Differentiation, Characterization And Expansion of Murine Pluripotent Stem Cells To Cardiomyocytes

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Quality is never an accident;
it is always the result of
high intention, sincere efforts,
intelligent direction
and skillful execution;
it represents the wise choice

of many alternatives……

William A. Foster

Dedicated to Mum, Dad and Himanshu…

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Kanika Jain

List of Abbreviations

bp	Base pair
kb	Kilo base pair
mM	Millimolar
mV	Millivolts
ng	Nanogram
µM	Micromolar
µg	Microgram
ATP	Adenosine tri phosphate
bFGF	Basic Fibroblast Growth Factor
BM	Bone Marrow
BMP	Bone Morphogenetic Proteins
BMSCs	BM-derived Multipotent Stem Cells
BMT	Bone Marrow Transplantation
BSA	Bovine Serum Albumin
cDNA	complementary DNA
CD C	Cluster of differentiation antigen
CMV	Cytomegalovirus
CVD	Cardiovascular disease
CSCs	Cardiac Stem Cells
cTnT	Cardiac Troponin T
DKK1	Dickkopf-related protein1
dNTP	Deoxyriboucleotide
DPBS +/+	Dulbecco's Phosphate Buffered Saline with $CaCl_2$ & $MgCl_2$
DPBS -/-	Dulbecco's Phosphate Buffered Saline without $CaCl_2$ & $MgCl_2$

dpc	Days post coitum
DSB	Double-strand Break
DTT	Dithiothreitol
EBs	Embryoid Bodies
EDTA	Ethylenediaminetetraacetic Acid
EGF	Embryonic Growth Factor
END-2	cells Endoderm-like Cells
EPCs	Endothelial Progenitor Cells
ESCs	Embryonic stem cells
FACS	Fluorescence-Activated Cell Sorting
FBS	Fetal Bovine Serum
FgF	Fibroblast Growth Factor
FHF	First Heart Field
FITC	Fluorescein Isothiocyanate
FSC/SSC	Forward scatter/Side scatter
g	Gravity force
GFP	Green Fluorescent Protein
GFP^+	Positive Green Fluorescent Protein
hyg3	Hygromycin
HSCs	Haematopoietic Stem Cells
HR	Homologous Recombination
ICM	Inner Cell Mass
Ig	Immnoglobulin
IGF-1	Insulin-like Growth Factor-1
IL	Interleukin
iPSCs	Induced pluripotent stem cells
IRES	Internal Ribosome Entry Site
IU	International Units

LB	Luria Bertani
LIF	Leukemia Inhibitory Factor
LM	Liquid Marble
LPM	Lateral Plate Mesoderm
LVEF	Left Ventricular Ejection Fraction
MEFs	Mouse Embryonic Fibroblasts
MI	Myocardial Infarct
mRNA	messenger RNA
MSCs	Mesenchymal Stem Cells
NaOH	Sodium Hydroxide
NEAA	Non Essential Amino Acids
NHEJ	Non-homologous End Joining
OG2	Bl6 mice with an Oct4-EGFP reporter transgene
p	Probability value
PCR	Polymerase Chain Reaction
PFA	Paraformaldehyde
PMSG	Pregnant mare serum gonadotropin
PSCs	Pluripotent stem cells
PTFE	Polytetrafluroethylene
RNA	Ribonucleic Acid
RPM	Rounds Per Minute
RVD	Repeat Variable Diresidue
SD	Standard deviation
SHF	Secondary Heart Field
TALENs	Transcription-Activator like Effector like Nucleases
Taq	Taq polymerase
TGF	Transforming Growth Factor
TMRM	Tetramethylrhodamine Methyl Ester Perchlorate

UTR	Untranslated Regions
UV	Ultraviolet
V	Volts
v/v	Volume/volume
w/v	Weight/Volume
ZFNs	Zinc-finger Nucleases

ABSTRACT

Pluripotent stem cells (PSC) such as embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs) represent a potential source for cell therapy, as they are known to differentiate into multiple cell lineages. Since mature cardiomyocytes are known to have extremely limited proliferative capacity, PSC derived adult cardiomyocytes may provide a reliable source of cells for transplantations if large-scale production can be achieved in bioreactors. However, generation of mature cardiomyocytes from PSCs remain a major challenge due to the heterogeneous population of cells obtained following differentiation, therefore limiting their utility. Another challenge that needs attention is obtaining a clinically significant number of cardiomyocytes that could be used for cardiac transplantations.

We need to remember that the differentiation process *in vitro* is never 100% pure, and the heterogeneous pool of cells obtained post differentiation poses a major hurdle to obtain specific cell types relevant for transplantation studies. In order to address this issue, **chapter 3** is dedicated to target the endogenous progenitor cardiac gene Nkx2.5 with a selection cassette comprising of IRES-mCherry-IRES-CMV-IRES-hyg3 using the newly developed TALEN approach. The chapter concludes with successful generation of the left, right TALEN along with the Obligate Ligation-Gated Recombination (ObLiGaRe) donor plasmid that can be used to transfect the ESCs/iPSCs eventually generating cardiac reporter cell lines. The generation of the reporter knock-in cell lines would allow easy identification of the generated cardiomyocytes at their progenitor and mature stages and provide a very simplified method of their purification and enrichment.

Furthermore, the ability of a novel micro bioreactor “Liquid Marble” (LM) to generate cardiomyocytes *in vitro* was studied intensively in **chapter 4**. The marble strategy is based on the principle that the culture media suspension drop (comprising ESCs) rolled over a powder bed can prove to be an efficient system to generate embryoid bodies (EBs). Here, in the LM, murine ESCs were allowed to aggregate in suspension for 5 days. Beating cardiac bodies were generated from the EBs obtained from the LM system. Furthermore, the differentiation process was progressively monitored for the expression of cardiac lineage markers by immunocytochemistry and real-time PCR. Cells obtained were analysed for the expression of Gata4, Flk1 and Mef2C (mesendoderm), followed by the expression of Nkx2.5, Mlc-2a, Mlc-2v, cTnT and α-actinin; indicating progression to the progenitor and mature cardiac cells. Immunocytochemistry analysis for Nkx2.5 and cTNT confirmed the generation of cardiac progenitors and mature cardiomyocytes. This study has the potential to set

paradigm for future studies by investigating the conditions for developing cardiomyocytes as a starting population for transplantation and also for research and drug discovery. This study was published in a peer review journal **Advanced Healthcare Materials, under the title "Cardiogenesis of Embryonic Stem Cells with Liquid Marble Micro-Bioreactor."**

Further, I envisaged using murine iPSCs in the similar LM micro-bioreactor to generate cardiomyocytes. Being derived from adult cells, e.g. skin fibroblasts; iPSCs bypass the ethical issues regarding the use of embryonic tissue to cure disease because the starting material can be obtained from the patients themselves. The cardiomyocytes obtained from this system holds a greater promise for transplantation studies as they overcome the major issue of immune-histocompatibility. The results obtained in **chapter 5** clearly indicate that LM is not only supporting the culture of iPSCs but it also allows the formation of functional EBs. This was supported by the reverse transcrption (RT-PCR) analysis, where the EBs expressed markers of all the three germ layers, ectoderm (Nestin), mesoderm (Brachyury, Nkx2.5) and endoderm (Gata4, FoxA2). Upon further differentiation, these EBs expressed proteins of cardiac progenitor (Nkx2.5) and mature (cTnT) genes as well as neural progenitor (Nestin) genes as was revealed by the immunocytochemical analysis. Thus, this study holds immense therapeutic potential as it can be used to differentiate patient specific iPSCs that can generate either cardiomyocytes/neurons and these cells once introduced into the patients will help to overcome the immune rejection issues.

Keeping in mind that the differentiated mature cells will be used for clinical applications, it becomes imperative to develop defined and efficient *in vitro* protocols, which would then provide the stringent levels of safety and quality control making stem cell transplantation therapy realizable. My research outcomes will provide a step up the ladder in the area of cardiac therapy.

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List of Publications

Journal publication

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  Equal First Authors **(Chapter 4)**

- **Kanika Jain**, Jun Liu, Wei Shen, Peggy P.Y. Chan, Kerry Hourigan. *Embryoid Body differentiation of induced pluripotent stem cells with Liquid Marble Micro-Bioreactor. (Manuscript in preparation).* **(Chapter 5)**

Book Chapter

- **Isolation and Handling of Mouse Embryonic Fibroblasts.**

  Kanika Jain, *Paul J Verma, Jun Liu.*

  Mouse Genetics, Methods in Molecular Biology, Volume 1194, 2014, pp 247-252 **(Appendix 1)**

Chapter One

Introduction and Objectives

1.1 Introduction

Cardiovascular disease (CVD) is the leading cause of death in Australia, with 43,946 deaths attributed to CVD alone in 2012 [1]. It kills one Australian every 12 minutes. Looking at those statistics, CVD is considered to be one of Australia's largest health problems. Despite improvements over the past few decades, it remains a burgeoning public health issue. CVDs generally include heart, stroke and blood vessel disease. It is one disheartening fact that the lives of 45,500 Australians (31.7% of all deaths) in 2010 were lost due to CVD, which was largely preventable. More so, the highest rate of hospitalization and death included people of the lower socioeconomic groups, Aboriginal and Torres Strait Islander people and those living in remote areas. Each year, around 55,000 Australians suffer a myocardial infarct (MI) (heart attack). This equates to one heart attack every 10 minutes. The human left ventricle has 2-4 billion cardiomyocytes and a MI can wipe out 25% of these in a few hours [2]. Coronary artery occlusion induced MI results in: loss of beating cardiomyocytes (by apoptosis or necrosis) [3]; heart remodelling and heart failure [4]. Angioplasty and thrombolytic agents can relieve the cause of infarction, but are unable to mitigate the injury to the myocardium resulting from the MI [5]. Fibroblasts divide and migrate into the damaged area to replace the dead cardiomyocytes but form a scar tissue, which is a thin ventricular wall unable to contract properly. The electrical conduction system of the heart is disrupted due to scarring and this starts to affect the surrounding viable ventricular myocytes, thus, making them susceptible to spontaneous depolarization. Eventually, this myocardial scar initiates a cascade of events, leading to heart failure and more cell death.

A transition from hyperplastic to hypertrophic myocardial growth is observed shortly after birth. This transition is well characterized by an increase in myofibril density and the formation of bi-nucleated cardiomyocytes [6, 7]. Soonpaa et al. (1996) demonstrated that cardiomyocyte DNA synthesis in mice passes through two temporally distinct phases. Cardiomyocyte proliferation is observed exclusively in the first phase (foetal life) of the synthesis, whereas the second phase (postnatal) was associated largely with bi-nucleation. Radiolabeled thymidine incorporation studies suggested that cardiomyocyte proliferation ceases before birth in mice, indicating that the adult cardiomyocytes show very limited capability of division in neonatal/postnatal hearts [6-8]. Unfortunately, as a consequence of this inability, adult cardiomyocytes are unable to replace the damaged myocardium in any clinically significant manner [9].

Because of the aforementioned transitions from the foetal to the postnatal myocardial growth, the heart has been categorised as one of the least regenerative organs of our body. The

regenerative response, if any, is very little in comparison to that seen in other tissues such as liver, skin, bones, skeletal muscle, etc. The regeneration of the diseased/injured myocardium to prevent or treat heart failure has proven to be a difficult challenge that has attracted many investigators over the past two decades. The field of cardiac repair has made substantial strides in recent years, where researchers have come a long way from conducting studies in experimental animals to clinical trials involving thousands of patients.

Presently, for end stage heart failure patients, the only treatment of choice that appears on the horizon is the allogeneic heart transplant. Heart transplantations are useful in extending the life of the patients suffering from terminal heart failures, but they suffer a major drawback. The ratio between the number of donor organs available and the recipients that need the transplant, severely limits this approach. Various other treatment opportunities, which are currently being deployed, include xenotransplantation [10], percutaneous coronary intervention combined with fibrinolytic drugs [11], artificial mechanical heart [12], life-saving devices such as defibrillators and left-ventricular assist devices [13-15]. Hansson et al. (2009) boast of a minor improvement in the left ventricular ejection fraction (LVEF) when transplanting non-cardiac stem cells such as skeletal muscle progenitors and bone–marrow-derived cells [16] *in vivo*. But, since these transplanted populations do not generate new cardiomyocytes [17], researchers are investigating alternative strategies for generating them.

The biggest challenge that has tantalized the researchers in cardiac tissue engineering is to generate, approximately, 1 billion cardiomyocytes that are lost during an infarct [18]. In order to obtain this astonishing number of cardiomyocytes, approximately, around one hundred standard 10 cm tissue culture dishes will be required [16]. Pluripotent stem cells (PSCs) are known for their ability to self-renew, proliferate, and differentiate into specialised cell types. The basic framework regarding using stem cell based therapies is quite simple in that they would be used as a replacement setting regenerating the injured/diseased myocardium, thereby replenishing the tissue lost. Since there is an inadequate internal repair mechanism within the diseased heart, the “holy grail” lies in addressing that by using embryonic stem cells (ESCs) or induced pluripotent stem cells (iPSCs). Ideally, PSCs, with the potential to robustly attain a functional cardiac phenotype, work in improving the cardiac function through direct electromechanical coupling with host tissue [4]. Currently, stem cells present the most promising source for generating the large amounts of functional cardiomyocytes needed for cellular-engineering therapies. PSCs, unlike multipotent adult stem cells, are capable of proliferating indefinitely, and can spontaneously differentiate into cells from any tissue and organ system in the body. Hence, they hold the key potential to generate the required quantity of cells for needed for cellular therapy. The recent breakthrough in the field

of iPSCs [19, 20] has demonstrated that these cells may provide an additional unlimited source for *in-vitro*-differentiated cardiomyocytes as they share striking similarities with their ESC counterparts.

In particular, it has been demonstrated that ESCs and iPSCs can unambiguously be differentiated into cardiomyocytes and they display spontaneous or induced contractile activity along with a proper action potential (AP) [21, 22]. Currently, cardiomyocytes can be differentiated from PSCs in multiple ways: (1) spontaneous embryoid body (EB) differentiation, (2) co-culture with mouse endoderm-like cells (END-2 cells), (3) directed differentiation, or monolayer culture [23-28]. The monumental success achieved in generating cardiomyocytes from PSCs has led to an explosion of *in vivo* studies of cell transplantation in animal models of myocardial infarct (MI). The convincing results of the animal studies have triggered a cascade of small pilot clinical trials [29-34] and also one large randomized, double blind, placebo-controlled trial [35] of cell transplantation in patients with acute MI. Although these clinical trials have generated some confidence in the feasibility of cell transplantation, a number of issues still need to be addressed before this cell therapy can be put into widespread clinical use. Some of these issues include: selection of the correct cell type for transplantation; optimal time and route for transplantation and enhancement of efficacy.

Another challenge faced by researchers over the last few years has been to develop robust enrichment/ isolation techniques that allow scalable purification of cardiomyocytes and their subtypes. Until now, the various methods that have been developed to sort pure cardiomyocytes include, but are not limited to, mechanical isolation [27, 36, 37], percoll density gradient centrifugation [38-41], genetic selection techniques [42, 43], etc. Most of the recently published literature on cardiomyocyte enrichment studies demonstrates the advantages of using the transgenic strategies (genetic manipulation) based on targeting the cardiac specific genes. The targeting strategies deploy either a specific drug resistance gene alone or in combination with some reporter genes like GFP or mCherry [44, 45].

Considering the present scenario, it is evident that the isolation of sufficient number of cells to generate millions of cardiomyocytes upon transplantation for a single patient is the need of the hour. Thus, robust, economical but scalable therapeutic paradigms need to be devised to obtain the same. The prodigious advances made in the field of stem cell biology, in the past few decades has created an aura of excitement and hope in the scientific community to quickly translate the bench work research into the clinical settings.

The objective of the present thesis is to tackle the major challenges in cardiac research. Evaluating the differentiation of pluripotent stem cells (murine ESCs and iPSc) using a novel liquid marble (LM) micro bioreactor to cardiomyocytes will be of outmost importance. In addition to this, tools to create cardiac specific reporter cell lines will be investigated, that will aid in sorting/enriching mature cardiomyocytes, hence helping in injecting a pure cardiac population for transplantation studies.

1.2 Heart development in vivo: Lesson to learn

Since cardiomyocyte differentiation recapitulates embryonic development, it becomes imperative to understand the cardiac lineage formation in the early embryo. How the developments occur *in vivo* would give tremendous insight into the feed-forward gene regulatory networks of cardiac development, which would ultimately help in deriving physiologically relevant cells [46]. The earliest events of organogenesis during the embryonic development involve the formation of the heart. Following fertilization, the zygote cleaves and forms blastocyst before implantation (-E3.5) comprising the inner cell mass (ICM) and trophoblast. It is this ICM that develops towards the whole body, and ESCs are isolated from the ICM. During the late blastocyst stage, the ICM segregates into the epiblast and the primitive endoderm lineages. On induction by Nodal, there is a migration of cells from the epiblast to the midline, giving rise to the primitive streak. The primitive streak is formed from the primitive endoderm and is the origin of many tissues [47].

The process of cardiomyogenesis has been best studied in the mouse and begins with the formation of the mesoderm via the process of gastrulation [48, 49] **(Figure 1)**. At mouse E5.0, Nodal signaling in the proximal epiblast induces the formation of mesoderm. The extraembryonic ectoderm, adjacent to the epiblast, maintains the expression of BMP4, which acts by inducing Wnt3 expression in the proximal epiblast. Mesoenodermal markers such as Brachyury and Eomes are induced by the expression of Wnt at E5.75. The genes involved in the mesoderm patterning, such as Fgf4 and Fgf8, are subsequently expressed in the developing primitive streak [46, 50, 51].

Cells migrating through the primitive streak differentiate into the mesoderm and endoderm. The remaining cells in the epiblast that do not migrate eventually form ectoderm. Parts of the mesoderm cells that migrate to the lateral side are called lateral plate mesoderm (LPM). Furthermore at E7.0, Brachyury and Eomes induce the expression of Mesp1, popularly known as the “master regulator” of cardiac progenitor specification [52, 53] and also the cardiac

mesoderm formation [54]. Mesp1 regulates cardiac differentiation via the DKK1- (Dickkopf-related protein1) mediated inhibition of Wnt signalling [55].

Cardiac development involves a complex series of events that starts with the induction of the mesoderm moving to the specification of cardiovascular progenitors which further expands and differentiates in culture eventually leading to cell maturation [56]. Naito et al. (2006) examined the role of Wnt signalling during cardio myogenesis in an ES cell based system. They concluded that Wnt/β-catenin signaling exhibits developmental stage-specific, biphasic, and antagonistic effects on cardiogenesis and appropriate modulation of Wnt signaling enables highly efficient cardiomyocyte differentiation [57]. A major point to remember is that Wnt signaling in the initial stages of PSC differentiation promotes cardiac progenitor specification, whereas in the later stages it acts in an antagonist way by inhibiting terminal differntiation. Further down the differentiation pathway, Mesp1 coordinates the up-regulation of a majority of the key cardiac transcription factors like Nkx2-5, Gata4, Mef2c etc. which form the core of the cardiac transcriptional machinery [52, 56] of cardiomyogenesis. Following the cardiac mesoderm specification, canonical Wnt and NOTCH signalling regulate the cardiac progenitor cell maintenance [46, 58, 59].

The first heart field (FHF) that forms the atria, left ventricle and the nodal conduction system; the secondary heart field (SHF) which forms the right ventricle, outflow tract, and part of the atria; and the proepicardial mesenchyme originate from the cardiac mesoderm [46, 49]. At the cardiac crescent stage, the FHF differentiates, whereas the SHF remains in an undifferentiated progenitor state as a result of the inhibitory Wnt signals from the midline [58]. The underlying anterior ectoderm exposes the cardiac crescent to the bone morphogenetic proteins (BMP) signaling, whereas the overlying anterior endoderm exposes it to the BMP, FGF, anti-canonical Wnt, and non-canonical Wnt signals [60]. To summarize, in the cardiac crescent, the Nkx2-5^{+} population is known as the FHF and the Isl1^{+} population is known as the SHF. The cells within the FHF and SHF appear to proliferate under neuregulin1, retinoic acid etc. signals [61, 62]. By E8.0, these cardiac crescent cells fuse in the midline forming the primitive heart tube, which consists of an internal layer of endocardial cells and an exterior layer of myocardial cells [46]. This primitive heart tube further loops rightward, forming the four-chambered heart. In order to develop technologies to generate reproducible cardiomyocytes, further study of maintaining and proliferating cardiac progenitors, along with in-depth analysis of the various signaling pathways and cardiac markers involved, seems imperative.

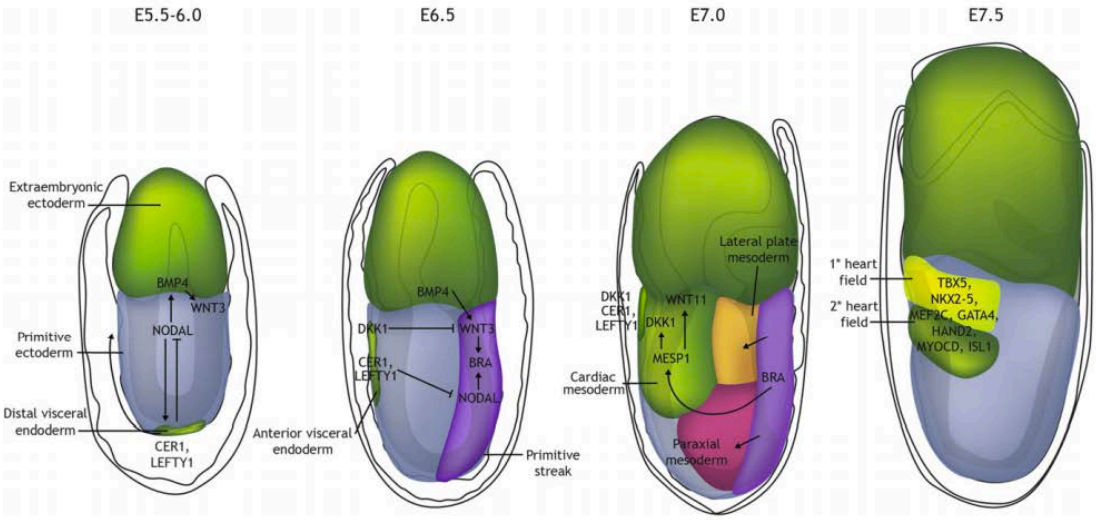


Figure 1 The mouse as a model for cardiogenesis.

