visualizing the beating activity under the microscope followed by luciferase assay and imaging by IVIS imaging system.
Screening for recombinant NCX1-fLUC ES cell line

As an alternate promoter to evaluate cardiac specific expression, we evaluated the cardiac promoter driving luciferase gene. We switched to NCX promoter driving luciferase gene for two reasons. NCX1 is sodium calcium exchanger transmembrane protein that was shown to be the earliest markers during cardiac development (Donald R.Menick et al, 2006). This promoter is well characterized and the size is1810 base pairs. This promoter was also shown to be regulated by adrenergic hormones (Donald R.Menick et al, 2005). To generate recombinant clones expressing luciferase in cardiac state, we developed this ES cell line same like the previous ones and picked approximately 144 stable antibiotic-resistant clones. The clones were then screened in pluripotent and cardiac differentiated state for luciferase expression. Of 144 stable clones only 23 clones have shown luciferase signal in cardiac induced state in primary screening. Out of these 23 clones showing luciferase signal in cardiac induced state in primary screening only 9 ES cells clones maintained stable luciferase signal in secondary screening. Eleven clones showed luciferase signal in cardiac induced state and undifferentiated state and these were not studied further. A representative figure showing screened luciferase signal in cardiac differentiated state (Figure 4). The 9 clones showing stable luciferase signal were further screened in pluripotent vs. cardiac induced state on the same plate (Fig.5). Among the 9 NCX1-fLUC clones, clone NCX1-43-fLUC showed 3 fold increase in luciferase activity after cardiac induction state compared to pluripotent state. Beating areas were visualized under the microscope to confirm cardiac differentiation before assaying for luciferase activity.
Six hundred cells /20ul were plated in hanging drops for 2 days followed by 5 days in suspension and seeding the embryoid bodies. Cardiac induction is confirmed by visualizing the beating activity under the microscope followed by luciferase assay and imaging by IVIS imaging system. Each clone is plated in duplicates with 8 embryoid bodies per well. Among the 24 different NCX1-luc clones only 33, 26, 43, 32 showed LUC expression following induction of cardiac differentiation.
Figure 6: Screening for stably transformed LUC recombinant mES clones.

(A) Various recombinant mES clones are listed for each row. Row 1, Parental (control) YFP-mES cells. Rows 2-3, GAPDH-fLUC clones #6 and #33. Rows 4-6, αMHC-fLUC clones #4, #5, and #17. (B) NCX1-fLUC clones. In both cases, cardiac differentiation was induced using the hanging-drop method and 6 EB’s per well are seeded into 4 wells per clone on the left side of each 48-well plate. Beating activity developed within 1-2 days after seeding. Pluripotent (undifferentiated) mES cells for each clonal cell line were seeded into the 4 wells on the right side of each plate in the same row as the corresponding EB’s for that clone (indicated to the left of each row).
In vitro characterization of stable luciferase ES cell lines

As seen from Fig.5 the top row is YFP-mES cells (i.e. No luciferase gene). As expected there was no luciferase expression in either pluripotent or cardiac-induced stage. In subsequent two rows are GAPDH–fLUC ES clones. GAPDH-fLUC-6, GAPDH-fLUC-33 in which luciferase is under the control of housekeeping gene. Luciferase signal is found in pluripotent and the cardiac-differentiated state. In the next 3 rows αMHC-fLUC clones are shown. αMHC-fLUC-4, αMHC-fLUC-5, αMHC-fLUC-17 were the three brightest LUC clones screened in this experiment. We noticed differential and selectivity in luciferase expression. Myosin heavy chain is structural protein that is abundantly expressed in heart. Even though it is cardiac specific αMHC-4-fLUC has higher luciferase signal in pluripotent state. In NCX1-fLUC clones several clones showed increase in luciferase activity after cardiac differentiation such as NCX1-33-fLUC, NCX1-43-fLUC, and NCX1-83-fLUC. Among these NCX1-fLUC clones, we characterized NCX1-43-fLUC for further evaluation.

Side note:                      Cell line                     Marker
1.                             GAPDH-LUC                   =                       Ubiquitous
2.                             αMHC4-LUC                   =                       Pluripotent +tumors
3.                             NCX1-43-LUC                  =                       Cardiomyocytes
Figure 7: Quantitative analysis of fLUC activity derived from pluripotent (non-differentiated) versus cardiac induced (differentiated) of stable recombinant mES a clonal cell lines.