Formation of the primitive streak begins gastrulation. An EMT transition by the anterior primitive ectoderm cells passes through the primitive streak. Cells in the mid primitive streak form the embryonic tissues such as heat and blood. Lateral and ventral progression of the midstreak mesoderm happens in case of cardiac progenitors giving rise to the LPM, forming the first heart field. These progenitors form the cardiac crescent, where secondary heart field progenitors are found [46].

1.3 Stem cells and cell therapy

Studies from multiple laboratories have shown that transplantation of donor cells (foetal and ES cell- derived cardiomyocytes, skeletal myoblasts, smooth muscles, and adult stem cells) can improve the function of the diseased heart [63]. In order to restore the cardiac function in an injured myocardium, the first experimental attempts that were designed focused on the use of two extra-cardiac sources of cells: progenitors from skeletal muscle and bone marrow (BM).

1.3.1 Skeletal Myoblasts

Adult skeletal muscle contains myogenic precursor cells, called skeletal myoblasts or satellite cells, which normally remain quiescent. In response to injury, these cells are rapidly mobilised, proliferate and differentiate, replacing damaged skeletal myocytes. Myoblasts can be easily obtained from a small skeletal biopsy and amplified in culture.

In the first approach, satellite cells, skeletal muscle-specific stem cells (‘‘myoblasts’’), were transplanted to the injured heart to understand the transdifferentition methodology that would result in an increased contractile capacity [16]. Reinecke et al. (2002) demonstrated that even though satellite cells transplanted to hosts infarcted hearts matured into fully differentiated skeletal muscle fibers but no evidence of conversion to a cardiomyocyte fate was observed

[64]. No electromechanical coupling of the engrafted cells with the host myocardium was observed [65]. Even though few groups reported some positive effects on the rodent hearts [66, 67], no trans-differentiation into cardiomyocytes or functional electromechanical coupling was observed. The functional benefit was, therefore, most likely a result of paracrine effects on native tissue from the grafted cells rather than a result of any contribution from the myotubes.

Despite the fact that not much is understood regarding the mode of action of skeletal myoblasts, a phase I clinical trial was initiated that transplanted satellite cells to the hearts of patients with chronic heart failure. The transplantation study was not conclusive of any functional benefits of skeletal myoblasts as it was of small size and uncontrolled. These sort of transplantation studies needs to be evaluated in large-scale randomized placebo-controlled clinical trials such as the myoblast autologous grafting in ischemic cardiomyopathy (MAGIC) trial [68]. Differences in the human and mouse cardiovascular systems explained the disappointing results of the clinical trials, in spite achieving successful results in animal models [69]. In short, there is a need for a better and a validated animal model system for cardiac regenerative therapy.

1.3.2 Bone marrow (BM) derived cells

Moving on from using skeletal muscle, studies have been conducted to transplant BM cells to the injured heart. BM is considered to be a residing place for primitive stem cells. It contains a complex assortment of stem/progenitor cells, including haematopoietic stem cells (HSCs), mesenchymal stem cells (MSCs), endothelial progenitor cells (EPCs), and the newly identified BM-derived multipotent stem cells (BMSCs) [70, 71]. These subsets of BM-derived stem/progenitor cells are multipotent and have the capacity to trans-differentiate into multiple cell types, including cardiomyocytes.

Bittner et al. (1999), in their findings, demonstrated for the first time that in adult, immune-competent mdx mice suffering from an inherited muscular dystrophy, BM-derived cells are recruited by skeletal and cardiac muscle and undergo muscle specific differentiation. In their study, normal and dystrophic (mdx) female mice received bone marrow transplantation (BMT) from normal congenic male donor mice. Following histological analysis, in normal BMT recipients, no Y chromosome-containing myonuclei were detected, either in skeletal or in cardiac muscle. All samples from dystrophic mdx skeletal muscles expressed Y chromosome specific signals. The study concluded that circulating BM cells can be recruited to the heart and differentiated into cardiomyocytes [72].

Following this observation, multiple studies have been conducted in mice, rats and pigs demonstrating that transplantation of BM-derived cells were able to improve cardiac function and in some cases lead to newly differentiated cardiomyocytes in scar tissue [73]. Morrison et al. (2001) provides a comprehensive review of the developmental potential of the autologous stem cells, including the bone marrow stem cells following transplantation [74].

Recently, there have been documented facts stating that what was originally interpreted as transdifferentiation, seemed to be the result of cell fusion [75-77]. It is a widely accepted fact that cell fusion events do not generate new cardiomyocytes, and according to that analogy, it seems highly unlikely that new cardiomyocytes are being generated by cell fusion events. This has raised inevitable questions regarding the transdifferentiation capability of BM cells, and re-evaluation of the previous reports seems to be necessary.

Taken together, these clinical trials indicated that using bone marrow and satellite cells for transplants may have very limited effects when administered to the failing heart. At the same time, the effects clearly cannot be contributed to the fact that any cardiac regeneration has occurred.

The ideal candidates for myocardial regeneration should be the cells that are easy to obtain in sufficient number, capable of proliferation and differentiation into functional cardiomyocytes. Given the fact that neither skeletal myoblasts nor BM-derived cells, the two cell types currently used in clinical trials, meet the above demands the search for the best-fit candidate continues.

1.3.3 Pluripotent Stem Cells (PSCs) (ESCs/ iPSCs)

1.3.3.1 Derivation and maintenance of ESCs/iPSCs

ESCs

The cardiac potential of ESCs and iPSCs is indisputable, but their unique origin and pluripotency presents a new set of challenges [78]. ESCs are pluripotent cells derived from the inner cell mass of preimplantation-stage blastocyst. Murine ESCs were first isolated in 1981 [79, 80] and their human counterparts in 1998 [81].

The two major features of these ESCs are: first, they can be cultured indefinitely as a self-renewing population and second, that they have a pluripotent phenotype, meaning given the right conditions these cells have the ability to differentiate into cell types from all 3 primary germ layers *in vitro* [82] and *in vivo* [79-81]. ESCs have generally been cultured on top of a feeder layer of mitotically inactivated primary mouse embryonic fibroblasts (MEFs). The ES colonies have been observed to be smooth with rounded edges and refractile borders [4, 22].

Leukemia inhibitory factor (LIF) has been used as a supplement in the culture medium to maintain the murine ESCs in an undifferentiated state [83, 84]. LIF acts via the well-studied JAK–STAT3 pathway [85], and BMP-4 (present in FBS of the culture medium), which acts via the SMAD induction [86]. Self-renewal of mouse ESCs in feeder-free and serum free conditions is allowed by a combination of these two cytokines. Mouse ESCs are known to have characteristic cell surface markers, which include SSEA-1, LIF receptor (CD118) and alkaline phosphatase [4, 87]. The specific lineage commitment and germ line contribution of different ESC lines (D3, E14 and TT2 to name a few) are different as it is very much cell line dependent [88]. ESCs maintained in an undifferentiated state are diploid and can proliferate indefinitely without senescence possessing normal karyotype even after multiple passages. In the absence of conditions that maintain them in an undifferentiated state, ESCs will spontaneously differentiate to form multicellular aggregates known as EBs [4]. These EBs harbor all the three germ layers: ectoderm, mesoderm and endoderm. Consistent with their *in vitro* pluripotent nature, injection of mouse ESCs in syngeneic or immune-compromised animals can give rise to tumors containing derivatives of all three germ layers (teratomas) [18]. The ease of manipulation of mouse ESCs makes them an invaluable tool for gene targeting experiments where germline transmission is necessary.

Human ESCs differ from their mouse counterparts in several significant ways. The human cells are positive for surface markers like SSEA-3 and SSEA-4 and alkaline phosphatase. They do not express the mouse cell surface markers SSEA-1 and CD118 [87, 89, 90]. Also, reports showed that in contrast to mouse ESCs [91, 92] that do not contribute to the formation of extraembryonic placenta in chimeric animals, human ESCs are able to differentiate into trophoblast-like cells [93] in the presence of BMP-4 [94].

Furthermore, it was reported that human ESCs do not require LIF in order to proliferate or to maintain their pluripotent state [81, 95]. Taking into account the LIF studies, it was understood that human ESCs do not express the LIF receptor and only weakly express gp130, both of which are required for the activation of the JAK-STAT3 pathway by LIF [87].

Bendall et al. (2007) reported the role of bFGF in maintaining the pluripotent state of human ESCs in an indirect manner by inducing the production of insulin-like growth factor-1 (IGF-1) from human ESC fibroblast like cells, which are present in culture, along with the human ESCs. This secreted IGF then acts on human ESCs, helping in maintaining their pluripotent state [96].

Despite the obvious advantages of pluripotency and unlimited *in vitro* propagation capacity, *in vivo* use of ESCs in myocardial regeneration has lagged far behind that of autologous adult stem cells. This is due to the ethical/political issues surrounding the allogenic use of ESCs as well as concerns over immunological response and teratoma development. As a result of these caveats, so far only a few studies have been published on the application of ESCs in experimental myocardial regeneration. A short- and a long term improvement in ventricular contractility was observed either shortly after coronary ligation or after 8 weeks, in rat models of MI, when undifferentiated mouse ESCs were injected directly into the injured heart. Morphological and immunohistological examinations revealed differentiation of mouse ESCs transplanted into cardiomyocytes similar to host cardiomyocytes [97-99]. Surprisingly, no rejection response was observed although no immunosuppressive treatment was given in these studies, suggesting immune privilege of ESCs. Ménard et al. (2005) observed engraftment and differentiation of cardiac-committed mESCs in a large animal model of post-MI heart failure. When cardiac committed mouse ESCs were injected along the borders and in the centres of the infarcted area at 2 weeks after MI, regenerated myocardium and improved cardiac function were observed in both immunocompetent and immunosuppressed sheep [100]. Besides mouse ESCs, human ESCs have also been investigated recently regarding their *in vivo* myocardium-regenerating capacity. When injected into the uninjured left ventricular wall of athymic rats, differentiated cardiac-enriched human ESC progeny were found to form human myocardium in the rat heart [39, 40].

Contrary to the belief that human ESCs will not enter clinical trials in the foreseeable future due to existing ethical and practical hurdles, the generation of induced pluripotent stem cells (iPSCs), which are ES-like cells generated by forced expression of pluripotent factors, in 2006 by Yamanaka and colleagues has revolutionized the field of ES biology [19].

iPSC technology has been propelled to the forefront of experimental regenerative medicine due to the multiple benefit it carries. Being derived from adult cells, ethical issues are bypassed in using iPSCs. These cells can thus be used to cure human diseases, as immunocompatibility is not an issue because the starting material, skin fibroblasts, can be obtained from the patients themselves.

iPSCs

One of the major biological advances of the 21st century is the reprogramming of somatic cells to a pluripotent state by adding a mixture of a few transcription factors. The finding that these iPSCs can give rise to every cell type of the body triggered an explosion of interest in using patient-specific iPSCs and their differentiated derivatives to model simple and complex clinical presentations of disease. This technique pioneered by Yamanaka and colleagues relies on the reprogramming of fully differentiated cells to ESC-like cells known as iPSCs. These were first established by the retrovirus-mediated transduction of four transcription factors (c-Myc, Oct3/4, Sox2, and Klf4) into mouse fibroblasts [19]. These reprogrammed cells, which were selected based on the expression of beta-geo cassette (a fusion of the beta-galactosidase and neomycin resistance genes), failed to contribute to the adult chimeras. Further selection based on the expression of pluripotency factors, Nanog or Oct3/4, successfully contributed to adult chimeras and also showed germline transmission [101-103].

The human iPSCs were established by the transduction of the same set of transcription factors (c-Myc, Oct3/4, Sox2, Klf4) or another set of transcription factors (Oct3/4, SOX2, Nanog, Lin28) into human fibroblasts [20, 104]. These human iPSCs are similar to their ES counterpart in morphology, gene expression and the epigenetic status of pluripotent cell-specific genes. Furthermore, they even differentiated into the cell types of the three germ layers *in vitro* and *in vivo* [20, 104].

In spite of the overwhelming success in generating iPSCs, however, there are currently caveats with the iPSC reprogramming procedure that need to be addressed before this elegant technology can be put to clinical use. One important aspect is that the original protocol for reprogramming of mouse/human ESCs to iPSCs relies on the use of viruses integrating into the genome of cells undergoing the reprogramming process. Even though recent work utilizing non-integrative approaches - transposon [105, 106], episomal [107], direct protein delivery [108] - has been devised, still their similarity and clinical efficacy to the retroviral generated iPSCs needs to be evaluated. Furthermore, critically assessment of the long-term performance of differentiated cells derived from iPSCs needs to be carried out. A detailed comparison between cardiomyocytes derived from iPSCs and cardiomyocytes derived from ESCs is mandatory before iPSCs can be considered for regenerative therapy. Although still in its infancy, it appears that cellular reprogramming may provide important tools for translational scientists to generate specific cell type for cell therapy.

Thus, by far, ESCs and iPSCs seem to be the best-fit candidates as they can provide an indefinite source of cells that can be differentiated to produce functional cardiac phenotypes and fulfill the numeric need of therapeutically relevant cells needed for transplantation.

1.3.3.2 PSCs as a robust source of functional cardiomyocytes

The ability of different multipotent stem cell types to differentiate into functional cardiomyocytes has been assessed *in vitro.* The methodologies applied to generate these cardiomyocytes have either been by using different cytokines or growth factors or by coculturing with neonatal cardiomyocytes [4, 109, 110]. However, although these protocols generate cardiomyocytes that demonstrate few cardiac markers, these 'cardiac-like' cells typically have poor sarcomeric organization and lack the ability to contract [109, 110].

Adult stem cells are unable to generate large numbers of cardiomyocytes. The use of ESCs or their more recently developed 'man-made' counterpart, iPSCs do not possess this limitation as they can be cultured indefinitely and that they retain their capacity to differentiate into almost all cell types. They thus, could prove to be a continuous source of generating cardiomyocytes. Furthermore, PSCs such as ESCs and iPSCs are known to differentiate into functional cardiomyocytes [21, 39, 111-113]. An interesting study by Ieda et al. (2010) has reported for the first time a "master- regulator" (comprising three developmental transcription factors Gata4, Mef2C, Tbx5) for directly reprogramming postnatal cardiac or dermal fibroblasts into differentiated cardiomyocyte-like cells [114]. Efficient differentiation protocols were derived for ESCs that were applied directly to iPSCs, and ranged from the use of EBs to guided differentiation systems **(section 1.5)** [4] . The differentiation system that governs the generation of cardiomyocytes from PSCs is very well characterized. Early cardiac transcription factors such as Nkx2.5 is expressed along with the sarcomeric proteins and calcium-handling proteins [78]. The functional properties of these PSC derived cardiomyocytes are similar to those reported for cardiomyocytes in the developing heart and hence show similar excitation and contraction couplings [36, 113, 115, 116]. The detailed view as to how the cardiomyocytes arise from ESCs is shown in **Figure 2**.

At least in theory, existing differentiation protocols can be scaled up through the use of highly efficient differentiation approaches **(section 1.5.2)**, as well as bioreactor technology **(section 1.8)**, so that the numeric demand of therapeutically relevant cardiomyocytes could be met for cell replacement therapy after MI [39, 117-119].

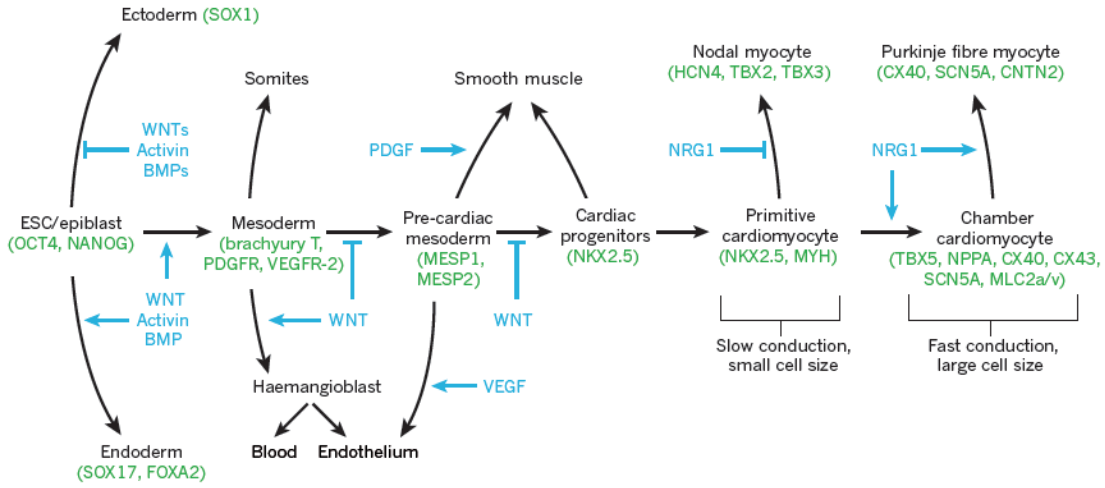


Figure 2 Cardiovascular Lineages during embryonic development and ESC differentiation .

Cardiac differentiation from ESCs closely mimics cardiac development in the embryo. the cardiovascular lineages involves a transition through a sequence of increasingly restricted progenitor cells, proceeding from a pluripotent state to mesoderm and then to cells committed to cardiovascular fates. Growth factors that regulate fate choices are listed at branch points (blue), and key transcription factors and surface markers for each cell state are listed under the cell types (green). The growth factors are useful for directing the differentiation of ESCs, whereas the markers are useful for purifying cells at defined developmental states. Although the field has made considerable progress towards determining the early events of cardiogenesis, a better understanding of how pacemaker and chamber-specific cardiac subtypes are formed is required for clinical applications. BMPs, bone morphogenetic proteins; CNTN2, contactin-2; CX, connexin; FOXA2, forkhead box protein A2; HCN4, potassium/sodium hyperpolarization-activated cyclic nucleotide-gated channel 4; MESP, mesoderm posterior protein; MLC2a/v, myosin light chain 2a and/or 2v; MYH, myosin heavy chain; NPPA, natriuretic peptide precursor A; NRG1, neuregulin 1; PDGF, platelet-derived growth factor; PDGFR, PDGF receptor; SCN5A, sodium channel protein type 5 subunit α; SOX, SRY-related high-mobility-group box; TBX, T-box transcription factor; VEGF, vascular endothelial growth factor; VEGFR-2, VEGF receptor-2 [78].

1.4 *In vivo* to *in vitro* lesson

Cardiac development is a very complex process that involves the sequential expression of multiple signal transduction proteins and transcription factors, working in harmony with each other. As explained in **section 1.2**, a number of signaling pathways and growth factors involving the BMPs, Wnts, Activins and FGFs have been employed to understand the developmental progression of cardiac cells [120-123]. Also, it is important to understand that these factors work in favor of cardiogenesis only during a certain time frame whereas elsewise it might act in an antagonist way. Thus, it seems imperative to carefully optimize the timing of the addition of these factors to guide the PSC differentiation towards the desired cardiac lineage.

By now, it is clear that the progression of the lateral plate mesoderm gives rise to the cells of the cardiovascular lineage, and that these cells migrate to form the primary and secondary

heart fields [124]. In a similar fashion, cardiac differentiation *in vitro* from PSCs proceeds in highly distinct stages with Brachyury and Mesp1 (mesodermal markers) being transiently up regulated at around day 5 and Gata-4, Nkx2.5 being expressed immediately after at around day 6 [125-127]. At this stage, the cells are still bi-potential, meaning that they can still differentiate towards the vascular (smooth and endothelial) lineages apart from the cardiac lineage. Once beyond this stage, i.e., from day 8 onwards, mature cardiac muscle markers start expressing, such as α- and β-MHC, MLC2a, MLC2v; cardiac α-actinin, cTnT, coincident with the observation of spontaneous cell contractions [125].

Based on our understanding of the above literature, it would be very interesting to pattern the different stages of cardiac differentiation. According to Rajala et al. (2011), the first step (mesoderm induction) in cardiac differentiation is very well characterized. It has been clearly demonstrated that Wnts, BMPs, and TGF β-family member Nodal (or Activin-A as a substitute of Nodal) plays a major role in the induction of mesoderm [128, 129]. Mechanisms guiding the patterning to cardiogenic mesoderm and the formation of cardiac mesoderm, the next two steps in cardiac differentiation, have been very well characterized in the animal and the chick embryos, but how these mechanisms apply to the PSC cardiogenesis is still in its infancy. It has been demonstrated in Xenopus and chick embryos that the inhibition of Nodal and Wnt signaling is mandatory for the generation of cardiomyocytes [57, 130-133]. An important Wnt antagonist Dkk-1 is deployed, to inhibit the Wnt signaling, during the differentiation protocol at this stage [118]. Furthermore, the transmembrane receptor “Notch” induces the expression of a combination of growth factors Wnt5a, BMP6 which increase the amount of cardiac progenitors from an PSC-derived mesoderm subpopulation [134]. The last step in cardiac differentiation involves the differentiation of the cardiac progenitors to generate beating cardiomyocytes. The beating process, even though not fully understood, is generally known to occur spontaneously *in vitro,* and is controlled through Wnt11 signaling [133].

Taking this patterning together, it seems obligatory that more research needs to be focused into understanding the two critical steps in cardiogenesis from PSCs; that is, promotion of mesendoderm to form committed cardiac mesoderm and factors that give rise to cardiomyocytes.

1.5 Cardiac differentiation of pluripotent stem cells

Figure 3 [28] summarize the differentiation approaches currently used for cardiomyocyte differentiation from PSCs. Given the scope of this thesis, spontaneous and directed cardomyocyte differentiation strategies will be dealt with in detail ahead.

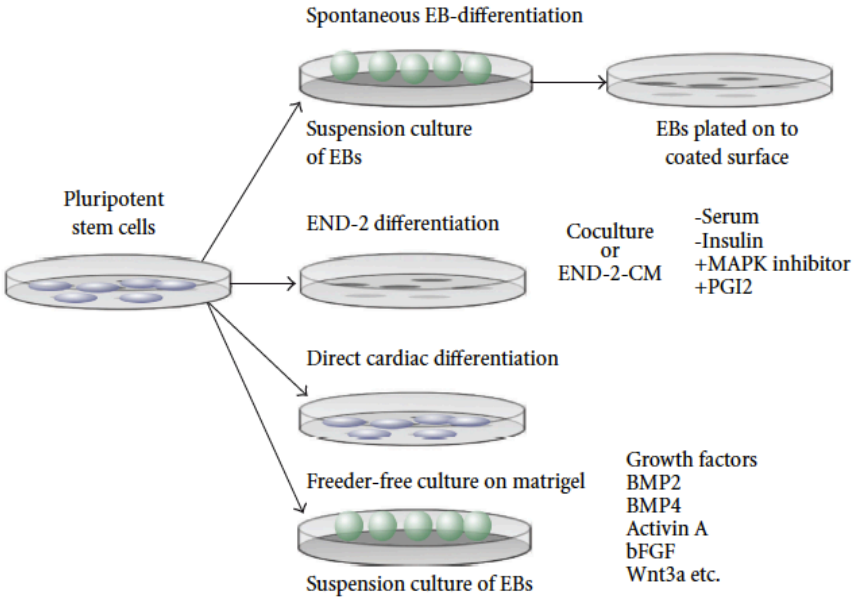


Figure 3 Schematic Overview outlining differentiation approaches currently used for cardiomyocyte differentiation from PSCs.

The embryoid body approach has, thus far, been the most utilized way to obtain beating cardiomyocytes from pluripotent stem cells and the formation of three-dimensional cell aggregates initiates and facilitates the differentiation process. Generally, cells are transferred to suspension cultures, or in order to obtain more stable and reproducible cell aggregates, the embryoid bodies are formed using the hanging-drop or the forced aggregation method. Using the END-2 approach, cardiomyocyte differentiation is triggered either by coculture of pluripotent stem cells, with END-2 cells or by embryoid body formation in suspension culture using END-2 conditioned medium. The depletion of serum and insulin has been shown to facilitate cardiogenesis in this approach, and inhibiting p38MAPK pathway by a specific inhibitor or by adding prostaglandin I2 can further enhance it. In the guided differentiation approach, undifferentiated pluripotent stem cells are cultured under feeder cell-free conditions or in suspension culture after embryoid body formation. Cardiac differentiation is induced with various growth factors, such as BMP2, BMP4, Activin A, bFGF, and Wnt3a [28].

1.5.1 Embryoid body formation and spontaneous cardiomyocyte differentiation

The first report of murine differentiation of ESCs into cardiomyocytes *in vitro* came in 1985. [135]. *In vitro* differentiation of ESCs requires an initial aggregation step (in this case hanging drops (HD)) to form 3D structures called EBs. Spontaneous development of the derivatives of the three germ layers (ectoderm, endoderm and mesoderm) occurs within the EB. Hence, within these mixed populations of cells, contracting areas with functional properties of cardiomyocytes can be detected (**Figure 4**). Various parameters like starting number of cells

in EBs, type of media and fetal bovine serum (FBS) used for culturing, addition of growth factors, type of PSC line and time of EB plating etc. affect the differentiation potency of PSCs to form cardiomycytes [136]. The cardiomyocytes are generally located between an epithelial layer and a basal layer of mesenchymal cells [137]. These cardiomyocytes within 1 to 4 days after EB plating start to appear as spontaneously contracting clusters. Upon further differentiation increase in the rate of contraction can be observed in the beating cluster, which decreases as the developing cardiac cells reach the mature stages [138]. The presence of these spontaneous contractions continues from several days to more than a month. Mature cardiomyocytes can stop contracting in culture but can be maintained as adult cells for many weeks [136, 137].

Further studying the electrophysiological measurements, the heterogeneous population of cardiomyocytes shows a transition from early stage cardiomyocytes with pacemaker activity to terminally differentiated atrial-/ ventricular-like cells. The low myofibrillar content of the small rounded immature early cells becomes organized in mature elongated cardiomyocytes [137]. Thus, the cardiomyocytes derived from EBs can range from; early, small and round cells (no or rarely developed sarcomeres) to pacemaker like-cells (rare myofibrillar content) and further to Purkinje-like cells (with oval nucleus, well organized tension-orientated myofibrils) spanning the long axis of the cells [137]. It is evident from the above description that as the ES cell derived cardiomyocytes mature, they become elongated with well-developed myofibrils and sarcomeres [139]. Terminal differentiation to cardiomyocytes will have densely packed well-organized bundles of myofibrils and the sarcomeres have clearly defined A bands, I bands, and Z disks [139, 140]. Also, nascent intercalated discs, gap junctions and desmosomes can be observed [135, 139, 141, 142]. Overall, given the structure, shape and myofibrillar organization of the PSC derived cardiomyocytes, it can be concluded that they are very similar to the neonatal cardiomyocytes obtained from the rodent hearts.

The gene expression profile of various lineage and cardiac markers over the whole course of differentiation protocol follows the same pathway as seen *in vivo* **(refer Figure 2)**.

The methodology of spontaneous differentiation of PSCs to generate functional cardiomyocytes is highly cell line dependent [36]. Nearly all cell lines have been known to form contracting outgrowths in the range of 5%–15% in the plated EBs [143]. Various growth factors have been known to increase the efficacy of the protocols like by adding BMP4 during d0–d4 [144] or by the addition of Wnt3A during d0–d2 [145]. Factors such as Wnt3A [146] and L-ascorbic acid [147] can improve cardiac differentiation by enhancing cardiac progenitor cell proliferation **(section 1.5.2)**.

Thus, a highly controlled culture methodology is presented by EB formation in which the generation of cardiac lineages follows a highly discrete pattern as found during *in vivo* cardiac development. This is also based on the fact that the molecular and protein expressions profiles, myofibrillar organization and the ultrastructure of the EB derived cardiomyocytes resemble the adult counterparts. Furthermore, the ease of spontaneous differentiation strategy has provided investigators with an immensely simple and valuable technique to understand cardiac developmental patterns.

Even though the differentiation to cardiomyocytes via EB methodology remains the most traditional of all the technologies until now, it is still one of the most convenient and economical technologies. Recent works on manipulating PSCs with reporter genes/ constructs have made the purification and isolation of ES cell derived cardiomyocytes from EBs much easier **(section 1.7)**.

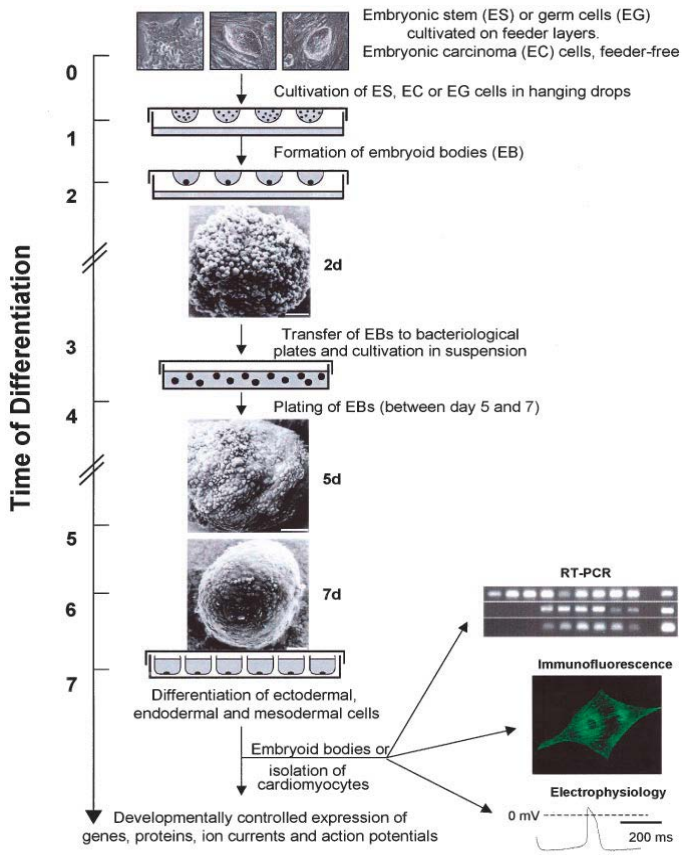


Figure 4 EC cell-derived cardiogenic differentiation (adapted from Wobus et al, 2002)

When allowed to form aggregates, EC, ES and EG spontaneously form EBs harbouring cells of all the three germ layers (ectoderm, endoderm and mesoderm). Undifferentiated ES cells cultivated on feeder cells of primary cultures of mouse embryonic fibroblasts were cultivated as embryoid bodies (EBs) in hanging drops for 2 days and in suspension for another 3 to 5 days before plating onto gelatin (0.1%)-coated tissue culture plates. The morphology of the 2-, 5-, and 7-day-old EBs is shown by scanning electron microscopy (bar = 50 mm) [136].

1.5.2 Directed cardiomyocyte differentiation

As mentioned in section 1.5.1, addition of specific growth factors such as BMPs, Wnts and FGFs has led to the development of the directed differentiation strategies, recapitulating the process of cardiogenesis in cell culture. Combination of BMP4, Wnt3a and Activin A has shown to induce gastrulation-like events and meso-/endoderm development in PSCs [41, 42, 148]. The anterior endoderm's cardioinductive activity is activated by exposure to superfamily of growth factors like the transforming growth factor – β (TGF β) and then the guided differentiation using BMP4 and Activin-A is further carried out. In this kind of methodology, PSCs are allowed to culture in medium supplemented with factors in a feeder free system to allow the induction of cardiomyocyte differentiation [28, 149] and eventually, allowed to culture in normal medium with factors removed.

In one of the protocols devised by the Keller lab, suspension EBs were formed by sequential addition of BMP4, FGF2, and Activin A (day1 and day4), DKK1 (day4 to day8) and FGF2 from day 8 onward. Maintaining these EBs in hypoxic (5% oxygen) conditions for 12 days produced around 70% of contracting EBs [118]. Also, Elliot and colleagues [150] have demonstrated a similarly high differentiation efficiency by employing a forced aggregation protocol that used BMP4, Activin A, Wnt3a in the culture medium during the first three days. Another study by Yang et al, 2008 demonstrated the induction of the cardiac mesoderm Activin-A, BMP-4, bFGF, and VEGF followed by cardiac specification by Wnt inhibhitor, Dkk-1. This protocol has been described to produce populations consisting of >40%–50% cardiomyocytes [118].

Many chemicals provide methodologies to understand the signaling mechanisms that promote cardiogenesis, therefore intense molecular screening technology has been exploited to search these compounds. Few studies have been published that have identified novel small molecules using these technologies that appear to stimulate the generation of cardiomyocytes from PSCs. Some of these molecules like cardiogenols, ascorbic acid, DMSO etc. that were discovered, had ability to upregulate late stage-markers for cardiogenesis [28]. The mechanisms through which these factors work in promoting cardiogenesis, which in turn activate cardiac development and other cell populations, still needs to be addressed.

Collectively, these studies demonstrate that exposing ESCs and iPSCs to various growth factors, at specific times and in specific doses, is essential for directing differentiation from early mesoendoderm via mesoderm towards a more specific cardiac fate.

1.6 Characteristics of pluripotent stem cell-derived cardiomyocytes

A number of studies have been published that describe the basic characteristics of PSC derived cardiomyocytes. In these reports, the analysis is based on specific molecular, structural and functional characteristics of the generated cardiomyocytes.

1.6.1 Molecular analysis of PSC derived cardiomyocytes

Once PSCs to cardiomyocytes differentiation initiates, the cells express several markers on a molecular level at the various initiation and intermediate stages of differentiation. Expression of early cardiac-specific transcription factors involved in cardiogenesis - such as Flk1, Gata-4, and Nkx2.5 are generally the first ones to up regulate [36, 151-153]. They are the indicators of the formation of the mesoderm, pre-cardiac mesoderm and eventually the cardiac progenitors [78]. Structural proteins like α -actinin, cTnT, MLC-2a and MLC-2v along with the gap junction proteins like desmin and tropomyosin [27, 38, 113] are also expressed. The positive expression of these factors indicate the formation of the primitive and mature cardiomyocytes [78]. Other cardiac and muscle-specific protein like atrial natriuretic peptide (ANP) was upregulated in the atrial and ventricular cardiomyocytes in the developing heart along with creatine kinase-MB and myoglobin [21, 28, 38]. The troponin complex that regulates the muscle contraction, happening in response to the alterations in intracellular calcium ion concentrations, is located on the thin filament of striated muscles [28]. Understanding the various molecular markers up and down regulated throughout the course of cardiac differentiation will provide tremendous cues in understanding the methodology and give answers to increase the efficiency of cardiomyocyte production.

1.6.2 Electrophysiological characteristics of PSC derived cardiomyocytes

The ES derived EB strategy allows, for the first time, the study *in vitro* of all subsequent stages of AP development in developing cardiomyocytes. Cardiac cells that are differentiated from murine PSCs can be classified as pacemaker, atrial, ventricular, nodal, His, Purkinje-like cells based on their AP [154]. Meanwhile, the cardiomyocytes of an early differentiation stage mostly reveal pacemaker-like AP, terminal differentiation stage cardiomyocytes reveal three major types of AP [155, 156]. A stable resting potential of -75mV and AP of high amplitude and upstroke velocity are observed in the cells depicting atrial and ventricular phenotypes (elongated cells with orientated myofibrils). Atrial AP differs from the ventricular AP by a less pronounced plateau and by an acetylcholine-induced hyperpolarization [157]. Another

type of AP, found in ES derived cardiomyocytes (small, roundish cells with rare and disorientated myofibrils), shows all the characteristics of sinus nodal pacemaker cells, including the typical shape and the hormonal regulation [158]. Functional expression of adrenoceptors, cholinoceptors and L-type Ca^{2+} channels were demonstrated upon chronotropic measurements [111].

1.6.3 Functional heterogeneity of PSC derived cardiomyocytes

Given the fact that cardiomyogenesis in ES cultures is a fairly random process, it is very likely that EBs at a given culture stage would contain cardiomyocytes from different stages of development. The most prominent current component found throughout the entire differentiation period of ESC derived cardiomyocytes is the voltage-dependent $\boldsymbol{I_{ca}}$. The whole-cell ***I*** exhibits all major biophysical properties and pharmacological characteristics inherent to the L-type Ca^{2+} current of the heart [157, 159, 160]. In some cardiomyocytes of intermediate and late stage, T type Ca^{2+} currents with negative threshold of activation, insensitivity to dihydropiridines and complete block by 50 μM Ni^{2+} were detected. Rise in the levels of the intracellular calcium in PSC-derived cardiomyocytes is known to be the trigger for contraction/beating of the cells [27, 115]. Studies have shown a difference in the regulation of intracellular calcium handling between PSC derived cardiomyocytes and mature adult cardiomyocytes. It is known that the PSC-derived cardiomyocytes have immature sarcoplasmic reticulum, due to the de-regulated expression of phospholamban and calsequestrin, the two main intracellular calcium handling proteins [115, 161].

Furthermore, these cardiomyocytes have been known to respond to β-adrenergic and muscarinic agonists, suggesting that the phenotypes of these cardiomyocytes are reminiscent of mouse heart cardiomyocytes at E8.75-9, where the heart chamber formation has just started [154]. The reason that these cells respond to alpha/beta-adrenergic- and muscarinic stimuli, has been attributed to the various studies wherein it has been demonstrated that drugs such as isoproterenol and carbachol could affect the contractile activity of the PSC derived cardiomyocytes.

Taking together the understanding of the molecular gene expressions, structural and electrophysiological properties of the PSC derived cardiomyocytes, it can be assessed that these cardiomyocytes possess immature phenotype and appear to have an immature sarcoplasmic reticulum function [28]. Even though the mature cells of the adult myocardium and the PSC derived cardiomyocytes differ in some functional properties, still, the generation

of *in vitro* cardiomyocytes set a basal line of functionality to be used as a model system for cardiac studies.

1.7 Enrichment strategies of pluripotent stem cell-derived cardiomyocytes

Despite the tremendous progress in improving the cardiac differentiation protocols, none of the currently available protocols results in homogenous populations of cardiomyocytes. One of the biggest challenges over the last few years has been to develop robust isolation techniques that allow scalable purification of cardiomyocytes and specific cardiac cells. Spontaneously contracting cardiac cells have been used for the purification of cardiomyocytes using the traditional methods that involve mechanical isolation based on manual dissection. Even the protocols generating low quality cardiac cell populations contain up to 70% cardiomyocytes [27, 36, 37]. Another fairly exploited approach presenting significant improvements to the enrichment process is the Percoll density gradient centrifugation where the cardiomyocytes are separated based on their buoyant properties. This method can be applied to enzymatically-dispersed cells from EBs and guided differentiation protocols, and results in approximately three to seven fold enrichment [38, 40, 41, 149]. A non-genetic method that has been generally employed to detect cardiomyocytes is based on the use of the mitochondrial dye tetramethylrhodamine methyl ester perchlorate (TMRM) [162]. This dye functions only in cardiomyocytes with high mitochondrial density, and it does not detect the most immature cells that develop in the cell cultures [163].

Cell surface markers and their respective antibodies have the advantage that they need not be genetically modified and can be used or the enrichment purposes. Based on the expression of receptor tyrosine kinases KDR (Flk1/ VEGFR2) and PDGFRA, FACS has the ability to sort multiple surface markers simultaneously making the possibility of using this technology to isolate cardiac progenitors [164]. To date, the highest level of cardiac purity has been obtained using the genetic selection techniques. This strategy utilizes the ability of the ESCs to be easily amenable to genetic manipulation and mutation/insertion events can be easily studied in EBs or in isolated cells post differentiation [125, 165]. ESCs are generally modified to carry reporter genes (like EGFP, mCherry) or mammalian selection gene (like antibiotic selection) under the transcriptional control of cardiac specific promoter. Furthermore, when these transgenic cells are allowed to differentiate and be selected based on the activation of the cardiac specific promoter one can obtain pure cardiomyocytes. Various studies have been carried out with genetic selection based on the expression of a selectable marker driven by cardiac specific promoter such as Nkx2.5 [150], Myh6 [166], MLC2V [167] and Isl1 [146].

Thus, cardiac cells can be selected with a very high purity rate when transgenic strategies are carried out in parallel to using antibiotic selection [46]. To sum up, most of the recently published cardiomyocyte enrichment reports suggest the advantage of using the transgenic strategy based on cardiac specific drug resistance selection, either alone or in combination with the reporter gene approach.

The recent finding of Transcription Activator like Effector like Nucleases (TALENs) and Clustered Regularly InterSpaced Short Palindromic Repeats (CRISPR) will help us to take this strategy one step further, as they have developed into a major technology for targeted genome editing. Primarily, zinc-finger nucleases (ZFNs) have be used to induce a double-strand break (DSB) precisely at a pre-determined position in the genome [168]. The break once generated activates the cells repair mechanism to heal the break via one of the two highly conserved repair pathways: non-homologous end joining (NHEJ) to drive targeted gene disruption or through the homologous recombination (HR) /homology-directed DNA repair pathway using an exogenous donor plasmid as a template. The donor design holds tremendous importance as the repair reaction can be used to generate gene deletion (knockout), gene disruptions, DNA addition (knock in) [168] and even single-nucleotide changes [169].

1.7.1 TALENs

TALENs organisational structure is very similar to that of ZFNs. The DNA-binding domain in case of TALENs comes from TAL effector proteins and the transcription factors from plant pathogenic bacteria [170]. TALENs are simply the proteins from the bacteria that are injected into infected plant cells via a type III secretion system by the pathogens of the genus *Xanthomonas* [171, 172]. This bacterial protein comprises the N-terminal translocation domain, a central repeat domain and a C-terminal transcriptional activation domain. Once inside the cell, these proteins have direct recognition ability for their specific DNA targets in the host genome. Upon successful insertion the expression of genes necessary for pathogen multiplication and spreading are activated [173].

The TALE has a DNA-binding domain, which is basically a repeat of an array of amino acid repeats, each about 34 residues long. The repeats are very similar to each other; only differing at two positions (amino acids 12 and 13, called the repeat variable diresidue, or RVD). Each RVD has a preferential binding to one of the four possible nucleotides, meaning that each TALE repeat binds to a single base pair [170] **(Figure 5)**. The specificity of a particular

repeat to a single nucleotide is established by this RVD, hence establishing a simple 1:1 code for protein-to-DNA interaction (i.e., 1 repeat unit to 1 nucleotide) [174]. The DNA binding domain's modular nature is unique throughout the kingdoms of life; thus, RVDs with different specificities can be assembled into arrays using methods such as the 'Golden Gate' cloning strategy, in order to target a user-defined DNA sequence [175-178]. Thus, since it was established that it is very simple to generate customised TALE arrays, this feature was combined with the ability of an endonuclease domain called Fok1 (at the C-terminal end); in order to create TALE based nucleases (TALENs). Dimeric form of Fok1 is the only active from of this endonuclease [179] hence, TALENs are composed of active pairs in which two monomers of Fok1 bind adjacent to the target sub-sites separated by a DNA spacer allowing for the formation of an active dimer to cleave the target locus [180]. Once the DSB has been made by TALENs, either NHEJ or HR gets activated as mentioned earlier in this section. The HR based repair uses the homologous sequence as a template to faithfully reconstitute the DSB. In eukaryotic cells, the NHEJ pathway is generally preferred over HR, but the frequency of the latter can be substantially increased when transferring high amounts of a homologous template sequence to the cell [181].