Open bars represent pluripotent undifferentiated cells mouse ES cell clones, and solid bars represent cardiac-induced. Notice that only NCX1-43-fLUC clone showed increased luciferase activity following induction towards cardiac differentiation. In contrast, LUC activity decreased from the αMHC-4-fLUC clone and GAPDH-6-fLUC clones following cardiac differentiation.
Comparison of α-MHC4-fLUC and NCX1-43-fLUC ES cell line

Figure 8: Quantitative analysis of LUC activity derived from pluripotent and cardiac-induced differentiation of stable recombinant mES clonal cell lines.

Green bars represent αMHC-4-fLUC clone #4 and brown bars represent NCX1-43-fLUC clone#. 43. Note that both have similar levels of base line luciferase activity in pluripotent state but only NCX1-43-fLUC clone showed increased luciferase activity following induction towards cardiac differentiation. In contrast LUC activity virtually disappeared from αMHC-4-fLUC clone following cardiac induction.
Figure 9: In vitro evaluation of NCX1-43-fLUC mES clonal cell line.

(A) Photograph of luciferase assay. The cells were induced to differentiate into beating cardiomyocytes via the hanging drop method. At 7+0 days, 8 Ebs per well were seeded into 48 well plates (left), with an equivalent number of pluripotent cells (right). Luciferase activity was measured using an in vivo imaging system in lysed cells. N=8well/group. (B) Quantitative analysis of luciferase activity: NCX1-43-fLUC clone showed increased luciferase signal in the cardiac- induced state compared to pluripotent state. (P<0.01)
In vitro characterization of NCX1-fLUC -43 ES cell line

We compared the luciferase signal in both pluripotent and cardiac induced states of the three recombinant cells and did quantitative comparison by maintaining approximately the same number of populations and normalizing the protein values and scintillation data. The luciferase signal increased in the cardiac-induced state in NCX1-43-fLUC cell line, as expected (Fig.7) but the LUC signal in cardiac-induced state decreased in α-MHC-4-fLUC cell line which was not expected Fig.6+7). Therefore, analyze the NCX1-43-fLUC clone further.

A new ES cell line was found that could potentially be used for in vivo cardiac bioluminescent imaging. This NCX1-43-fLUC clonal ES cell line was further characterized by plating approximately same number of cells of both pluripotent and cardiac- induced on the same plate to confirm true differential expression of luciferase in cardiac- induced state (Fig. 9). Our results show that there was a significant increase in LUC activity following induction of cardiac differentiation compared to the pluripotent states. The total protein levels were measured in both cardiac-induced and pluripotent states and the region of interest were measured using the IVIS, NCX1-43-fLUC clone showed significantly higher (p<0.01) bioluminescent activity in the cardiac-differentiated state compared to the pluripotent state. There was a threefold increase in LUC activity in the cardiac- induced state compared to undifferentiated cells. Cardiac induction was confirmed by observing the beating areas before the assaying for luciferase was done. This NCX1-43-fLUC ES cell line displayed morphology, stable LUC expression and growth characteristics similar to ES cells and significant LUC expression when induced to differentiate into beating cardiomyocytes. The unique advantage of this cell line is that in vitro LUC assessment can be done before injecting the cells. In addition they can be really identified in tissue sections because of the presence of YFP protein.
Figure 10: In vivo image of α MHC-4-FLUC transplanted cells in adult mice at 100,000 cells/20ul injection volume

(A). IVIS imaging photograph showing no luciferase expression (B). Detection of YFP-mES cell in adult mouse heart. The mouse was sacrificed 3 days post transplantation and heart was isolated, fixed and sectioned for histological examination. The image represents an overlay of phase contrast and fluorescent images from same filed of view. A prominent YFP expressing cell is observed within myocardial layer of left ventricle. (C). In vitro confirmation of cell viability after injecting the cells with syringe in the plate and assaying by in vivo luciferase assay and imaging by IVIS system.
**Invivo imaging of α-MHC-fLUC transplanted ES Cells in adult mice.**