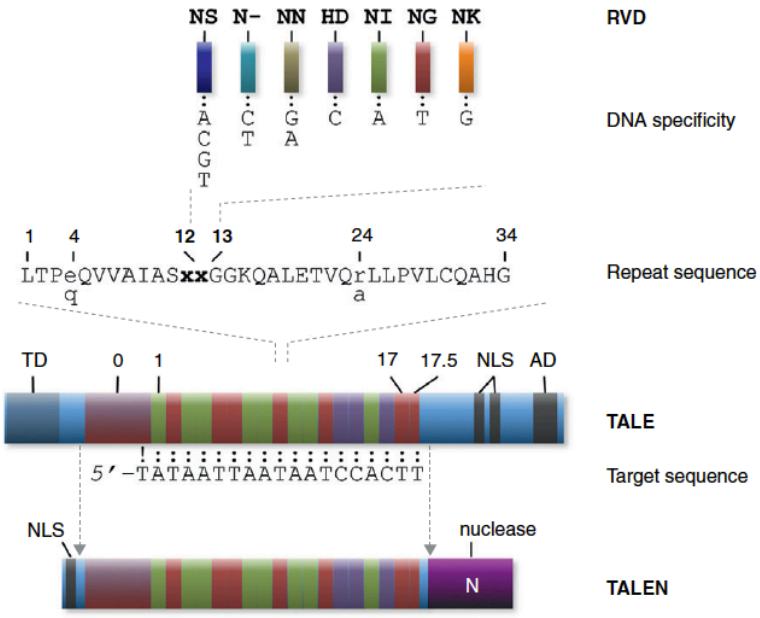


Figure 5 DNA recognition code of transcription activator-like effectors (Mussolino and Cathomen)

A prototypical transcription activator-like effector (TALE) consists of an N-terminal translocation domain (TD), the central repeat units (0–17.5) that mediate DNA binding and a C-terminal region encompassing nuclear localization signals (NLS) and a transcriptional activation domain (AD). The central TALE repeat units can be fused to a nuclease domain to generate a sequence-specific TALE-nuclease (TALEN) [174].

Even though the DNA binding of TAL effector is not very well understood than that of ZFNs, their seemingly simpler code could prove very beneficial for engineered-nuclease design. As explained earlier, TALENs cleave as dimers, target sequences are long (the shortest reported so far binds 13 nucleotides per monomer) [170], and appear to have less stringent requirements than ZFNs for the length of the spacer between binding sites.

TALENs will permit the generation of transgenic cell lines with two major advantages over standard genetic manipulation techniques: minimal integration site-dependent effects on the transgene and secondly, no change in the functional set up of the host-cells transcriptional machinery [182]. In transgenic/regenerative biology, TALENs will aid in generating reporter cell lines that will help in the establishment of protocols for cardiac gene therapy. This would allow the production of highly enriched and pure population of transplantable stem cells that would make the realisation of cellular therapy to become a clinical success.

1.7.2 CRISPR/Cas9 system

Manipulating the DNA or RNA for human disease prevention or treatment is the most important component of cell therapy. This involves rectifying, replacing or deleting the target genes in genetic diseases, thus producing disabling mutations to combat infectious diseases or inducing therapeutic mutations. Recently, a new RNA guided genome editing tool CRISPR-Cas9 technology was added to the list of programmable nucleases, holding huge therapeutic potential. This technology was first introduced into the mammalian organisms in 2013 and since then has proven to be highly advantageous over its existing counterparts in being an easy to use, simple in design, multiplexing and a cost effective tool [183-185]. A DSB is generated by this nuclease at a specific target site in the genome locus which activates the repair pathways of the cell either by error prone NHEJ or HDR. In the absence of a template, NHEJ is activated, resulting in insertions and/or deletions (indels) that disrupt the target loci. In the presence of a donor template with homology to the targeted locus, the HDR pathway operates, allowing for precise mutations to be made.

The CRISPR/Cas9 system was first described as an adaptive immune system in bacteria and archeaeons but now has been modified as RNA-guided endonucleases for genome editing. As shown in the **Figure 6**, the core component Cas9 comprises of the two catalytic active domains RuvC and HNH along with the single guide RNA (sgRNA) that is derived from CRISPR RNA (cr RNA) and the trans acting CRISPR RNA [186].

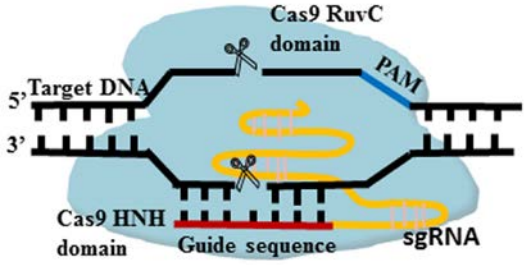


Figure 6 Mechanism of CRISPR/Cas9 genome editing

Site-specific DNA cleavage by nuclease Cas9 directed by complementary between a single guide RNA (sgRNA) and the target sequence on the presence of a PAM on the opposite strand. sgRNA derives from crRNA and tracrRNA. Wild-type Cas9 possesses two catalytically active domains termed HNH and RuvC, each of which cleaves a DNA strand [187].

sgRNA is able to guide the Cas9 to its target site by the presence of a protospacer-adjacent motif (PAM) on the opposite strand, by RNA-DNA base pairing, resulting in the Cas9-generated site specific DNA DSBs. These breaks are eventually repaired by HDR if the homologous sequence is available (leading to precise gene correction or replacement) or elsewise by NHEJ (leading to small insert or indel mutations) [184, 185]. Furthermore, Cas9 can thus be re-directed towards almost any target of interest in the immediate vicinity of the PAM sequence by altering the guide sequence within the sgRNA. This ease of implementation and multiplexing ability has been exploited further to generate engineered eukaryotic cells. A couple of very interesting studies have been carried out where direct injection of the sgRNA and mRNA encoding Cas9 into the embryos was carried out [188, 189]. These studies enabled the rapid generation of transgenic mice with multiple modified alleles and the results hold enormous profit for editing organisms that are otherwise genetically difficult to manipulate.

Cas9 system holds several potential advantages over the conventional genome editing tools like the ZFNs and TALENs. The first and foremost is the ease of customization referring to the fact that in order to target multiple sites using the Cas9 system, it requires only to design a complementary sgRNA whereas the nuclease Cas9 remains the same in all cases. This is much simpler than generating bulky guiding proteins in ZFN and TALEN based tools. The second very important advantage of the CRISPR/Cas9 system is multiplexing where multiple target sites can be edited in parallel using multiple sgRNA [185, 186].

Despite holding multiple advantages, this system does suffer a few drawbacks when compared to TALENs. The first one being that the target sites of CRISPR/Cas9 is restricted by the presence of PAM sequence and a guanine at the 5' end 11, whereas the only restriction of TALEN targets is presence of thymine at the 5'end [190], which means that TALENs are capable of targeting more genome sites than the CRISPR/Cas9 system. Second, a very important benefit of TALENs is the production of low number of off-target effects in comparison to the CRISPR/Cas9 system [191, 192], due to the heterodimeric nature of the Fok1 nuclease in TALENs. Further, TALENs can be re-engineered to target and cleave mitochondrial DNA in patient-derived cells [193], whereas this area has not been explored in the case of CRISPR/Cas9 system.

CRISPR/Cas9 is a new and a versatile tool for various genome modifications and it holds a great promise in cell therapy, but given the fact that this technology still being standardized and optimized, using TALENs for manipulating the ESCs /iPSCs remain the undisputed choice for this thesis.

1.8 *In vitro* expansion strategies

Cell transplantation therapy has attracted increasing attention as an alternative to organ transplantations [194]. A whole plethora of future biomedical applications has been opened up by the recent stem cell and tissue engineering based technologies. Nevertheless, to realise the full therapeutic potential of PSCs it will be required that their large-scale generation be carried out in a robust system without any limitation [195, 196]. This calls for the need of *in vitro* expansion of stem cells to be used for cell therapy prior to their commitment into tissue/lineage specific directions.

PSCs (both ESCs/iPSCs) being pluripotent have the capacity of unlimited self-renewal and are promising cell donor sources to provide a sufficient number of cells for cell-based therapy. However, the expansion and differentiation of these cells is still under question as being complex procedures it gets difficult to manage their scale up production systems [24, 197]. In order to solve these issues, the bioreactor culture system offers attractive advantages of ready scalability and relative simplicity [198, 199]. The potential of bioreactors to address these issues is demonstrated by their capacity to support robust and well-defined scale-up platforms for expansion of PSCs [200], EB formation [201, 202], as well as differentiation [203]. A huge number of studies have been carried out recently in the area of bioreactors involved in the large-scale production of PSC-derived cells. A bioreactor is a device in which

biological processes such as cell expansion, differentiation etc. are carried out in an immensely controlled environment *in vitro*, that includes exchange of oxygen, nutrients and metabolites [204].

At the present time, EB formation in hydrodynamic conditions has been achieved by (1) spinner flasks; (2) rotating cell culture system (RCCS); (3) rotary orbital culture; and (4) complex methods combining these techniques, but all these technologies suffer from a major disadvantage of generating shear forces during the culture process thus damaging the cells [205]. The selection of bioreactors for stem cell expansion and differentiation beyond the bench scale is reliant on whether the cells are adherent, suspension as single cells or aggregate to form EBs [206]. Therefore, it becomes imperative that the design of the bioreactor is according to the applications.

1.8.1 Bioreactors

With the recent realization of the potential of stem cells, robust techniques for their large scale *in vitro* expansion will be required [195]. This highlights the requirement for the *in vitro* expansion of stem cells used for therapy prior to their commitment into tissue-specific applications. The theory of selecting bioreactors for stem cell expansion and differentiation beyond bench scale is largely reliant on whether the cells are adherent, suspension grown as single cells or aggregates for EB formation [206]. Hence, it is very important that the bioreactors are designed according the application that they will be used for. All of the techniques mentioned above generally improve ES cell aggregation and form EB faster and more homogeneously in size compared to typical static suspension cultures.

Spinner Flasks

They have one of the most promising in vitro expansion systems for stem cells especially the ESC/iPSCs and also for the EB cultivation and differentiation into multiple cell types [207]. This methodology provides attractive benefits since there is design is very simple, they have scalable configuration and provide an easy and continuous monitoring for tight regulation of the culture environment [208]. Furthermore, the scaling up is relatively simple because of the improved mass transport achieved by stirring. However, the system causes sheer stress on the impellers thus rendering the flow environment inappropriate for culturing for long term [209]. Also, the observation that the high rate of paddle-impeller stirring can be harmful for the cells, thus an optimal fluid velocity promoting suitable sheer stress for the cell types being cultured is critical [210].

RCCS

Another approach for controlling the EB agglomeration uses the RCCS technology where a slow turning lateral vessel (SLTV) and a high aspect rotating vessel (HARV) is involved that forms a milder bioreactor [211]. This methodology hold significant advantage as the rotation mechanisms causes an extremely low fluid sheer stress and also that the vessels are equipped with membrane diffusion gas exchange to optimize oxygen levels [210]. This type of rotating vessel has known to have impacted on the process of human EB formation and agglomeration where EBs were formed without any necrotic centres [211]. It was also found that the ESC grown in the SLTV bioreactor were of higher quality and yielded nearly 4-fold increase in the number of EB particles. These EBs also showed enhanced cardiac differentiation in comparison to the static suspension cultures [212].

Complex methods

In other cases, encapsulation of ESCs was combined with transferring them into a bioreactor. For example encapsulation of ES cells in defined conditions (i.e. number of cells per EB and capsule size); alginate [213], agarose [214], Matrigel [215], Hyaluronic acid [216] etc. were used to control the agglomeration of cells. Then, once the initial EB formation was initiated, all encapsulated ESCs were transferred to the spinner flasks. This method allowed a 61 fold expansion in the number of cells which was significantly higher than the stirred non-encapsulated system. This was realised that the combination of encapsulation along with bioreactors enhanced the differentiation ability of the cells [217, 218]. Also murine ESCs were expanded as aggregates on micro carriers in the stirred vessels where they retained their stem cell makers and could form EBs. Thus, the combination of the carriers/scaffolds along with the stirred vessels led to higher growth and proliferation of the ESC/iPSCs [210].

1.8.2 Liquid Marbles

Recently, “Liquid marbles” (LM) have been used as a micro bioreactor system for generating EBs. The EBs obtained off a LM has been found to retain their differentiation ability [219, 220]. Aussillous [221] introduced the concept of LM and was used to describe an interfacial phenomenon whereby upon the contact of a small drop of liquid with hydrophobic powder particles, the drop becomes covered by a multilayer of powder particles of micro to nano-sizes Even though the focus of most of the studies for liquid marbles have been in investigating the fundamental interfacial properties, many other studies have harnessed its potential as a micro bioreactor ranging from blood typing to culturing cancer spheroids [219,

222, 223]. These works clearly demonstrate the huge biomedical potential that these liquid marbles hold.

Poly tetra-fluoro ethylene (PTFE) is a fluorocarbon solid consisting completely of carbon and fluorine atoms. PTFE is hydrophobic, which means that neither water nor water containing substances wet PTFE, as there is high electronegativity present due to the presence of fluorine. It is also known to have one of the lowest coefficients of friction against any solid. PTFE is used to generate liquid marbles where, when a small drop of liquid comes in contact with the powder, the drop becomes covered with a multilayer of powder particles of micro to nano sizes [224]. These marbles have the flexibility of a liquid drop and retain their near spherical shape on the liquid or solid surface. This interfacial phenomenon forms a very critical aspect to the generation of the liquid marble micro bioreactors. Many studies have investigated the fundamental interfacial properties of LMs [225, 226] and several studies have exploited LMs as miniaturized reactors [222, 227-229].

The first study demonstrating the use of LMs as miniature bioreactors was carried out by Arabatan et al, wherein they cultured the HepG2 (hepatocellular carcinoma) cells to form cell spheroids *in vitro*. LM consisting of HepG2 cells was generated using chemically inert polytetrafluroethylene (PTFE) hydrophobic powder. The method was successful in generating numerous cell spheroids, via a facile and straightforward route, given the fact that internal structure of LM is constrained and the shell is non-adhesive. This study opened avenues of using LM for biological systems and proved that the system is able to sustain mammalian cells [227].

Furthermore, another study of using marbles to generate EBs was reported by Sarvi et al. [219]. The study reported the generation of EBs from Oct4B2 ESCs. They studied the effect of cell number and LM size in generating viable and functional EBs. It was found out that a single marble generates multiple EBs, which is in contrast to the hanging drop methodology where a single drop produces one EB. Formation of EBs itself is the first step of deducing the differentiation potential of the stem cells. Molecular analysis (RT-PCR) of the EBs generated by LM revealed the expression of various lineage markers like Nestin, Brachyury and FoxA2. This gave a conclusive proof of the ability of the LM to support the culture and differentiation of PSCs.

The research area of LM is still in its infancy and a lot more needs to be studied to understand the working mechanics of this system. The ability of the LM to function as a miniature bioreactor is very exciting and needs more exploration. Even though studies have been carried

out using cancer cells [227] and ESCs [219], still, a lot needs to be explored like using iPS cell lines and even different ES cell lines to see if the LM system can be adopted across different cell types and become a global setting.

This thesis intends to harness the ability of this LM system in understanding the differentiation capability of the LM generated EBs to form cardiomyocytes. Furthermore, it also intends to use an ES and iPS cell line in the marble reactor system to understand its ability of sustaining both embryonic and patient specific iPS cell lines that will provide a hope for patients awaiting cellular transplantation therapies.

1.9 Applications of PSC derived cardiomyocytes

The need for the production of stem cell derived cardiomyocyte production is to provide a continuous source of donor cardiomyocytes to make cellular therapy realizable. Many of the heart diseases are associated with the loss of terminally differentiated cardiomyocytes that cannot be regenerated by the body. In the case of end-stage heart failure, heart transplantation remains the last resort. Replacement of 1 billion cardiomyocytes lost post infarct is potentially one of the biggest challenges faced by researchers [40], hinting on the development of simple, economical and reproducible methodologies for cardiomyocyte production. Transplantation of cardiomyocytes generated from PSCs seems to be the best approach for repairing the injured myocardium with new working myocardium. In spite of the tremendous progress in generating cardiomyocytes, many issues like immune rejection, cell survival, electrophysiology, arrhythmia of the generated cells still needs to be addressed [230].

It is a well-documented fact that there is a dearth of economical and reliable methods to accurately mimic the human cardiac physiological response in animal models, and, coupled with the fact that the process of drug discovery is long, arduous, and expensive, it would be highly beneficial to utilize this technology in cardiac drug discovery programs and safety testing. The pharmaceutical industry currently invests approximately $1.5 billion to develop a candidate drug [46, 231]. The generated cardiomyocytes would provide an indispensible tool for preclinical drug screening of potential drugs to treat multiple heart issues [232].

The last and the most important application of PSC derived cardiomyocytes would be in disease modeling and personalized medicine. With the advent of iPSC technology, it is now possible to generate patient specific (suffering from cardiac diseases) iPSCs, differentiating them into cardiomyocytes, and then performing the functional (electrophysiological) and

molecular analyses providing a powerful tool to understand the progression of the disease at a molecular level [233]. Novel drug discovery and efficacy testing along with the potential of personalized medicine is potentially a platform of hope for the cardiac disease sufferers.

1.10 Future directions and conclusion

ESCs and iPSCs, from a variety species including mice and humans have the potential to differentiate into cardiomyocytes and other cell types. Challenges remain before these applications could be widely used to develop regenerative medicine strategies for treating cardiovascular diseases.

In vitro experiments have mostly relied on spontaneous differentiation of PSCs in EBs to form contracting outgrowths containing cardiomyocytes. Nodal, Atrial and Ventricular-like cell types can be obtained from EBs from mouse or human ESCs. The use of cytokines and growth factors has impacted the differentiation of PSCs to cardiac lineages. Further, techniques are evolving to enrich and purify the different populations of cardiomyocytes obtained. Given the fact that these PSCs can be genetically engineered/manipulated will provide ample opportunities to study disease-specific mutations in cardiomyocytes as well as other basic research studies. Also, as explained in section 1.9, these ESC derived cardiomyocytes can be used for drug development and cardiac toxicity testing.

Currently, monolayer differentiation protocols (in humans) have resulted in the production of 2 X 10^8 enriched cardiomyocytes per T-225 flasks. Also it has been noticed that culturing in multilayer tissue culture flasks or on suspended micro carriers promises greater scalability. The basic EB methodology can be adapted to high throughput using flasks with rotating paddles, or by using a simple suspension culture to produce iPSC-derived cardiomyocytes in commercial quantities [234]. Increase in scalability and reduction in the cost of production can be achieved by eliminating growth factors from the culture medium [235].

Other major considerations, such as classifying the type of cardiomyocyte (ventricular, atrial, nodal) based on AP [234], and selecting relevant populations (either based on differentiation strategies or based on electrophysiology) [45] still needs to be addressed. The issues surrounding donor cell death post cell transplant, tumorigenic ability of the pluripotent cells remaining in the pool of differentiated cells still needs further research. Thus, keeping in mind that the generated cardiomyocytes will be used for clinical applications, it becomes imperative to develop defined and efficient *in vitro* protocols, which would then provide the

stringent levels of safety and quality control making stem cell transplantation therapy realizable. Overall, ESC/iPSC research to generate enriched cardiomyocytes is still in its infancy, but the promise of these cells for cardiovascular research and therapy is great.

1.11 Overall Aims

Given the literature, this study aims to fill in the various caveats that still exist in understanding the differentiation and expansion of PSCs to generate functional cardiomyocytes. One major challenge that is realised over the past few years is the development of robust isolation/enrichment techniques to allow scalable purification of cardiomyocytes and specific subtypes.

Considering the **hypothesis**, "Nkx2.5, being a cardiac progenitor gene will allow selection of cardiac progenitors from a pool of heterogeneous cells post differentiation", the **AIM** 1 of this thesis was to devise an enrichment strategy by targeting the Nkx2.5 gene in the stem cells genome using TALENs and further introducing IRES-mCherry-CMV-IRES-hyg3 downstream the Nkx2.5 gene. Once transfected into the PSCs this would eventually allow the expression of mCherry (red) to be controlled by the expression of Nkx2.5 thereby allowing the sorting of cardiac progenitors. The PSCs when differentiated to cardiac lineage would allow all cardiac progenitors would fluoresce red (due to the expression of mCherry) and then can be separated from the pool of heterogeneous cells thus enriching the cardiac population.

Moving further, expansion of lineage specific cells that would give a respectable number of therapeutically relevant cells for transplantations is another major challenge that has been tantalizing the researchers since decades. This thesis explored the possibility of a novel micro bioreactor "Liquid Marbles" for *in vitro* expansion of generating cardiomyocytes from ESCs. Keeping in mind the **hypothesis** that the "Liquid marble micro bioreactor system allows gases (CO_2 and O_2) to freely transport across the powder shell that will encourage the culture, propagation and differentiation of ESC/iPSCs inside the marble core" this novel system generated higher number of EBs, which allowed the generation of a higher number of cardiac specific cells upon differentiation. Thus, **AIM 2** was exploring the "Cardiogenesis of Embryonic Stem Cells with Liquid Marble Micro-Bioreactor."

The same micro bioreactor was further explored to culture and differentiate iPSCs, since the induced pluripotent stem cells overcome the ethical concerns presented by the use of ESCs.

This strategy will hold a high therapeutic value since patient specific cell lines can be used to differentiate cells in liquid marbles and then can be injected back into the patients without any problems of immune rejection. Thus, **AIM 3** of this thesis was "Standardizing a novel liquid marble system for the generation of embryoid bodies using induced pluripotent stem cells."

In conclusion, the specific aims of each chapter are as follows:

1. Generation of Nkx2.5 TALENs to enrich the cardiomyocytes generated from pluripotent stem cells.
2. Cardiogenesis of Embryonic Stem Cells with Liquid Marble Micro-Bioreactor.
3. Standardizing a novel liquid marble system for the generation of embryoid bodies using induced pluripotent stem cells.

Chapter Two

Materials and Methods

2.1 Animals- Biosafety and Ethics statement

All mice were housed at the Monash Medical Centre Animal Facilities, Clayton. Australia. The mice used for experiments were maintained and treated according to the guidelines established by the Monash University Animal Ethics Committee. The project was covered for the ethics approval under MMCA2010/13 and satisfied Australian National Health and Medical Research Council (NH&MRC) guidelines for animal experimentation.

2.2 Tissue Culture

2.2.1 ESc/iPSC culture medium

Murine ESC/iPSC medium was prepared based in Dulbecco's Modified Eagle Medium (DMEM) supplemented with FBS 15% (JRH Bioproducts),GlutaMAX™, 2mM Non-Essential Amino Acids (NEAA), 10μM (Gibco™), 1 x 10^3 IU/mL of Leukaemia Inhibitor Factor (LIF) (ESGRO™ LIF), 2-Mercaptoethanol, 55 μM (Gibco™) and Penicillin-Streptomycin (25 units and 25 μg respectively; Invitrogen™). After sterilization through 0.22 μm filter, the medium was stored at 4°C for no more than two weeks and warmed at 37°C prior to use.

2.2.2 Fibroblast Basic Medium

A **book chapter** on "Isolation and handling of mouse embryonic fibroblasts (MEFs)" is published in "Methods in Molecular Biology." **(Appendix 1)**
Briefly, mouse embryonic fibroblast (MEF) medium consisted of Dulbecco's Modified Eagle Medium (DMEM) (Invitrogen™) and Foetal Bovine Serum 10% (JRH Bioproducts) enriched with GlutaMAX™, 2 mM Non-Essential Amino Acids (NEAA), 10 μM (Gibco®), and Penicillin-Streptomycin (25 units and 25 μg respectively; Invitrogen™). Medium was filtered through 0.22 μm filters and stored at 4°C for no more than two weeks. Medium was warmed in water bath prior to use.

2.2.3 Differentiation medium (DM)

The differentiation medium consisted of high glucose DMEM (Gibco) supplemented with 15% FBS (JRH Biosciences), 1% NEAA, 1% GlutaMAX™ (Gibco), 0.5% penicillin-streptomycin (Gibco), 0.1 mM β-mercaptoethanol (Gibco) but without mLIF.
Medium was 0.22 μm filter sterilised and stored at 4°C for up to a fortnight.

2.2.4 Dulbecco's Phosphate Buffered Saline (DPBS)

All washings were carried out in DPBS +/+ (Gibco) with $CaCl_2$ and $MgCl_2$ salts. During the trypsinization of the cells, DPBS -/- (Gibco) without the $CaCl_2$ and $MgCl_2$ salts was used.

2.2.5 Matrix Support

The stock solution of Gelatin (Sigma) (2%) was diluted in DPBS +/+ and the final working concentration used was 0.1%. The plastic tissue culture plates (BD Falcon, BD Biosciences) were coated with Gelatin for a minimum of 20 minutes prior to culturing the cells. The solution was sterilized using 0.22 μm filter and stored at 4°C for further use.

2.2.6 Oct4B2 ESCs

Feeder free Oct4B2 ESCs contain Oct4-GFP-IRES-puromycin and hygromycin resistance cassettes. The cassette has regulatory elements of the Oct4 promoter driving the eGFP expression. When Oct4 is expressed in the undifferentiated ES cells, GFP is expressed along, further being resistant to puromycin (1.5 μg/ml). Oct4B2 cells are known to be constitutively resistant to hygromycin (22.5 μg/ml) as well. Oct4B2 cell line was derived from ESD3 cells after transfection with OCT4DE-TK-EGFP-IRES-PURO^R and co-transfected with PGK-HYGRO^R and was kindly provided by BersaGen.
0.1% Gelatin **(section 2.2.5)** was used to treat plastic tissue culture plates as the matrix support for culturing these cells.

2.2.7 MEF iPSC#6 iPS cell line

The cell line was generated in the lab wherein OG2 MEFs were transfected with pMX-based retroviral vectors encoding the mouse cDNA of Oct4, Sox2, Klf4 and c-myc. The OG2MEFs are derived from the OG2 Bl6 mouse [236], which possesses an Oct4-GFP transgene under the control of the endogenous Oct4 promoter. This feature allows the cells to fluoresce green when the endogenous Oct4 gene is expressed especially in the pluripotent/undifferentiated state. The MEF iPS#6 cell line was sourced from Prof. Verma's lab stocks. The iPSCs cells are feeder dependent **(section 2.2.8)**.

2.2.8 Mouse Embryonic Fibroblasts (MEFs) and Feeder cell preparation

A **book chapter** on "Isolation and handling of mouse embryonic fibroblasts" was published in "Methods in Molecular Biology". Detailed protocol can be referred to from **Appendix 1**.

Briefly, 4-6 weeks old QS or CD1 female mice were super ovulated with intra-peritoneal injections of 5 IU of Pregnant Mare Serum Gonadotropin (PMSG) followed by 5 IU of Human Chorionic Gonadotropin (hCG) 24 hours apart followed by mating with QS or CD1 male mice. Female mice were checked for seminal plugs the day after mating separated. Pregnant female mice were sacrificed at 13.5 dpc (days post coitum), in CO_2 chamber (>70% in air) followed by cervical dislocation. The abdominal section was cut open and uterine horns were dissected, rinsed with 70% (v/v) ethanol and placed into petri dishes with DPBS-/- (Gibco) with 1X penicillin-streptomycin. Each embryo was separated, and their head and abdominal sections were cut away. The remains of the carcass were washed with DPBS, and blood was removed. Pieces of the embryo were finely minced and homogenized by triturating in TrypLE™ Express (Invitrogen). Once obtained, the homogenized tissue was incubated with gentle shaking at 37°C for 30 minutes. The resulting suspension was transferred into T-175 flasks containing MEF medium and cultured until 90% confluence. The cells were cryopreserved and stored for subsequent use.

Feeder cell preparation

Passage 3 or 4 MEFs prepared as in **section 2.2.8** and were cultured in MEF medium until 90% confluent. 8-10 μg/ml Mitomycin-C (Sigma-Aldrich) was used to mitotically inactivate MEFs for 3-3.5 hours. After the Mitomycin-C treatment, MEFs were washed twice with DPBS +/+ and trypsinized as per MEF passaging protocol (see chapter "Isolation and handling of mouse embryonic fibroblasts" **Appendix 1**). Following the mitomycin treatment, cells were detached and stored at -80°C until use as feeder layers for no more than two months or preserved in liquid nitrogen for long-term storage.

In order to facilitate cell adhesion, surfaces from plates/flasks were incubated in a solution of 0.1% gelatin for 20 minutes. Immediately, the solution was aspirated and feeders were plated at a cell density of $2.5x10^4$ cells/cm^2.

2.2.9 Maintenance of murine ES/iPS cells

Feeder free murine Oct4B2 ESCs were plated onto 0.1% gelatin coated tissue culture plates at a density of 1.5 x 10^4 cells/cm^2 in ESC medium **(section 2.2.1)**. Cells were passaged when 70% confluent as per passaging protocol **(section 2.2.10)**.

Feeders **(section 2.2.8)** were plated onto 0.1% gelatin coated dishes. Feeder dependent murine MEF iPSC#6 were cultured in iPSC medium onto already prepared feeder plates. Cells were passaged when 70% confluent as per passaging protocol **(section 2.2.10)**. Care was taken to passage the iPS cells further onto feeders only. Plating them without feeders will cause the cells to lose pluripotency and start to differentiate.

2.2.10 Cell Passage

Passaging by trypsinisation was done when the cells reached around 70% confluency. After aspiration of the culture medium, a wash with DPBS -/- was given. A sufficient amount of TrypLE (stable trypsin-like enzyme) was added to cover the entire dish surface and left in the incubator at 37°C for 3-4 minutes. TrypLE was inactivated by the addition of ESC/iPSC culture medium at 3 times the volume of TrypLE. The entire cell suspension was transferred to a 15 ml centrifugation tube and centrifuged for 5 minutes at 400 g (Eppendorf Centrifuge 5702). The supernatant was aspirated and the cell pellet was resuspended in appropriate amount of culture medium, counted and replated at an appropriate dilution.

2.2.11 Cryopreservation of cells

Cells were frozen by the addition of 10% DMSO (cryoprotectant) to the total volume of the cell suspension. The cryo-freezing container “Mr Frosty” (Nalgene) contained isopropanol (BDH) that was equilibrated at 4°C for approximately 1-hour prior to use. The volume of the prepared cell suspension was adjusted using the appropriate medium, and an equal volume of freezing medium (filtered) that consisted of 10% DMSO and 90% fetal bovine serum (FBS) was very slowly added to the cells. Cell stocks were prepared in 1.5 ml cryo-vials (Corning) and slowly frozen in cryo-freezing container, which was kept at -80°C overnight. For long-term storage of cells, cryo-vials were transferred to liquid nitrogen tanks.

2.2.12 Thawing of cells

The cells were obtained from either liquid nitrogen tanks or -80°C and kept in dry ice box before transferring them to water bath at 37°C. Care was taken as to not to submerge the entire vial into the water bath. Cells were then transferred to a 15 ml centrifugation tube and were very slowly (drop wise) diluted with 10 ml culture medium. The cell suspension was then centrifuged at 400 g for 5 minutes (Eppendorf Centrifuge 5702). The pellet was redissolved in appropriate amount of culture media and plated in culture dishes. The culture medium was changed after 24 hours.

2.2.13 Immunostaining

Cells were cultured in 0.1% gelatinised 24-well plate (BD Falcon) for 1-2 days prior to fixation. For fixation, cells were washed 3 times with DPBS+/+ supplemented with 0.1% Bovine Serum Albumin (BSA) (Fraction V) (Invitrogen). Each wash was kept for 5 minutes and then aspirated. After washing, the cells were incubated at room temperature with 4% Paraformaldehyde (PFA, Sigma-Aldrich) for 30-40 minutes. PFA was aspirated and three washes with DPBS+/+ supplemented with 0.1% Bovine Serum Albumin (BSA) (Fraction V) were given again, each wash being for 10 minutes.

Fixed cells were incubated overnight at 4°C with antibody diluent (5% goat serum or donkey serum (Sigma), 0.2% Triton X-100 and 1% BSA (Fraction V) (Invitrogen) in DPBS comprising the appropriate primary antibody at appropriate dilutions as mentioned by the manufacturers. Care was taken to keep the cells in humidified chamber to prevent drying of the cells.

The next day, cells were given three washes with DPBS+/+ supplemented with 0.1% Bovine Serum Albumin (BSA) (Fraction V), each wash being of 10 minutes. The appropriate secondary antibody was added at a concentration of 1:1000 in respective antibody diluent and incubated at room temperature for 1 hour in dark or wrapped in foil. Finally, three washes with DPBS+/+ supplemented with 0.1% Bovine Serum Albumin (BSA) (Fraction V) was given, each wash being of 10 minutes. After incubation, nuclear stain Hoechst 33342 (Sigma) was added for three minutes; washed away three times with washing buffer. Stained cells were visualised using a fluorescent inverted microscope (1 x 71 Olympus, Melville, NY). Photographs were taken using a DP70 camera (Olympus, Melville, NY) attached to the microscope.

Primary antibodies used were: anti-Nkx2.5 (Santa Cruz, ThermoFisher Scientific, Australia), anti-cTnT (Abcam, Sapphire Bioscience, Australia), anti-Nestin (Chemicon, Millipore, Australia). Secondary antibodies were: goat anti-rabbit IgG Alexa- Fluor-594 and goat anti-mouse IgG Alexa- Fluor-594 (Molecular Probes, Life technologies, Australia).

2.2.14 Flow Cytometry

Flow cytometry was performed at the Monash University Biochemistry Imaging facility. For Fluorescence-activated cell sorting (FACS) analysis, cells were trypsinized and an appropriate cell number was resuspended in DPBS+/+. Analysis was performed using a BD FACS Canto

II Analyser™. Ideally, 100,000 cells (minimum 10000) were analyzed. From the total number of acquired cells, the analyzed cells were taken from an adjusted region 1 (R1) established accordingly to each cell type. This R1 parent population was then analyzed for percentages of GFP positives cells based on their FITC (fluorescein isothiocyanate) spectral emission at 525 nm. Data was analyzed using a forward scatter/side scatter (FSC/SSC) dot plot and then analyzed with the appropriate channel.

2.2.15 Liquid marble and Embryoid body formation

ESC/iPSCs were cultured to 70-80% confluency; cells were then washed with DPBS and dispersed into single cells using Tryple^{Tm} express. To form liquid marbles, 2×10^4 ESC/iPSCs were suspended in 300µl of differentiation medium **(Section 2.2.3)**. This drop of cell suspension was placed onto a polytetrafluoroethylene (PTFE, 35 µm particle size, Sigma, Australia) hydrophobic powder bed inside a petri dish (60 mm diameter) using a micropipette. When a drop of cell suspension was rolled on the hydrophobic powder bed, the powder particles wrapped the surface of the drop, thus leading to the formation of a liquid marble. The petri dish was then placed inside a larger petri dish (100 mm diameter) containing sterile water to minimise evaporation from the liquid marbles, following which the set of dishes was covered and kept inside a humidified incubator. The ESCs were allowed to aggregate to form EBs within the liquid marble over a period of 5 days.

Detailed information for the generation of liquid marbles and EBs can be obtained from **chapter 4 (section 4.2.2)**.

2.2.2 Molecular Biology Materials and Methods

2.3.1 Luria-Bertani (LB) Broth

The dry contents were added to a measuring cylinder and 900 ml of water was added to it. Care was taken to completely dissolve the contents in water. The pH of the media was adjusted to 7.5 with 1M NaOH and finally the volume was made up to 1L. The media was autoclaved at 15 psi at 121°C for 20 minutes.

2.3.2 Luria-Bertani (LB) Agar

To the recipe mentioned for LB broth (section 2.3.1) 1.5% w/v agar (Oxid) was added. Similar procedure for autoclaving was followed (section 2.3.1).

2.3.3 Genomic DNA isolation

Samples used for genomic DNA extraction were frozen and stored at -80°C. A total of 5 x 10^6 cells were trypsinized and digested with proteinase K, followed by silica-based DNA extraction was performed using the DNeasy blood and tissue kit (Qiagen) according to the manufacturer's instructions.

2.3.4 Plasmid DNA isolation

Plasmid DNA isolation was performed for mini and maxi preps using QIAGEN Plasmid Maxi Prep and QIAprep Spin Miniprep Kit (Qiagen) respectively. After re-suspending in endonuclease free water, DNA was quantified and purity assessed.

2.3.5 DNA/RNA quantification

DNA and RNA purity was assessed and concentration quantified in 2 µl of the dilution using a NanoDrop™ 1000 Spectrophotometer with extinction coefficients of 50 ng-cm/µl and 40 ng-cm/ µl respectively.

2.3.6 Agarose gel electrophoresis

DNA was resolved on 1% Agarose gels containing Syber safe (Life Technologies). 6X gel loading buffer was added to the DNA sample to a final concentration of 1X and then the sample was loaded onto the gel.
Gels were visualised using the Bio-Rad Universal hood II Gel Doc system (Bio-Rad Laboratories Inc.) and images taken using the Quantity One version 4.4.3 basic software.

2.3.7 RNA isolation and clean up

Total RNA was extracted from cell pellets frozen at -80°C using the Qiagen RNeasy Kit as per the manufacturer's instructions.

The RNA sample was cleaned of genomic DNA contaminants by DNase treatment (DNA-free kit, Applied Biosystems) for half an hour and subsequent inactivation of the enzyme. The cleaned RNA was then assessed for any DNA contamination by performing a PCR for the β-actin gene. The samples of cleaned RNA showed no band of β-Actin as this gene is largely presently only in DNA. This was used as a control for all the Reverse Transcription-RT-PCR reactions.

2.3.8 First strand cDNA synthesis

The cleaned RNA samples were used further to generate complementary DNA (cDNA) from 1 µg of cleaned RNA samples. For cDNA synthesis, the SuperScript™ III System (Invitrogen) was used according to the manufacturer's instructions. Oligo d(T) or random hexo-nucleotides were used to initiate retro-transcription with total mRNA as a template. A PCR for housekeeping gene was set up per sample to assess retro-transcription in parallel with a PCR of the negative reaction to discriminate DNA contamination.

2.3.9 Polymerase chain reaction (PCR)

Standard PCR reactions were performed using GoTaq® Green Master Mix (reaction volume 25 µl) using between 20 to 250 ng of cDNA as a template. Primers were used at 1 µM. PCR conditions are summarized in **Table 1** and a comprehensive list of the primers used during this study is provided in **Table 2**.

Table 1 Standard PCR conditions for RT-PCR analysis

Step	Temperature ^{0}C	Time	Cycles
Denature	92	10 minutes	1
Denature	92	30 seconds	30
Anneal	58	30 seconds	
Extend	72	30 seconds	
Final Extend	72	5 minutes	1
Terminal hold	4	∞	1

Annealing temperatures were adjusted according to the primers used.

Table 2 General PCR primer sequences used in the study

Gene	Primer Sequence	Annealing Temperature (^{0}C)	NCBI Accession number
Oct3/4 Transgene (F) pMXs (R)	F: TTG GGC TAG AGA AGG ATG TGG TTC R: GAC ATG GCC TGC CCG GTT ATT ATT	58	NM_001252452.1
Sox2 Transgene (F) pMXs (R)	F: GGT TAC CTC TTC CTC CCA CTC CAG R: GAC ATG GCC TGC CCG GTT ATT ATT	58	NM_003106.3
Klf4 Transgene (F) pMXs (R)	F: GCG AAC TCA CAC AGG CGA GAA ACC R: GAC ATG GCC TGC CCG GTT ATT ATT	58	NM_010637.3
cMyc Transgene (F) pMXs (R)	F: CAG AGG AGG AAC GAG CTG AAG CGC R: GAC ATG GCC TGC CCG GTT ATT ATT	58	NM_010849.4
Brachyury	F: CAT GTA CTC TTT CTT GCT GG R: GGT CTC GGG AAA GCA GTG GC	55	NM_009309.2
Nestin	F: TCT GGA AGT CAA CAG AGG TGG R: ACG GAG TCT TGT TCA CCT GC	58	NM_016701.3
Oct4	F: GTT CAG CCA GAC CAC CAT CT R: CCA GGG TCT CCG ATT TGC AT	58	NM_013633
Flk-1	F: GGC GGT GGT GAC AGT ATC TT R: CTC GGT GAT GTA CAC GAT GC	56	NM_010612.2
Gata4	F: TCT CAC TAT GGG CAC AGC AG R: GCG ATG TCT GAG TGA CAG GA	56	NM_008092
Nkx2.5	F: ACACCCACGCCTTTCTCAGTCAAA R: CGACAGGTACCGCTGTTGCTTGAA	58	NM_008700.2
MLC2a	F: TCA GCT GCA TTG ACC AGA AC R: AAG ACG GTG AAG TTG ATG GG	58	NM_022879.2
MLC2v	F: AAA GAG GCT CCA GGT CCA AT R: CCT CTC TGC TTG TGT GGT CA	58	NM_010861.3
α-actinin	F: ATG AGG ATT GGC TGC TTT R: TGT TCC ACC CGG TCT TG	58	NM_013456
β-actin	F: CAC CAC ACC TTC TAC AAT GAG C R: TCG TAG ATG GGC ACA GTG TGG G	58	NM_007393.3

2.3.10 Real time primer efficiencies

To determine qPCR primer efficiencies, before experimental analyses, five different cDNA concentrations ranging within 1-100 ng were evaluated in duplicate. Concentrations for further analysis were chosen based in those ranges with a slope <1 after linear regression.

2.3.10 Quantitative RT-PCR (qPCR)

qPCR reaction were performed using Power SYBR® Green PCR Master Mix (Invitrogen). Analyses were performed in triplicate (technical replicate). mRNA values were expressed as ΔΔct values after normalization against the calibrator (endogenous gene β-Actin), an example of a reaction is given in **Table 3.**

Table 3 Standard qPCR conditions

Reagent	Volume	Final conc'n
2x Power SYBR® Green	5 µl	1X
1µM Forward primer	0.5µl	50 nM
1µM Reverse primer	0.5µl	50 nM
dH_2O	3 µl	NA
cDNA*	1 µl	1-100 ng

***cDNA concentration was adjusted based in the corresponding primer efficiency.**

2.3.11 DNA sequencing

The Gandel Charitable Trust Sequencing Centre, Monash Health Research Precinct, Clayton, Australia performed DNA sequencing. DNA was diluted in total volume of 16 µl with 3.2 pmol primer.

SEQUENCE ANALYSIS:

The analysis for sequences was performed using the BLAST engine on the NCBI website (http://www.ncbi.nlm.nih.gov/blast/Blast.cgi) [237], or ClustalW2 program on the EBI website (http://www.ebi.ac.uk/Tools/Cclustalw2/index.html) [238]. BLASTn was use to compare sequences with genomic databases. Bl2seq was used to align 2 sequences identified as highly similar in Blastn results. ClustalW2 was used to align multiple files simultaneously.

2.3.12 Statistical Analysis

Data is presented as mean (M) ± standard error (SEM). qPCR data is presented as ΔΔct values

using the stated calibrator. When comparison between treatments was pertinent, data was analysed using a two-way ANOVA, Mann-Whitney U test or Student t-test. Differences were considered statistically significant where $p<0.05$.

2.3.13 Software

Statistical analysis was performed using the package IBM® SPSS® Statistics Version 20 or, where indicated, the software GraphPad® Prism 5 for windows version 5.03. qPCR data collection was done using SDS 2.4.1 Standalone version 1.6.0, qPCR data calculation and analysis were done using Microsoft® Excel® 2010 version 14.0.6123.5001 (32 bit). Plasmid maps and sequences were analysed and edited using GENtle v 1.9.4 (Magnus Manske, Cologne University). Images of the cells were acquired using the programs DPManager Version 1.2.1.107 and DPController Version 1.2.1.108 (Olympus) on a DP70 digital camera. Gel analysis was done using Quality One Version 4.6.3. Photographs, images and figures were edited and organized using Adobe® Photoshop® CS5 extended Version 12.0 (64 bit).

Chapter Three

Generation of Nkx2.5 TALENs to enrich the cardiomyocytes generated from PSCs

Section 3.1 Introduction and Aims

Generation of cardiomyocytes from PSCs post differentiation provides a powerful system to investigate the various cellular interactions and molecular regulators that govern the specification, commitment and maturation of cardiac lineages in parallel to providing a unique and unlimited source of donor cardiomyocytes for drug testing and various regenerative therapies [118, 124, 163, 164]. To be able to translate this potential into practice, however, will depend on the development of technologies that will allow the generation of a reproducible number of enriched cardiomyocytes, as contaminating cell types could interfere in the understanding of drug responses and other functional properties *in vitro*. It also increases the risk of abnormal growth and teratoma formation post transplantation *in vivo* [231]. PSCs can easily generate beating cardiac muscle cells by forming embryo like cell aggregates called EBs [125]. The cardiomyocytes formed post differentiation express cardiac specific genes and proteins in a highly controlled fashion [137, 156, 239]. However, EBs at any given points in time contains cardiomyocytes at different stages, thus posing a major hurdle in characterizing the differentiation of cardiac lineages. A highly efficient strategy to assess a highly enriched cardiac lineage would be to isolate cardiac progenitors from EBs and further carry out the differentiation of those progenitors *in vitro*. Thus, it seems imperative to identify and isolate the intermediate stages in EB-derived cardiomyocyte differentiation in order to completely elucidate the mechanism of cardiac development. Another difficulty that seems to intrigue researchers while studying ES cell derived cardiomyocytes is that the EBs contain cell types with different chamber specifications [137].