The α-MHC-4-fLUC clonal ES cell line was tested in vivo in early experiments because this clone has the brightest LUC activity. This ES cell line was used for initial studies of *in vivo* bioluminescent imaging to see the feasibility of this ES cell line. As a preliminary study, pluripotent α-MHC-4-Fluc ES cells were injected at 100,000 cells/20µl injection volume in an adult mouse through open chest and allowed the animal to recover for 2 days before imaging. There was no expression at 2, 3, 8 days (4/4). After 8 days animals (Fig. 9B) were sacrificed and hearts were fixed and sectioned to see the presence of YFP. In (Fig.9C) the luciferase assay was done with luciferase substrate mimicking *in vivo* conditions without lysing the cells to see that injection syringe could not have caused damage to the cells while performing injections. The minimum amount of cells required to see LUC expression was also studied in vitro and also comparing the injection media either HBSS or ES cell culture media.
Figure 11: In vivo imaging of transplanted recombinant αMHC4-fLUC mES cells in neonatal mice.

The neonates were anesthetized on ice for 2 mins, and varying quantities of undifferentiated αMHC-LUC clone #4 mES cells were aseptically injected into the heart through the chest wall using a 1 cc tuberculin syringe. The injection volume was 20 μl per heart. In this experiment 100,000 cells were transferred into each mouse heart. The mice were then injected with the substrate, luciferin (150 μg/g), and imaged using IVIS (Xenogen Corp). Note the prominent light emission in the heart region. Control animals receiving vehicle (HBSS) did not display any light emission (not shown).
Acute in vivo imaging of transplanted α-MHC-4-fLUC ES cells in neonatal mice

There was no LUC expression found from transplanted adult hearts, possibly because luciferase activity was not bright enough to visualize from the adult hearts. To enhance the likelihood of being able to image ES cells in vivo, we began to use neonatal mice model (Christensen, et al. 2000) rather than adult mice because the light would have less tissue to penetrate and hence less loss due to scatter. This model has advantages over adult in being that no complicated open heart live surgery and having premature immune system. The animals are smaller and intramyocardial injection can be performed into the heart with ease. As an initial effort of cell transplantation in neonatal mice, I injected pluripotent α-MHC-4-fLUC-43 (n=6), YFP (n=6), and HBSS alone (n=5) into 3 separate litters. ES cells were injected into the neonatal hearts by intramyocardial injection at day 1-3 after birth. Injections were given at 3 varied concentrations of cells ranging from 100,000, 1 million and 1.6 million cells. In vivo imaging was done on the animals immediately after transplantation. Controls and animals injected with pluripotent YFP cells have no luciferase signal as expected because they have no luciferase genes. Luciferase expression in αMHC4-fLUC-43 injected cells was observed at 1 million and 1.6 million cell concentrations.
Figure 12: In vivo imaging of pluripotent NCX1-43-fLUC ES cells injected into neonatal pups, at 8, 18, and 24 days after transplantation.

(A, B, C) 100,000–cell transplantation. At higher cell numbers the luciferase signal was higher and the cells spread along the lungs and thoracic cavity, resulting in luminescent tumor formation. (D, E, F) 50,000–cell transplantation. At lower cell numbers, the expression looks more localized to heart. (G, H) 10,000–cell transplantation the expression is very week and disappeared by 24 day period.
Dose- response of undifferentiated NCX1-43-fLUC ES cells transplanted into neonatal mice and *in vivo* bioluminescent imaging:

A time course of bioluminescent imaging with pluripotent ES cell transplantation in neonatal mice was investigated. To test this, NCX1-43-fLUC 43 ES cells were injected into neonatal hearts at doses ranging from 10,000 to 200,000 cells, and imaged at 8, 18 and 24 days post-cell injection. Eight days was the starting point of bioluminescent imaging for *in vivo* studies because in *in vitro* it takes 8 days to form cardiac differentiated cells from pluripotent ES cells. Mice were analyzed for LUC expression and presence of tumors over a 24-day period. In neonates injected at 10,000 cell concentrations, *(n=5)* the ROI values ranged from 2343 to 16468 and at 18 days 1635.3 and lost after 18 days and showed weak luciferase signal. In neonatal mice injected with 50,000 cells *(n=4)* the ROI ranged at 6547 to 29651 photon.sec/cm$^2$/steradian at 8 days and 3546 to 20697 photon.sec/cm$^2$/steradian at 18 days and 1005 to 3967 photon.sec/cm$^2$/steradian at 24 days among different animals. Expression increased until 2 weeks and after decreased. In animals injected with 100,000 cells, there was a significant increase in expression over time and tumors were found *(Fig.11)*. The expression ranged *(n=7)* between 16,206 to 63304 photon.sec/cm$^2$/steradian at 8 days and 19172 to 67666 photon.sec/cm$^2$/steradian at 18 days and 4418 to 26625 photon.sec/cm$^2$/steradian at 24 days among the various litters. The expression gradually increased over time showing the cells were proliferating. In mice injected with 200,000 cells, the expression was higher and 100% tumor formation was noticed with increased mortality before the 24 day period. LUC activity increased until 3 weeks at different cell numbers and there was significant drop of luciferase activity after week 3. Control animals receiving a same volume of HBSS *(1X)* did not develop tumors or LUC expression as expected. Signal intensity was highest in mice injected with 200,000 cells.
compared to 10,000 cells as evidenced by in vivo bioluminescent imaging. In general the signal intensity increased from week 1 to week 3 and signal intensity decreased there after over time.

Figure 13: Evaluation of incubation time, recombinant cell line and cell number on in vivo luciferase signal:

(A) luciferase signal of recombinant ES cell line, differentiation state, and number injected into neonatal mice and imaged at 8, 18 and 24 days. GAPDH and MHC driven clones were the brightest of the respective recombinant clones. P- Pluripotent: C- Cardiac induced (7+3) at transplantation) (B) Percentage of recipient mice expressing thoracic tumors 4 weeks after injection of pluripotent NCX1-43-fLUC ES cells. 80% of animals formed tumors with injection of 100,000 cells: 100% formed tumors when injected with 200,000 and higher cells. Animals receiving less than 100,000 cells did not develop tumors. (C) representative H&E staining of tumor section from animal receiving 100,000 cells. (D) Representative fluorescent microphotograph of tumor demonstrating ES (YFP) origin of tumor cells.
Bioluminescent imaging of undifferentiated NCX1-43-fLUC ES cells

The initial study was to see the feasibility of recombinant ES-LUC cell lines in studying the proliferation, differentiation status of the transplanted cells in heart via \textit{in vivo} bioluminescent imaging strategy. The presence of tumor formation via bioluminescent imaging strategy can also be studied. Recipient mice injected with 100,000 cell number and higher concentrations formed tumors as evidenced by imaging and histological assessments. At concentration 50,000 cells and lower, tumor formation greatly reduced and was evident by imaging and histology at the end of 24 day period. This observation was consistent with several other reports that cell death occurs (Murry \textit{et al} 2007, Kolossov, \textit{et al} 2006). We noticed tumor formation even in cardiac-induced cells at 100,000 and higher concentrations. The cardiac induced cells were not enriched for pure cardiomyocytes populations and therefore, contain mixtures of other cells which might result tumor formation. There were dose-response relationship, with preferentially less tumors with less than 50,000 cells, and 80\% of animal developing tumors at 100,000 cells and above. Tumor formation was also seen cardiac differentiated cells at higher concentration of 100,000 cells and above. Post mortem analysis also confirmed the presence of tumors at higher cell number as evidenced by hematoxylin and eosin staining and the teratoma was visualized by fluorescence because these cells express YFP protein. LUC signal increased in cardiac-induced cells compared to pluripotent ES cells over time as seen from (Fig.12).
Figure 14: In vivo imaging of cardiac induced NCX1-FLUC cells (7+3) days following injection into neonatal hearts and imaged at 4, 8, 18, 24 days after transplantation. (50,000 cells per heart).
Transplantation of cardiac-induced NCX1-43-fLUC ES cells and in vivo bioluminescent imaging

The number of cardiac-induced cells that should be injected into the recipient neonatal mice to see noticeable luciferase expression without tumor formation was investigated. Cardiac-induced (7+3 day) cells were injected, which was assumed as earlier stage cardiomyocytes and imaged at 4 time-points. In vivo imaging was done at 8, 18, 24 days post-transplantation. At 50,000 NCX1-43-fLUC cells number there was noticeable luciferase expression as measured by in vivo imaging throughout the 24 day period. 1 litter of animals formed tumors out of 10 transplantation experiments at 50,000 cells transplantation. Tumors formed from cardiac differentiated cells were harder compared to tumors that were formed when NCX1-43-fLUC pluripotent cells were transplanted. At 50,000 cell number LUC expression looks more localized to the heart. Day 8 was our initial imaging day because cardiac induction occurs after 7 days from ES cells and the animals were too small to handle them at 4 days. The earliest imaging day of transplanted cardiac differentiated (7+3) cells was at 4 days as seen from Fig.12. representative sections of ventricle showing the presence of YFP and α-actinin showing that few cells were differentiated into cardiomyocytes.(figure 14).