In the case of *in vivo* cardiogenesis, diversification into chamber specific cardiomyocytes is believed to occur at a very early stage in cardiac development where many cardiac genes are expressed along the anterior posterior- axis of the cardiac mesoderm [240]. A similar pattern of chamber diversification seems to occur in EB-derived cardiomyocytes despite the absence of anterior-posterior positional information. But, electrophysiological examination of the PSC cell derived cardiomyocytes has identified sino atrial node-, atrium-, and ventricle like cells in the later stages of the differentiation [137, 155]. Nonetheless, it remains to be determined when the chamber-specific diversification occurs in the EBs.

It is a well-known fact that the expression of transcription factors generally precedes that of structural genes. A number of transcription factors express in the heart primordium, where they play a highly crucial role in heart development by controlling the expression of many cardiac muscle-specific genes [241]. Amongst these, Nkx2.5 is the one that is expressed throughout the course of development in the heart primordium as well as in the

cardiomyocytes [242, 243]. The expression of Nkx2.5 precedes the onset of myogenic differentiation, and continues in cardiomyocytes of embryonic, foetal and adult hearts [242]. Positive Nkx2.5 expression plays a very critical role in the transcriptional regulation of cardiac specific genes by acting in sync with other transcription factors and the expression data suggests commitment to and/or differentiation of the myocardial lineage [114, 242, 244]. In mice, the Nkx2.5 gene expression begins in the cardiac mesoderm at around E7.5, several hours before the mature cardiac markers like α- cardiac actin or β-myosin heavy chain [242] express. Furthermore, it has been demonstrated that Nkx2.5 expression is induced in ES-cell derived EBs at least two days before even the contractile activity is observed. The data further suggest that cardiac progenitors can be distinguished at the molecular expression levels [242]. Thus, analysis of Nkx-2.5 expression and function may provide an early entry point for experimental dissection of the molecular basis of cardiac myogenesis.

Putting the above information together, if all Nkx2.5+ cells include all cardiac precursor lineages during the heart development, Nkx2.5 expression can be used to identify the cardiac progenitors in a mixture of differentiating EBs, thereby helping us to sort and generate a purified population of cardiomyocytes.

To enrich for cardiomyocytes from differentiation cultures, generating cardiac reporter cell lines seemed to be an urgent need. Given the fact that Nkx2.5 gene expression is the first one to be observed in the EBs differentiating to the cardiac lineage and that the expression is maintained all through the process, targeting the endogenous Nkx2.5 loci with a reporter marker seemed like a plausible strategy. Hence, a genome-editing tool that is safe and efficient is highly sought after.

The discovery of the simple code of TALEs, Transcription Activator Like Effectors [179, 245], and rapidly increasing knowledge of TALE nucleases (TALENs) presented genome-editing researchers with a facile tool to modify the genes in their chromosomal contexts. TALEs are modular DNA binding proteins discovered in the plant pathogen *Xhanthomonas* spp. during pathogenesis [179]. The most interesting aspect of these proteins is their repetitive central DNA binding domain, which contains a number of highly conserved 33-35 amino acid sequences. These repeats are different in amino acid positions 12 and 13 (RVDs: Repeat Variable Di-residues). Based on the RVDs, there is a one-to-one correspondence between a repeat in DNA binding domain of TALE and a base on the targeted sequence of DNA [175]. By engineering the central DNA binding of TALEs, new proteins with the ability of recognizing and binding a specific sequence throughout the genome can be produced [246]. TALENs are DNA cutting proteins, which are artificially generated by replacing the native

activation domain of TALEs with a non-specific nuclease domain, usually of *Fok*1. As the *Fok*1 nuclease domain needs to be dimerized for cutting the DNA, a pair of TALENs can be easily customized to introduce a double strand break (DSB) at the targeted locus [247]. TALEN-induced DNA lesions can be repaired by one of the DNA repair machinery pathways; the dominant NHEJ and the less frequent HR. Using the donor plasmid ObLiGaRe [248] approach, it is highly possible to knock-in a reporter gene at the target site in the genome by insertions occurring during the NHEJ repair.

The main aim of this study was to generate the following: a pair of TALENs (Nkx2.5 DD and Nkx2.5 RR) that would target the murine Nkx2.5 gene loci and an ObLiGaRe donor plasmid carrying the reporter and the antibiotic resistance genes (IRES-mCherry-CMV-IRES-hygro), which would be inserted where the TALENs induces the break. These TALENs and the donor plasmid hold the advantage that they can be used to transfect mouse ESCs and iPSCs in order to generate cardiac reporter cell lines. Herein, we used the Golden-Gate cloning method [175] with modifications to generate TALENs targeting a conserved region in the second exon of the Nkx2.5 gene. This chapter will describe two major achievements: 1) Successful generation of the DD and RR TALENs and 2) Successful generation of the ObLiGaRe donor plasmid comprising the Nkx2.5 DD and RR homologous sequences, m-cherry reporter gene and hygromycin antibiotic resistance.

In summary, the differentiation process *in vitro* is never 100% pure, and the heterogeneous pool of cells obtained post differentiation poses a major hurdle to proceed with the transplantation studies. This heterogeneous pool of cells if injected into a diseased model causes tumours that can prove lethal for the recipients. The generation of the knock-in cell lines using TALENs would allow easy identification of the generated cardiomyocytes at their progenitor and mature stage and provide a very simplified method of their purification and enrichment.

Section 3.2 Materials and Methods

All plasmid DNA extractions were performed using Mini-prep kit (Qiagen). Sequencing was performed by the Gandel Charitable Trust Sequencing Centre facilities, Monash University, Australia. Primers were obtained from Genework, Australia, and are listed in **Table 4**.

Table 4 PCR primer sequences used in generating TALENs

Gene	Primer Sequence	Annealing Temperature (^{0}C)	Reference
Nkx2.5-Set1	F: AGA GCC AGC TTG GGA TTC AC R: ATG CCA ACA TCA GCT GAC CA	64	Self-generated
pCR 8	F1: TTG ATG CCT GGC AGT TCC CT R1: CGA ACC GAA CAG GCT TAT GT	55	Cermak et al, (2011)[175]
TAL	F1: TTG GCG TCG GCA AAC AGT GG R2: GGC GAC GAG GTG GTC GTT GG	55	Cermak et al, (2011)[175]
SeqTALEN_5-1	CAT CGC GCA ATG CAC TGA C	-	Cermak et al, (2011)[175]

3.2.1 TALEN target site selection

The sequence corresponding to the murine Nkx2.5 coding region was downloaded from NCBI and saved using CLC Main Workbench software 6. The region downstream of the stop codon (approx. 150bp) in the second exon was selected to be the target site for the TALENs. A region of around 100 bp was chosen around the desirable cut site and pasted in the sequence box available in TALEN Targeter 2.0 software [249] (https://tale-nt.cac.cornell.edu/node/add/talen).

Further, the "Provide Custom Spacer/RVD Lengths" tab was selected and the parameters were set to be as: Minimum Spacer Length: 15, Maximum Spacer Length: 16, Minimum Repeat Array Length: 17, Maximum Repeat Array Length: 20, G Substitution: NN/NH, Filter Options: Show TALEN Pairs (hide redundant TALENs), Off-Target Sequence: select Mus musculus, Upstream Base: T only (Recommended). One site having the minimum number of off target sites and higher percentage of HD and NN/NH RVDs was selected.

A typical TALEN binding site (5'-$TN_{(17-20)}$ $spacer_{(15-16)}$ $N_{(17-20)}$A-3') is composed of a left target sequence (17-20 bp), a spacer sequence (15-16 bp) and a right target site (17-20 bp). The nucleotide just 5' to the first position of the TALEN binding site should ideally be a T for both left and right TALENs. For each right or left TALEN monomers two rounds of Golden-Gate cloning must be performed.

3.2.2 TALEN construction

Golden-Gate TALEN construction library comprises a collection of modules, array, last repeat (LR), and backbone plasmids. For each plasmid, culture the corresponding *E. coli* provided by Addgene. Use 10 ml of LB medium containing appropriate concentration of the antibiotic. It is cultured overnight (16-18 hours) in a shaking incubator at 37 °C.

For preparing the glycerol stock, mix 500 µl of the culture with 500 µl of the 80% autoclaved glycerol and immediately store them in -80 °C freezer after mixing with brief shaking.

Extract the plasmids by using QIAprep Spin Miniprep Kit, according to the manufacture instructions, and quantify the quantity of the isolated plasmids by using NanoDrop spectrophotometer. Adjust the quantity of all plasmids to 150 ng/µl and store them in -20 °C freezer.

3.2.2.1 First Round of Golden-Gate Cloning

Prepared one tube for each reaction. Each left (DD) or right (RR) TALENs were divided into two separate reaction in the first round which here were named as:
left TALEN (DD): A.DD and B.DD
right TALEN (RR): A.RR and B.RR.

For example: In case of our Nkx2.5-DD TALEN:

A.DD composed of 10 modules (NG_1 NG_2 HD_3 NG_4 HD_5 HD_6 NN_7 NI_8 NG_9 HD_{10}) and pFUS_A as the destination backbone.

B.DD is composed of 7 modules (HD_1 NI_2 NG_3 HD_4 HD_5 HD_6 NI_7) and pFUS_A7 as the destination backbone.

Further, the instructions by for the Golden Gate cloning assembly (Addgene) [175] manufacturer was followed to construct the four sets (A.DD, B.DD, A.RR, B.RR) **(Appendix 3)**.

3.2.2.2 Second Round of Golden-Gate Cloning

1) Prepared one reaction each for the left (DD) and right (RR) TALENs. Pipetted 75 ng of the desired destination backbone (containing the *Fok1* domain), 150 ng of each respective pLR, A.DD/RR and B.DD/RR extracted plasmids (from Golden Gate 1) into 15 µL digestion reaction including 5 units *BsmB*I in 1x NEB buffer 4. Incubated the reaction for 15 min at 55°C.

2) Added the following reagents to the reaction: 2 µL ATP, 0.5 µL NEB buffer 4, 0.5 µL T7 DNA ligase, 1 µL DTT and 1 µL water. Incubate the reaction for 15 min at 25 °C and 15 min at 55 °C. ExonucleaseV treatment was not necessary for this step. In our experience, we usually transform the ligation product into DH5α competent cells without inactivation.

3) Transformed (Heat shock) the ligation product into a DH5α competent *E. coli* strain according to the protocol supplied with the cells.

4) After one hour shaking (250 rpm) at 37 °C, 100 µL of the culture was spread onto the ampicillin plates and incubated for 16 hours. Colonies were picked up from the DD and RR plates for further processing.

3.2.2.3 Colony PCR, Plasmid extraction and confirmation of the final TALENs generated

The colonies picked up from the DD and RR plates from the previous step were processed ahead for colony PCR using the **primer set Tal F1 and TAL R2**.

1) Picked up 3 colonies and re-suspend in 40 µL sterile water separately. Added 2 µL of each of this colony suspension as a template into the PCR reaction tubes containing 10 µL of 2x GoTaq Green PCR master mix, 1 µL of 5 µM primer mix of TAL_F1 and TAL_R2 and 7 µL of PCR grade water.

2) PCR program and thermal conditions were set as follows: initial denaturation for 2 min at 95 °C, 35 cycles of amplification including 94 °C for 20 sec, 55 °C for 30 sec, 72 °C for 3 min, and a final cycle at 72 °C for 5 min.

3) Ran 5 µl of PCR products on a 1% agarose gel containing 1x SYBR Safe. The expected band was around 3 kb. A laddering effect is a sign of correct assembly.

4) Based on gel electrophoresis results for each left-DD and right-RR TALEN, one colony was selected and cultured for 16-18 hours (overnight) in 10 mL of autoclaved LB containing 10 µL of ampicillin in a 37 °C incubator on a shaking of 250 rpm.

5) Isolated the plasmids of DD (left TALEN) and RR (right TALEN) by using QIAprep mini kit (Qiagen) according to the manufacture's instructions.

6) Used the NanoDrop spectrophotometer for quantifying the quantity and quality of the extracted plasmids.

7) Sequenced plasmids using primers SeqTALEN_5-1 and TAL_R2, which will read from either end of the modules and should cover the full DNA-binding domain.

8) Generated reference sequence by using TALE reference sequence generator (Bao Lab, http://baolab.bme.gatech.edu/Research/BioinformaticTools/assembleTALSequences.html) and aligned with the sequencing results by using sequence analysing software.

3.2.3 ObLiGaRe donor construction

ObLiGaRe is a new method for site-specific gene insertions that uses the efficient NHEJ pathway and acts independently of HR. According to Maresca et al, this method is applicable with both ZFNs and TALENs and has enabled the authors to insert a 15 kb inducible gene expression cassette at a defined locus in human cell lines [248].

In the construction of our donor plasmid, the DD and RR homologous sequences will be inserted in the reverse orientation, further driving the expression of IRES-mCherry-CMV-IRES-hyg3. The plasmid map of the final product Nkx2.5 RR-DD-IRES-mCherry-CMV-IRES-hyg3 can be seen in **Figure 7.**

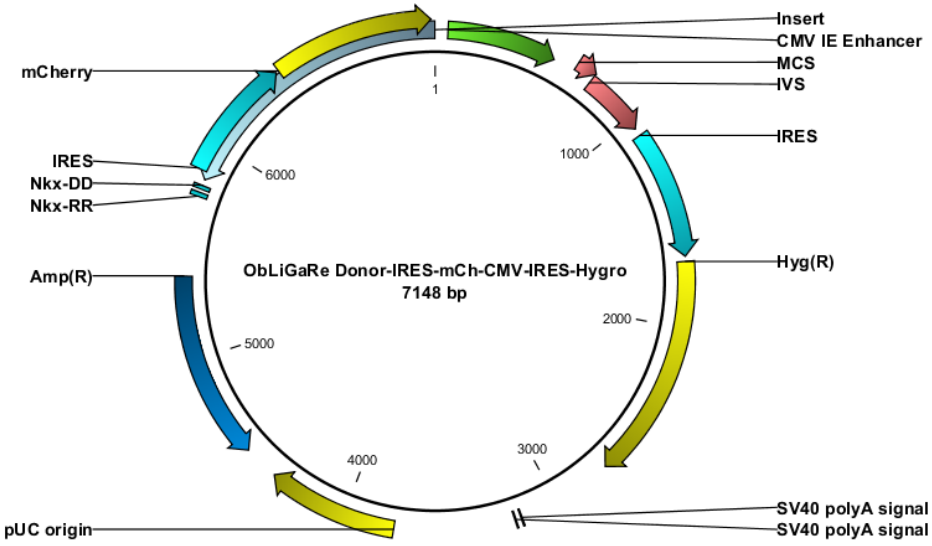


Figure 7 ObLiGaRe Donor plasmid

The final ObLiGaRe donor plasmid map comprises the Nkx-DD and Nkx-RR in the reverse orientation driving the expression of IRES-mCherry-CMV-IRES-hygro. The DD and RR sequences in the donor plasmid contain homologous sequences from the mouse Nkx2.5 genome that would allow the recombination to occur, once the Nkx-DD and Nkx-RR TALENs have created the DSB.

The steps involved in the generation of the **ObLiGaRe plasmid (Nkx2.RR-DD-IRES-mCherry-CMV-IRES-hyg3)** are as follows:

3.2.3.1 Digestion of the purified IRES-mCherry fragment

5µg vector plasmid 12_ABQSZP_IRES-mCherry (**Figure 8**), synthetized by GeneArt was digested using 5 units BsmBI (NEB #R0580L) at 55°C for 1 hour. Further, the digested product was run on 1% Agarose gel and was gel purified. The resulted fragment of IRES-mCherry (1330bp) had SpeI compatible ends.

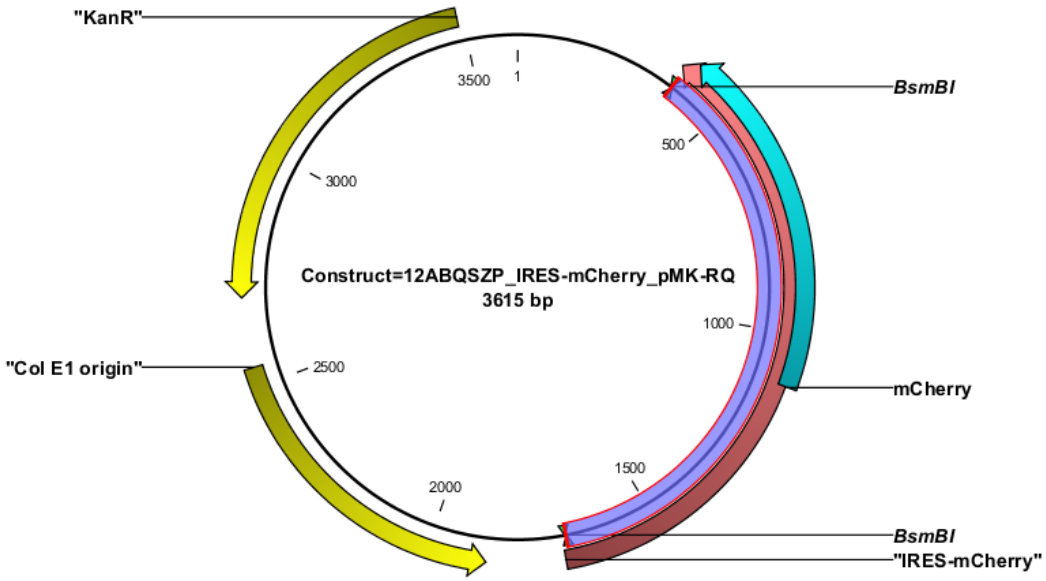


Figure 8 Plasmid map of 12_ABQSZP_IRES_mCherry

As evident from the figure, the plasmid comprised IRES-mCherry, which was restriction digested using BsmBI. The highlighted blue section shows the approximate 1330bp region that was digested from the vector.

3.2.3.2 Insertion of IRES-mCherry into pCMV-IRES-hyg3

The digested and gel purified fragment of IRES-mcherry **(from section 3.2.3.1)** was added to a digestion/ligation reaction containing pIREShyg3 (**see Figure 9**) (pIRES-mCherry: pCMV-IRES-hyg3 10:1), SpeI (NEB #0133S) and T7 DNA ligase in 1xNEB buffer4 supplemented with 1 mM ATP.

Inserting "IRES-mCherry" fragment at the SpeI position (located 60bp upstream of the CMV promoter site) killed the SpeI site in pCMV-IRES-hyg3 and resulted in the formation of a new restriction site for MluI, which is positioned 22 bp upstream of the IRES-mCherry-CMV-IRES-hyg3 (**see Figure 10**). The resulting digested mixture was treated with Exonuclease V and then transformed into DH5α Competent cells. Following transformation, a colony PCR was carried out and a colony containing the correct orientation of IRES-mCherry was minipreped (Qiagen) using the manufacturer's instructions.

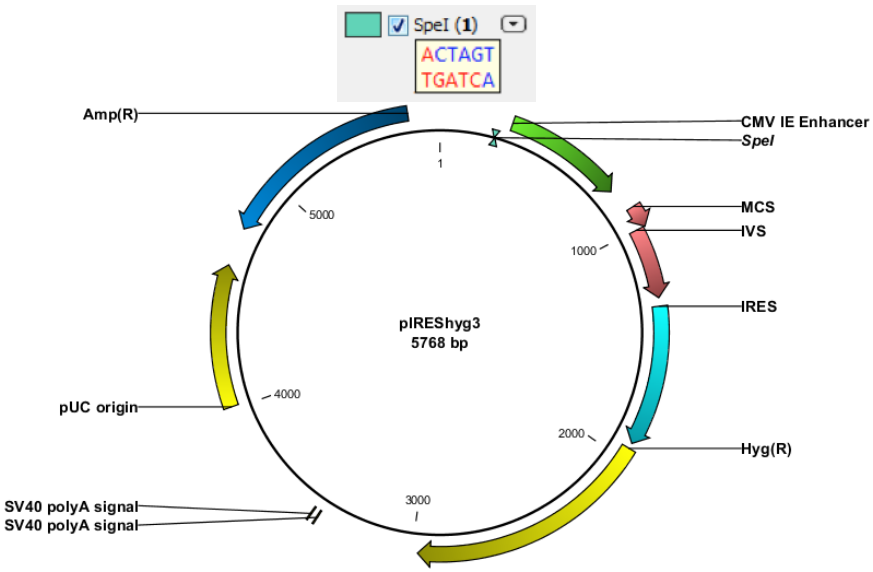


Figure 9 Plasmid map of IRES-hyg3

The SpeI site is located at 5' of the CMV enhancer region. IRES-mCherry fragment will be inserted at this position thereby disrupting the SpeI restriction site.

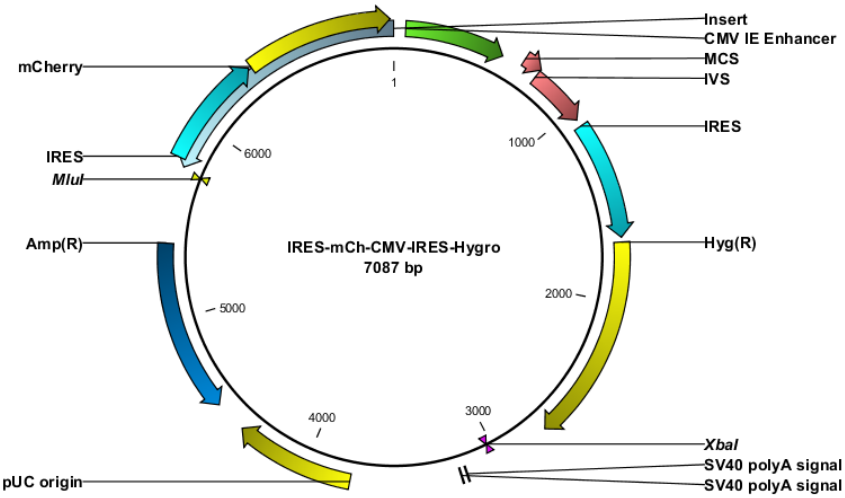


Figure 10 Plasmid map of IRES-mCherry-CMV-IRES-hyg3

Following, the SpeI digestion and insertion of the IRES-mCherry fragment, there is no longer recognition of the SpeI site. The resultant plasmid generated is IRES-mCherry-CMV-IRES-hyg3. There is a generation of 5'MluI site that will be the ObLiGaRe binding sites for DD and RR sequences.