Note: cardiac differentiated cells in this thesis refers to ES cell derived cardiomyocytes containing mixed population of other cells
Figure 15: Transplanted NCX1-43-FLUC cardiomyocyte in neonatal mouse heart at 14 days after transplantation.

Transplanted cell, identified by YFP expression, 14 days after transplantation stained for the cardiomyocyte striation marker α-actinin. (A, D) YFP in 543 nm excitation. (B, E) α-Actinin in 633 nm excitation. (C, F) Overlay of YFP and α-actinin. (A-C) 20x magnification. (D-F) 40x magnification.
CHAPTER 4
DISCUSSION

This study was designed to generate recombinant ES cell lines in which the luciferase gene is under the control of constitutive and cardiac-specific cell lines and to explore the feasibility of these ES cell lines for use *in vivo* bioluminescent imaging in a neonatal mice model. The major findings are:

1. Novel stable recombinant mES reporter cell lines were developed for *in vivo* bioluminescence imaging.

2. One of these cell lines was created using the glyceraldehyde 3-phosphodehydrogenase (GAPDH) promoter fused to the fLUC reporter and it showed similar levels of fLUC expression in undifferentiated (pluripotent) compared to cardiac-differentiated mES cells.

3. Another cell line was produced using the cardiac-specific sodium-calcium exchanger 1 (NCX1) promoter fused to the fLUC reporter and this cell line showed markedly increased fLUC expression following induction of cardiac differentiation in culture when compared to the pluripotent cells.

4. Transplantation of the recombinant fLUC-expressing cells into neonatal mouse hearts produced bioluminescent signals that persisted for at least 24 days, the maximum time point analyzed in this study.

5. Transplantation of 100,000 or more mES cells to the heart consistently produced teratoma and tumor formations, regardless of which recombinant clone was used or whether the mES cells were injected as pluripotent or cardiac-differentiated cells.
(6) Transplantation of between 10,000 and 50,000 cardiac-differentiated NCX1-fLUC mES cells per heart resulted in measurable bioluminescent image signals in vivo with low incidence of tumor formation.

(7) Some of the transplanted NCX1-fLUC mES cells were identified in ventricular muscle tissue in postmortem histological sections where it was found that they had developed cardiomyocyte characteristics.

As an advantage over cell types, pluripotent ES cells have the ability to differentiate into any cell type, including cardiomyocyte and endothelial cells. One major hindrance of these cells is the potential accompanying development of unwanted cell population and tumors. In this study, we were able to evaluate the survival and proliferation/differentiation of cells over a 4-week period and observed a decrease in LUC signal, consistent with other reports indicating cell death with time (Reinnecke and Murry, 2002).

A major advantage of the NCX1-43-fLUC cell line, is that in vivo assessments can be done prior to transplantation regarding the health and differentiation potential of the cell line (Menick, et al. 2006). The NCX1-LUC-43 cell line can serve as an effective marker for cardiac development. As the parental ES cell line was genetically engineered with the YFP gene, the cells can be visualized after fixation. Thus, the transplanted cells can be identified in the host tissue irrespective of the differentiation status.

**Comparison of promoter strength and specificity**

We studied the proliferation and survival of the three recombinant ES cells by transplanting these cells at various cell numbers into the neonatal mice model, followed by in vivo bioluminescent imaging over a period of 4 weeks post-transplantation. Even though the GAPDH promoter is of approximately same size as that of NCX1, used for this study it does not show a significant
increase in LUC activity with cardiac differentiation. From this, it is clear that size of the promoter is not correlated to reporter gene expression. The GAPDH promoter is well-characterized, and it contains two hypoxia responsive elements and hypoxia inducible factor (HIF-1) consensus binding sites (Graven, et al. 1999 and Shan Lu, et al. 2001), which can stimulate cardiac specific genes. NCX1 is earlier marker and plays an important role in cardiac development, and its promoter is well characterized. (Menick, et al. 2006). The size of NCX1 promoter is 1800 base pairs. It has been shown that 181 base pairs are sufficient for driving cardiac specific reporter gene expression and contain regulatory elements that can be stimulated by adrenergic drugs. (Menick, et al. 2005) The NCX1-43-fLUC ES cell line showed differential expression and a 3-fold increase in luciferase activity in the cardiac-differentiated state.