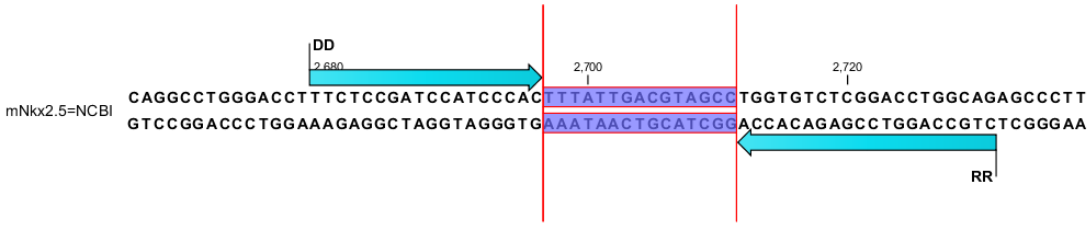
3.2.3.3 Insertion of ObLiGaRe binding site to the vector IRES-mCherry-CMV-IRES-hyg3

Generating the Nkx2.5-ObLiGaRe binding sites:

In order to obtain the Nkx2.5 ObLiGaRe DD and RR binding sites, the Nkx2.5 TALEN target site **(section 3.2.1)** was used.

NKx2.5 TALEN Target Site Sequence:

T **TTCTCCGATCCATCCCAC** tttattgacgtagcc **TGGTGTCTCGGACCTGGCAG** A



NKx2.5 ObLiGaRe Binding site

For ObLiGaRe approach, the donor construct must have reverse DD and RR recognition sites with non-changed spacer sequence between them.

In order to insert the ObLiGaRe DD and RR binding site at the MluI site 5' of IRES-mCherry-CMV-IRES-hyg3 (**see Figure 11**), a pair of single-stranded oligomers were synthesized having MluI compatible ends.

Oligomers

```
5' CGCGGTCTGCCAGGTCCGAGACACCATTTATTGACGTAGCCGTGGGATGGATCGGAGAAAC 3'
     ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
   3' CAGACGGTCCAGGCTCTGTGGTAAATAACTGCATCGGCACCCTACCTAGCCTCTTTGGCGC 5'
```

After annealing and phosphorylation, oligomers were added to a digestion ligation reaction mix containing MluI and T7 DNA ligase in 1x NEBuffer4 supplemented with 1mM ATP. After transformation and colony PCR, a colony containing the correct orientation of insertion was minipreped and sequence confirmed (**see Figure 11**).

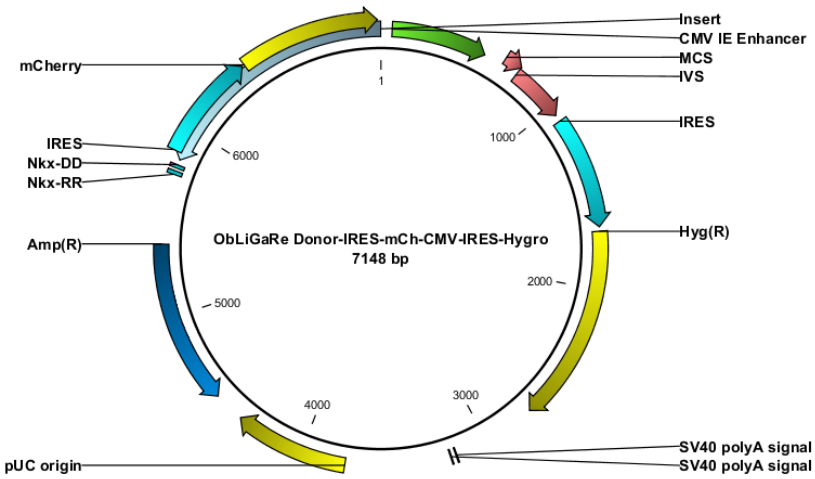


Figure 11 Plasmid map of the final ObLiGaRe donor plasmid Nkx2.5 RR-DD-IRES-mCherry-CMV-IRES-hyg3

The ObLiGaRe Nkx2.5 RR-DD sequences have been inserted in the reverse orientation 5'of the IRES-mCherry sequence. The resulting 7.148kbp ObLiGaRe donor plasmid will be used in conjunction with the Nkx2.5 DD and Nkx2.5 RR TALEN to generate reporter stem cell lines where TALENs create the DSB and the ObLiGaRe donor causes the NHEJ repair mechanism thereby knocking in the mCherry-Hyg3 cassette.

Section 3.3 Results and Discussions

3.3.1 Location of the Nkx2.5 gene in the mouse genome

The murine Nkx.5 gene is located on chromosome 17, - strand, comprises of 2 exons as seen in **Figure 12**. The gene was studied for generating TALEN target site in the 3' UTR region of the second exon.

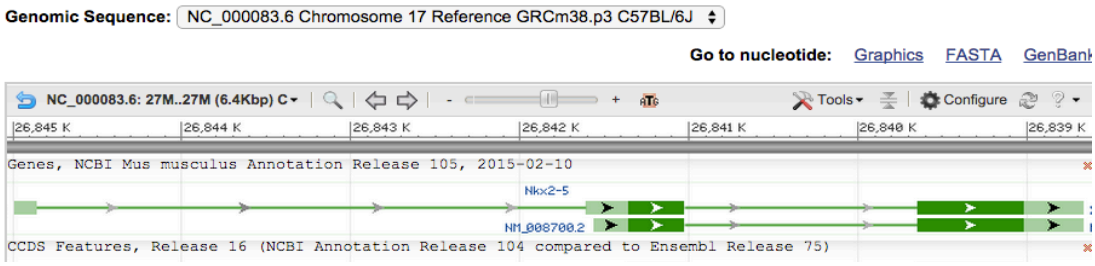


Figure 12 NCBI view of the Nkx2.5 gene exons

The Nkx2.5 gene resides in the – strand on chromosome 17.

Thus, a suitable position in the Nkx2.5 exon was successfully selected and the sequence was used for the further generation of the TALEN target site.

3.3.2 TALEN target site selection

Bioinformatics tool (TALEN-NT software) was successfully used for finding a TALEN target site with minimum off target-sites in the 3'UTR regions of the murine Nkx2.5 genome after the stop codon (TAG) (**Figure 13**).

Sequence Name	Cut Site	TAL1 start	TAL2 start	Spacer range	TAL1 RVDs	TAL2 RVDs	Plus strand sequence	Unique RE sites in spacer	% RVDs HD or NN/NH	Off-Target Counts
Kanika	195	170	222	188-202	NG NG HD NG HD HD NN NI NG HD HD NI NG HD HD HD NI HD	HD NG NN HD HD NI NN NN NG HD HD NN NI NN NI HD NI HD HD NI	T TTCTCCGAT CCATCCCA C tttattgacgtagc c TGGTGTCT CGGACCTG GCAG A	HpyCH4IV:A CGT	61	4 TAL1 TAL1: 0 TAL1 TAL2: 0 TAL2 TAL1: 1 TAL2 TAL2: 3

Figure 13 Off-target counts for Nkx2.5-TALENs predicted by TALEN Targeter 2.0 software

Off-target counts for Nkx2.5-TALENs predicted by TALEN Targeter 2.0 software [250] (https://tale-nt.cac.cornell.edu/). It is worth to mention that TALEN Targeter 2.0 doesn't take into the consideration where the input sequence is from. Off-target sites predicted for TAL2-TAL2 can be ignored due to the fact that obligate heterodimer TALENs cannot introduce DNA breaks. These results indicate that the selected TALEN target site is unique for introducing the reporter plasmid.

Hence, based on the selection made by the software [249] the following target site was selected since it had the minimum number of off target sites.

T **TTCTCCGATCCATCCCAC** tttattgacgtagcc **TGGTGTCTCGGACCTGGCAG** A

Finally, successful selection of the target site was made in the region after the stop codon in the 3'UTR region as can be seen in **Figure 14**. The green arrow represents the mRNA region comprising the 5' and the 3'UTR regions.

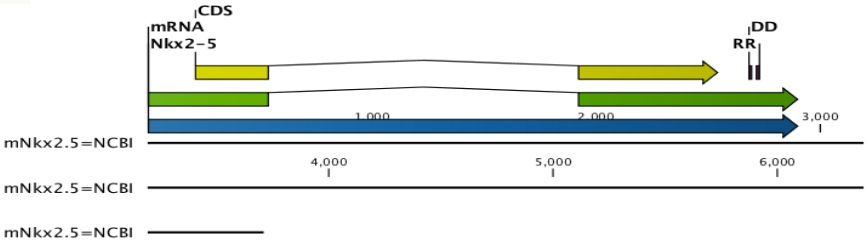


Figure 14 Position of the TALEN target site in the murine Nkx2.5 gene

The target site selection for designing the Nkx2.DD and RR TALENs was made in the region of the second exon approximately 100bp downstream of the stop codon in the 3'UTR region.

We know that the TALENs have been established as a second genome-editing technique besides zinc-finger nucleases [247, 251]. Although the binding of TALENs is highly specific, undesired *off-targets* in addition to the targeted genomic region remain an important issue [174, 252, 253] that may cause severe side effects. Hence, it is needless to say how crucial it is to have a TALEN sequence with minimum off target sites in the genome. The target site designed in this section has the least off target sites thereby implying the high target specificity of the TALEN sequence.

3.3.3 Generation of the Nkx2.5 DD and RR TALENs

3.3.3.1 Golden Gate assembly 1 (GGI)

The Golden Gate cloning system provides a cost and time efficient approach for constructing TALEN-encoding plasmids. In our hands, nearly all of the constructed TALENs were correctly assembled. Although the system was originally designed for use in yeast [175], further enhancements were made to construct vectors suitable for mammalian cell applications [254-258]. In this study, the **Nkx2.5-DD arm was split into GGI-A.DD and GGI-B.DD**. The reaction comprised assembling different along with their respective

destination backbone. Thus, both GGI-A.DD and GGI-B.DD plasmids were generated (**Figure 15**).

Each of the split plasmids comprised its individual set of modules. For example:
GGI-A.DD has 10 modules (NG_1 NG_2 HD_3 NG_4 HD_5 HD_6 NN_7 NI_8 NG_9 HD_{10}) and pFUS_A as the destination backbone.
Similarly, GGI-B.DD has 7 modules (HD_1 NI_2 NG_3 HD_4 HD_5 HD_6 NI_7) and pFUS_B7 as the destination backbone.

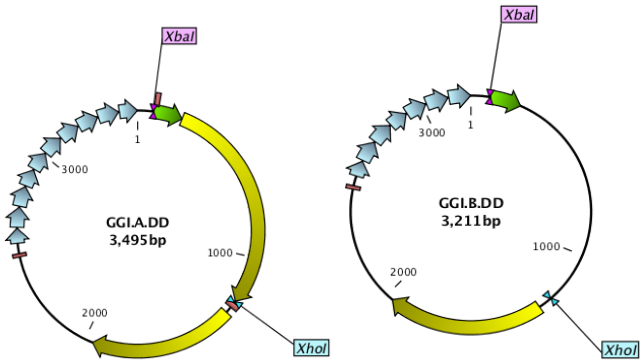


Figure 15 Generation of the GG1-A.DD and GG1-B.DD vector plasmids

Both GG1-A.DD and GG1-B.DD comprised individual modules along with their destination backbone. The GG1-A.DD had 10 modules and pFUS_A as the destination backbone whereas; GG1-B.DD had 7 modules and pFUS_A7 as the destination backbone.

Furthermore, the Nkx2.5-RR was split into GG1-A.RR and GG1-B.RR. In a similar fashion the reaction comprised multiple modules put together along with their respective destination backbone. Thus, both GG1-A.RR and GG1-B.RR plasmids were generated (**Figure 16**).

Each of the split plasmids comprised its individual set of modules. For example:
GG1-A.RR has 10 modules (HD_1 NG_2 NN_3 HD_4 HD_5 NI_6 NN_7 NN_8 NG_9 HD_{10}) and pFUS_A as the destination backbone.
Similarly, GG1-B.RR has 9 modules (HD_1 NN_2 NI_3 NN_4 NI_5 HD_6 NI_7 HD_8 HD_9) and pFUS_B9 as the destination backbone.

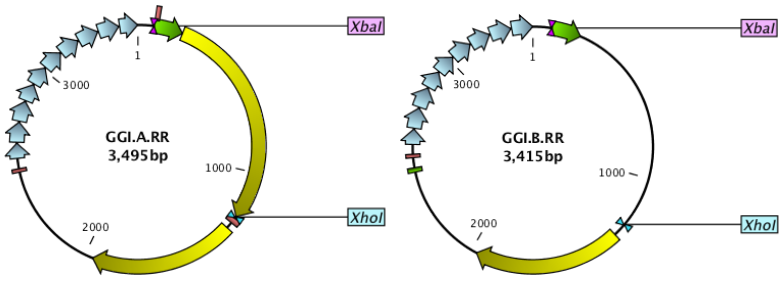


Figure 16 Generation of the GG1-A.RR and GG1-B.RR vector plasmids

Both GG1-A.RR and GG1-B.RR comprised of individual modules along with their destination backbone. The GG1-A.RR had 10 modules and pFUS_A as the destination backbone whereas, GG1-B.RR had 9 modules and pFUS_B9 as the destination backbone.

The initial step in the generation of the TALENs is the assembly the modules for both the TALENs. It is this modular assembly that will be taken for processing in the Golden Gate II reaction set-ups. Based, on the design of the modules for A.DD, B.DD, A.RR, B.RR **(see Figure 15 and Figure 16)**; we successfully generated the respective assembly.

3.3.3.2 Golden Gate assembly II (GII)

After assembling the modules in GGI, for the respective TALENs the, generated plasmids need to be processed further by adding their Left repeat (LR) module and another backbone vector comprising the Fok1 domain.

For example, in order to assemble the final Nkx2.5 DD (left TALEN) **(Figure 17)**

pGGI A.DD+ pGGI B.DD+ HD_{LR}+ pFok1 DD domain.

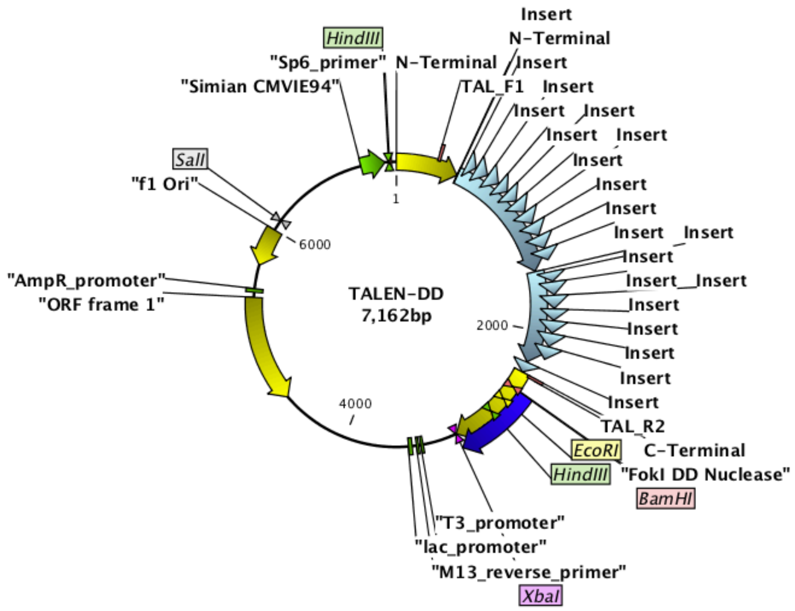


Figure 17 Generation of the final Nkx2.5 DD TALEN

The plasmid contains the complete modular assembly from GG1 A.DD and GG1 B.DD along with the final repeat array and the Fok1 DD domain at the C-terminal.

Successful generation of the Nkx2.5 DD left TALEN was carried out. The resulting final plasmid contained the complete modular assembly from GG1 A.DD and GG1 B.DD along with the final repeat array HD_{LR} and the Fok1 DD domain at the c-terminal.

Similarly, in order to assemble the final Nkx2.5 RR (right TALEN) **(see Figure 18)** pGGI A.RR+ pGGI B.RR+ NI_{LR}+ Backbone plasmid with Fok1 Domain.

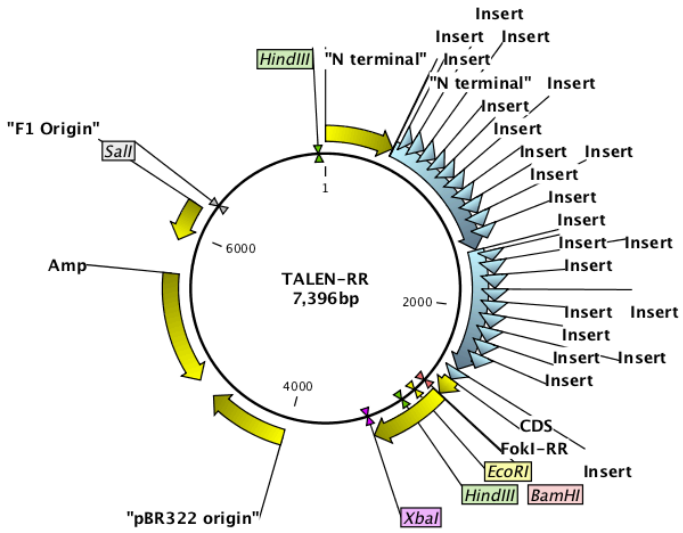


Figure 18 Generation of the final Nkx2.5 RR TALEN

The plasmid contains the complete modular assembly from GG1 A.RR and GG1 B.RR along with the final repeat array and the Fok1 RR domain at the C-terminal.

Also, we successfully generated the Nkx2.5 RR right TALEN. The resulting final plasmid contained the complete modular assembly from GG1 A.RR and GG1 B.RR along with the final repeat array NI_{LR} and the Fok1 RR domain at the C-terminal.

Final PCR using TAL F1 and TAL R2 primers was carried out to confirm the complete assembly of the DD and RR TALENs. The laddering effect and the smearing observed in the gel image (**Figure 19**) confirm the successful generation of the TALENs; based on those effects, clone 2 was selected as the final Nkx2.5 DD and clone 1 was selected as the final Nkx2.5 RR TALENs. Thus, respective clones were cultured further, plasmid was isolated, and glycerol stocks were prepared for storage.

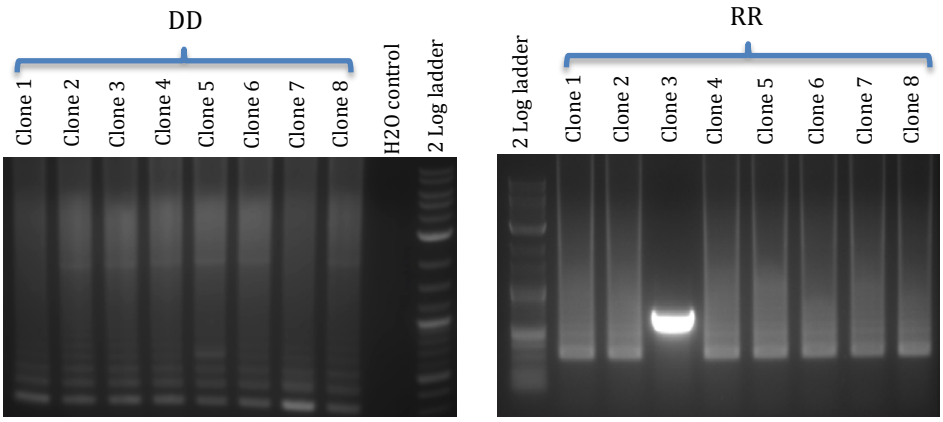


Figure 19 Screening of final Nkx2.5 DD and RR TALEN clones

Based on the laddering effect in DD and smearing effect in RR following PCR, successful generation of the Nkx2.5 DD left TALEN and Nkx2.5 RR right TALEN was completed. Clone 2 from DD and clone 1 from RR was selected and glycerol stocks were prepared for storage.

Successful generation of the TALENs is the first and foremost step in gene targeting strategies. Genome editing starts with efficient DSB break in the target DNA. Both the left and the right TALEN comprise a pair of DNA binding proteins fused to the Fok1 nuclease and can edit DNA through either HR or NHEJ. TALENs have high target specificity making them an obvious choice for the researchers to carry out gene alterations [247, 251]. Further, off target activity appears to be less of an issue for TALEN. TALENs are typically built with 18 repeats of 34 amino acids. The repeat, vary at amino acids 12 and 13, called the RVDs. A TALEN pair must bind on opposite sides of the target site, separated by a "spacer" ranging from 14-20 nucleotides. This offset design is necessary because Fok1 requires dimerization for activity. Therefore, such an extremely long (approximately 36 bp) DNA binding site is expected to be found rarely, if ever, in genomes other than the target [175, 246].

This characteristic of TALENs worked to our advantage while designing the Nkx2.5 DD and RR sequences as we obtained a minimum number of off target hits (**section 3.3.2**); this increased the probability of our TALENs targeting the desired Nkx2.5 sequence in the genome. This result is supported by the study conducted by Park et al., where iPSC lines were edited using TALENs and no mutagenic activity was detected at other genome sites homologous to the target site [259]. Based on the successful results obtained from **sections 3.3.3**, TALEN provides a valuable option, thanks to its relatively unconstrained target site requirements and high degree of specificity.

3.3.4 Generation of the ObLiGaRe donor plasmid

BsmBI digested IRES-mCherry plasmid produced SpeI overhangs. The digested product was inserted into the SpeI digested IRES-hyg3, resulting in the formation of the plasmid vector IRES-mCherry-CMV-IRES-hyg3. Furthermore, this plasmid was digested with MluI, allowing the insertion of the Nkx2.5 ObLiGaRe binding sites (**see section 3.2.3**).