MHC is a structural protein and cardiac-specific. The αMHC-4-fLUC ES line showed differential luciferase expression that strongly decreased following cardiac induction. This might be due to position effects and endogenous 5′ regulatory elements controlling the LUC gene as a result of non-targeted integration into the genome by electroporation procedure.

**Comparison of pluripotent vs. cardiac-differentiated ES cells**

We compared pluripotent and cardiac-induced cells at varied cell concentrations. We transplanted NCX1-43 ES cell at 10,000, 50,000 and 100,000 cells in both the pluripotent and cardiac-differentiated state and assessed bioluminescence over a 4-week period. The luciferase expression increased in the initial 2 weeks and decreased thereafter. Bioluminescence signal was lost. This signal loss might be due to cell death, consistent with the published reports (Murry, et al. 2007 and Befar, et al.). It has also been shown that immunogenicity increases upon differentiation and can result in cell death. We believe this is unlikely in these experiments.
because the ES cells were isolated from the same mouse strain as the transplantation recipients to minimize the immune response.

**Comparison of number of cells and tumorogenesity**

We hypothesized proliferation of cells may be dose-dependent and result in tumor formation at higher concentrations and cardiac induction at lower concentrations, consistent with other reports (Murry, *et al.* 2007) We also hypothesized that these outcomes can be revealed by noninvasive bioluminescent imaging. A dose-response relationship yielding tumors at concentrations of 100,000 and above and no tumors at concentrations of 50,000 and below is evidenced by bioluminescent imaging irrespective of the recombinant ES cell line used. At concentrations greater than or equal to 100,000 injected cells, we observed robust and spatially diffuse luciferase expression (Fig.11). The bioluminescent imaging data is correlated with postmortem analysis showing the YFP origin of tumor formation. At lower concentrations of less then 50,000 cells, the expression appears more localized to the heart, and no tumor formation was seen.

**Technical issues**

One major limitation in our studies is that bioluminescent and fluorescent reporter technologies use low energy and so cannot be feasible for use in larger animals. The neonatal model used has several advantages in that it does not require complicated open-heart surgery and the environment is more conducive to transplanted cell survival because of the immature organ and immune systems. One of the major limitations of the neonatal model is that the studies cannot be extrapolated to a disease model. Conventional technologies rely on postmortem assessments for cell visualization, whereas *in vivo* bioluminescent imaging allows for both noninvasive repetitive long-term monitoring of cells in the same animal with minimal animal handling and the study of more basic mechanisms such as proliferation and differentiation. Images can be obtained at
faster rates than to other imaging modalities like MRI and PET, and the technology is extraordinarily cost-effective compared to other imaging modalities.

**Future directions**

Transplantation of undifferentiated ES cells resulted in tumor formation and was found to be dose-dependent irrespective of the particular promoter strain, consistent with other reports (Murry, *et al.* 2007). We have seen dose-dependent tumor formation in cardiac-differentiated cells, but these were comparatively less frequent than pluripotent ES cells. This might be due to assumption that fewer than 5% of the ES cells will differentiate into cardiomyocytes, so transplanted cell population is not pure. It was also shown that immunogenicity increases upon differentiation, so preselection by either genetic selection or density gradients to enrich more pure populations of cardiomyocytes will be necessary for transplantation of these cells to use in cellular therapies.

There is strong preliminary evidence that the cardiomyocyte-enriched fraction from density gradients have higher luciferase activity, which is strong evidence that these are true cardiomyocytes. Future studies challenging the cells with adrenergic drugs can confirm that they are the true cardiomyocytes. Future studies transplanting these cells into a diseased model to assess the survival and differentiation status of these cells via *in vivo* bioluminescent imaging followed by MRI will examine functional improvements resulting from these cells.

In summary, ES-derived cardiomyocytes are a better source of therapeutic cells for further investigation. Bioluminescent imaging is a cost effective technology to study the survival and proliferation of transplanted cells in heart non-invasively, with minimal animal handling, and images can be obtained faster than other imaging modalities like MRI.
REFERENCES