Custom designed nucleases (CDNs) have made gene targeting feasible in cell lines that have low HR efficiency where NHEJ remains the dominant DSB repair pathway. As a result, generating a knock-in seems more plausible and the obvious choice for the moment [248]. An advantage of using the ObLiGaRe strategy is that it eliminates cloning the homology arms into the donor vector and does not require any previous knowledge of the overhangs generated by specific CDNs. The results outlined in the generation of the donor plasmid in this chapter is supported by the fact that ObLiGaRe strategy works very well with the AAVS1 TALENs as reported by Maresca et al. [248].

Given the results based on the sequences obtained after inserting the Nkx2.5 RR and DD ObLiGaRe binding site arms into the IRES-mCherry-CMV-IRES-hyg3 vector, we have been able to successfully generate a donor plasmid capable of working along with the TALENs, helping to insert the IRES-mCherry-CMV-IRES- hyg3 cassette downstream of the Nkx2.5 gene in order to create knock-in reporter cell lines.

3.3.5 Summary of Results

This chapter reports the successful exploration of the possibility of generating Nkx2.5 TALENs and an ObLiGaRe donor plasmid. These tools can be used manipulate the ESCs or iPSCs and hence will prove to be a powerful tool in generating knock in reporter cell lines wherein the cardiac progenitor gene Nkx2.5 would be targeted downstream to introduce mCherry fluorescent reporter and hygromycin resistance genes. Once the differentiation of the manipulated ESCs or iPSCs is carried out via EB formation, the cardiac progenitor cells would fluoresce red as the Nkx2.5 gene would be expressed, hence expressing mCherry (red).

This is a powerful method to enrich the heterogeneous population obtained post differentiation, as at this stage the cardiac progenitors can be selected and taken down further for differentiating into mature cardiomyocytes. This would allow the generation of a pure population of cardiac cells that will be clinically relevant and can be used for transplantation studies with no risk of teratomas or rejection.

Section 3.4 Conclusion and future directions

Targeted genome editing using TALENs is a cutting edge approach for efficiently modifying any sequence of interest in the living cells or organisms. This technology relies on the use of engineered nucleases, artificial proteins composed of customizable sequence specific DNA-binding domain fused to a nuclease that cleaves the DNA in a non-sequence specific manner. This technology provides a hope to tackle various genetic disorders, including monogenic diseases such as sickle cell anaemia or cystic fibrosis. Taking into account its broad importance, genome editing with engineered nucleases was named the 2011 'Method of the Year'. This chapter explored in detail the ability of the TALENs to target a cardiac progenitor marker Nkx2.5 with the idea of knocking in a reporter gene mCherry, thereby helping in the generation of PSC reporter lines. These lines when used for differentiation will aid in the enrichment of cardiac specific cell lineages.

Another advantage of using TALENs is that this technology may be used to assess the effect of inserting a disease mutation into a wild-type cell line. This will help in understanding the mutation or disease itself, and also the effect of removing a disease mutation from a patient-specific iPSC line can be studied. Certainly the rapidity and efficiency of genome editing with TALENs should make it feasible to test the effects of a disease mutation in a variety of genetic backgrounds.

The engineered TALEN technology holds immense potential to facilitate and enhance genetic manipulations in different organisms and cell types. The ease of their construction and the robust success rates has already spurred a huge acceptance of this genome editing technology. Although many challenging and interesting questions need to be answered yet, still this research hold humongous applications for future regenerative medicine.

Chapter Four

Cardiogenesis of Embryonic Stem Cells with Liquid Marble Bioreactor*

This study is published as a co first author publication **(Appendix 2)**

Fatemeh Sarvi*, **Kanika Jain***, Tina Arbatan, Paul J. Verma, Kerry Hourigan, Mark C. Thompson, Wei Shen, Peggy P.Y. Chan. *Cardiogenesis of Embryonic Stem Cells with Liquid Marble Micro-Bioreactor*. ***Advanced HealthCare Materials (IF 4.88)*** Article first published online: 12 MAY 2014, DOI: 10.1002/adhm.201400138.
* Equal First Authors.

Cardiogenesis of Embryonic Stem Cells with Liquid Marble Micro-Bioreactor

Section 4.1 Introduction and Aims

Adult hearts have a very limited capacity for self-regeneration after MI; heart attack. Transplanted stem cells or progenitor cells have the capacity to repair infarcted myocardium [260, 261]. Pluripotent ESC, isolated from the inner cell mass of a developing blastocyst [262] possess the ability to self-renew, and have the potential to differentiate into various cell lineages, including all three germ layers [263] and cardiomyocytes [264]. The ability to engineer these ESCs genetically, together with their ability to differentiate into cardiomyocytes *in vitro*, make them valuable and promising cell sources for cell therapy, tissue engineering and regenerative medicine [265]. Forming three-dimensional (3D) embryo-like cell aggregates, known as EBs, is a key step for the *in-vitro* differentiation of ESCs [266]. Indeed, an EB consists of three germ layers (ectodermal, mesodermal, and endodermal tissues) that emulate the features of a developing embryo [263, 267], thereby providing a valuable tool for various embryogenesis studies [268, 269].

Several methods have been employed to form EBs from ESCs and to subsequently differentiate them into cardiomyocytes. These include: hanging-drop culture, [268] spinner flask, [270] centrifuge-forced aggregation, [271] and suspension culture in a low-adherence vessel [263, 266]. The hanging-drop method is the most commonly used technique for EB formation, in which an ESC suspension was placed on the inner surface of a petri dish lid. EBs can be formed after inverting the lid due to the balance of gravitational and surface tension forces. Changing the droplet volume and seeding density can tune the size of the EBs. However, the hanging drop method is labour intensive and time consuming. It is also practically impossible to perform medium exchange using this method. In addition, the drop volume is limited to less than 50 μl, thereby making it incapable of supporting large-scale production [267]. Rotation-based methods, such as the spinner flask and centrifuge-forced aggregation methods can facilitate large-scale production. However, these methods require costly equipment; moreover, the shear stress induced by the rotation may reduce cell viability, and disrupt cell-cell signalling and the subsequent cell differentiation. The method based on suspension culturing using conventional low-adherence vessels has limited control over size, shape and uniformity of EBs [219].

The LM was first described by [221] and consists of a drop of liquid encapsulated by hydrophobic powder particles. These particles adhere to the surface of the liquid drop,

isolating the liquid core from the supporting surface, while allowing gas exchange between the interior liquid and the surrounding environment [272]. A LM can be rolled around similar to a droplet of mercury [273]. As the shells of LM are made from discrete particles, the shells can be opened, allowing materials such as reagents and products to be introduced into or extracted from the liquid marble; this unique property therefore facilitates chemical and biochemical reactions to be controlled within LMs.

In addition, reagent consumption can be reduced due to the small size of a LM. The chance of contamination is low as a result of the indirect contact between the liquid core and the supporting surface, thus providing an advantage for a variety of applications [272, 274-276]. The use of LMs as miniaturized bioreactors is particularly attractive because of the capability to contain chemical and biological reactions [275, 277-279]. Our previous studies reported the production of 3D cancer-cell spheroids using LM micro-bioreactors [278]. This LM method is advantageous for spheroid production, as it allows the production of spheroids with homogeneous size and shape at a larger scale compared with the hanging drop method, as well as facilitating medium exchange. Unlike rotation-based methods, the LM method does not induce shear stress on the spheroids, thereby producing viable spheroids [219].

Herein, we report the use of the liquid marble as a micro-bioreactor to produce EBs from ESC and, for the first time, we study the feasibility to further differentiate the EBs into lineage-specific cells. The *in-vitro* cardiac differentiation ability of the resulting EBs was assessed by examining gene expression, protein expression and contraction characteristics. We demonstrate that LMs provide a promising platform to facilitate EB differentiation into cardiac lineages.

Section 4.2 Materials and Methods

4.2.1 Tissue Culture

Feeder free murine Oct4B2-ESC (129/Sv) containing the Oct4-GFP-IRES-puromycin and hygromycin resistance cassette was used for this work. For expansion, ESCs were cultured in Dulbecco's modified eagle medium (Gibco) supplemented with 10% FBS (JRH Biosciences, Australia), 1% non-essential amino acids (NEAA) (Invitrogen), 1% GlutaMAX™ (Invitrogen), 0.5% penicillin-streptomycin (Invitrogen), 0.1 mMβ-mercaptoethanol (Invitrogen), and 1,000 U ml^{-1} ESGRO leukemia inhibitory factor (mLIF, Chemicon, Australia). The medium was filtered through a 0.22 µm filter for sterilization and was stored at 4°C for up to a fortnight. Cells were cultured on 0.1% gelatin-coated 6-wellplates (BD Falcon) at 37°C in a humidified 5% CO_2 incubator and were passaged every 2-3 days. The GFP expression of the cells was monitored using an IX71 Olympus epifluorescence microscope.

4.2.2 Preparation of cell containing liquid marble micro-bioreactor

ESCs were cultured up to 70-80% confluency; cells were then washed with DPBS and dispersed into single cells using Tryple^{Tm} express (Gibco, Life Technologies, Australia). To form liquid marbles, 2×10^4 ESCs were suspended in 300 µl of differentiation medium. The differentiation medium consisted of high glucose DMEM (Gibco, Life Technologies, Australia) supplemented with 10% FBS (JRH Biosciences, Australia), 1% non-essential amino acids, 1% GlutaMAX™ (Gibco, Life Technologies, Australia), 0.5% penicillin-streptomycin (Gibco, Life Technologies, Australia), 0.1 mM β- mercaptoethanol (Gibco, Life Technologies, Australia) and without mLIF. This drop of cell suspension was placed onto a polytetrafluoroethylene (PTFE, 35 µm particle size, Sigma, Australia) powder bed inside a petri dish (60 mm diameter) using a micropipette. When the drop of cell suspension was rolled on the hydrophobic powder bed, the powder particles wrapped the surface of the drop, thus leading to the formation of a liquid marble. The petri dish was then placed inside a larger petri dish (100 mm diameter) containing sterile water to minimise evaporation from the liquid marbles, following which the set of dishes was covered and kept inside a humidified incubator. The ESCs were allowed to aggregate to form EBs within the liquid marble over a period of 5 days. **Figure 20** illustrates the process of forming the liquid marbles.

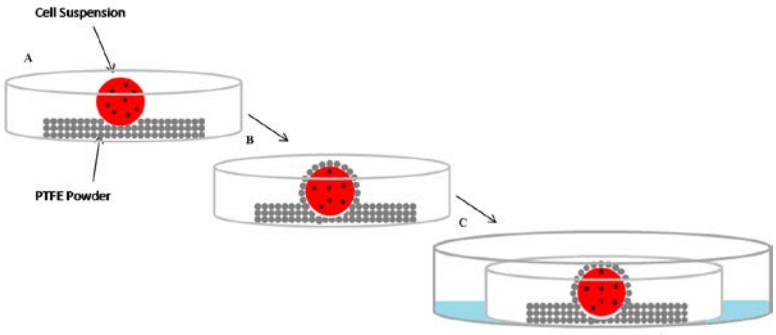


Figure 20 Schematic illustrations of the steps involved in the generation of the liquid marble micro bioreactor

(A) 300 µl of cell suspension is placed onto a hydrophobic PTFE powder bed; (B) The Petri dish was then rolled gently to allow the PTFE particles to cover the cell suspension to form the liquid marble. (C) Placing the marble dish inside a bigger petri dish with sterilized water to prevent evaporation.

4.2.3 EB Morphology and pluripotency characterization

ESC-containing LMs were prepared and allowed to incubate for a period of 10 days. EB samples were taken from the LMs at day 3, 7 and 10. The morphology and GFP expression of EBs were monitored using optical and epifluorescence microscopy (Olympus 1X70 microscope). Both phase-contrast and epifluorescent images were captured. The collected EBs were further dissociated into single cells using Tryple^{Tm} express; the GFP expression of these cells was quantified using fluorescence-activated cell sorting (FACS) at different time points during EB formation.

4.2.4 In Vitro Cardiac differentiation

For cardiac differentiation, 5 days old EBs were removed from the marbles and transferred to 0.1% gelatin-coated 24-well plates and cultured in differentiation medium for further analysis. The plated EBs were examined daily for contractile activity based on videos captured at 15 fps using a camera through an optical microscope. The detailed description of the protocol is illustrated in **Figure 21**.

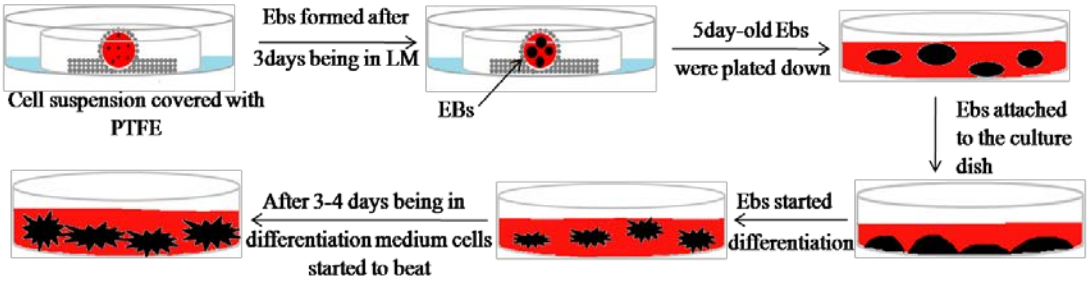


Figure 21 Schematic steps leading to cardiac differentiation

4.2.5 Reverse-Transcription and Real-Time Polymerase Chain Reaction

The gene expression analysis of the EBs and the cells undergoing differentiation was carried out quantitatively using the reverse-transcription (RT) polymerase chain reaction (PCR). LM suspended EBs and plated-down EBs were both subjected to RT-PCR analysis using various differentiation markers.

EBs were allowed to form inside the LMs for 3, 7 and 10 days, and were then retrieved from the LM for RT-PCR. To allow further differentiation, some EBs were retrieved from LM after 5 days and plated down on gelatin-coated wells for another 15 days. The plated EBs were analysed for differentiation markers at time points D6, 8, 10, 12 and 15 days. Cells were harvested with Tryple^{TM} express; the resulting cell pellets were snap chilled at -80 °C prior to analysis. For RT-PCR, ribonucleic acid (RNA) was isolated from cells using the RNeasy kit (Qiagen, Australia) according to the manufacturer's instructions. RNA quality and concentration were measured using a NanoDrop ND-1000 (NanoDrop Technologies, Australia). The isolated RNA was subjected to RQ1 DNase (Ambion, Australia) treatment to remove any contaminating genomic deoxyribonucleic acid (DNA).

Complementary DNA (cDNA) was generated using the Superscript III enzyme (Life Technologies, Australia) according to the manufacturer's protocols. The cDNA samples were subjected to PCR amplification with mouse cardiac specific primers. β-actin was used as an internal control. The primer sequences were obtained from the online NCBI Primer-Blast databank and are listed in **Table 5**. The PCR products were size fractionated using 1% agarose gel electrophoresis at 110 V for 1 h. For quantification, real-time PCR was performed. Real-time PCR analysis was performed using a 7900HT Fast Real-Time PCR system (Applied Biosystems, Australia) at standard reaction conditions using the Power SYBR Green PCR Master Mix (Applied Biosystems, Australia). Briefly, after 2 min denaturation at 95 °C, 35 PCR cycles were carried out at 95°C for 15 s, 58°C for 30 s and 72°C for 30 s, followed by a dissociation stage. Relative mRNA levels were calculated using

the ΔΔCT method [280] and were analysed using SDS Version 2.4.1 software. The experiment was replicated three times.

Table 5 PCR primers used for RT-PCR gene expression analysis

Gene	Primer Sequence	Annealing Temperature (^{0}C)	NCBI Accession number
Oct4	F: GTT CAG CCA GAC CAC CAT CT R: CCA GGG TCT CCG ATT TGC AT	58	NM_013633
Flk-1	F: GGC GGT GGT GAC AGT ATC TT R: CTC GGT GAT GTA CAC GAT GC	56	NM_010612.2
Gata4	F: TCT CAC TAT GGG CAC AGC AG R: GCG ATG TCT GAG TGA CAG GA	56	NM_008092
Nkx2.5	F: ACACCCACGCCTTTCTCAGTCAAA R: CGACAGGTACCGCTGTTGCTTGAA	58	NM_008700.2
MLC2a	F: TCA GCT GCA TTG ACC AGA AC R: AAG ACG GTG AAG TTG ATG GG	58	NM_022879.2
MLC2v	F: AAA GAG GCT CCA GGT CCA AT R: CCT CTC TGC TTG TGT GGT CA	58	NM_010861.3
α-actinin	F: ATG AGG ATT GGC TGC TTT R: TGT TCC ACC CGG TCT TG	58	NM_013456

4.2.6 Immunocytochemistry

The protein markers on the cells were characterized using immunostaining. Incubated cells were first fixed in 4% paraformaldehyde for 30 minutes and washed three times with 1% BSA in +/+DPBS. Fixed cells were incubated with blocking solution (5% goat serum, 1% BSA in +/+DPBS) for 1 hour at room temperature. Cells were then incubated overnight with Nkx2.5 primary antibody (Santa Cruz, ThermoFisher Scientific, Australia) and cTnT primary antibody (Abcam, Sapphire Bioscience, Australia) diluted (1:500) in blocking solution at 4°C. Next, the cells were washed three times with 1% BSA in +/+DPBS and then incubated at room temperature for 1 hour with Alexa 594 labelled secondary antibodies (Molecular Probes, Life technologies, Australia) at a concentration of 1:400 in blocking solution. Cells were washed three times with 1% BSA in DPBS for 10 minutes. Nuclei were counterstained with Hoechst (1 µg/ml, Sigma, Australia) dye. Cells were analysed by epifluorescence microscopy (IX71 Olympus microscope, Australia).

4.2.7 Image Analysis

After plating 5-day-old EBs for another 4 days in gelatin-coated wells, beating foci appeared in the outgrowing EBs. Videos of the beating foci were captured using the microscope camera system; these videos were converted into image sequences using NIS viewer elements software (Nikon, USA). The images were converted to grey scale. The contraction rhythm of the EBs was evaluated using a modified image processing method described in Arshi et al [281]. As the EB underwent rhythmic beating, the cell cluster colour changed from dark to light. Ten different areas were selected from each video, the contracting motion through changes in grey scale intensity in each area was analysed using ImageJ software (NIH, USA), and the resulting mean intensity was plotted against time.

Section 4.3 Results and Discussions

4.3.1 Pluripotency and propagation of EB

Formation of three-dimensional aggregates called EBs is an important step that precedes the initiation of *in vitro* differentiation of ESC into various cell types [282]. Under the *in vitro* conditions, an EB is known to simulate the events of a developing embryo. In a previous study, we reported the possibility of using LMs as a facile and efficient micro-bioreactor for *in-vitro* EB formation [219]. In that study, ESC were cultivated inside liquid marbles and cell aggregates were obtained from day 3. All three germ layers developed spontaneously within the cell aggregates, indicating that the ESC formed EBs successfully inside the liquid marbles [219]. In our present study, we use LMs as micro-bioreactors to generate EBs from Oct4B2-ESC and investigate, for the first time, the capability of liquid marble to facilitate cardiac differentiation. The morphology of cells harvested from the liquid marbles was assessed using optical microscopy. The Oct4B2-ESC contain a pluripotency marker that drives the expression of GFP, hence GFP expression is a direct indicator of cell pluripotency [283].
Examining their GFP expression monitored the pluripotency of cells forming the EBs. Representative images in **Figure 22** show that after cultivating Oct4B2-ESC in liquid marbles for 3 days, these cells aggregated to form EBs, and that these EBs exhibited a compact and round shape with relatively uniform size.

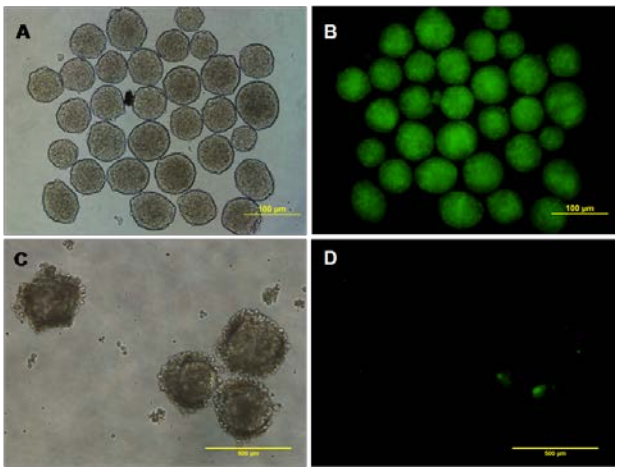


Figure 22 Representative phase contrast and fluorescence microscopy images showing morphology of EBs

The EBs were formed by allowing Oct4B2-ESCs to aggregate inside liquid marbles for (A,B) 3 days (scale bars represent 100 μm) and (C,D) 10 days (scale bars represent 500 μm). Fluorescence microscopy images (B,D) show the expression of GFP under the control of Oct4.

As evident from **Figure 22 A, B**, the ESCs, while aggregating to form clusters destined to form EBs, retained their GFP expression, indicating that the cells do not lose their pluripotency. The Day 10 analysis of the EBs revealed an increase in size suggesting the proliferation of cells within the LM. A decrease in the GFP fluorescence was observed, stipulating the loss of pluripotency and that there was an initiation of differentiation of ES cells within the LM (**Figure 22 D**).

Oct4B2 cells were allowed to form EBs in LM for 10 days; samples were collected from the LMs at day 0 (control), 3, 7 and 10. FACS analysis was performed on the dissociated cells to quantify the number of GFP^+ cells in an EB in order to examine their pluripotency. Representative FACS analysis profiles in **Figure 23** show that 98.8% and 92.4% of cells expressed GFP at day 0 and 3, respectively. The GFP expression gradually reduced to 20% and 9.4% at day 7 and 10, respectively. Thus, the loss of GFP expression as suggested from the FACS analysis and the fluorescence data implies the ability of LM to allow proliferation and differentiation of Oct4B2 cells. This novel system is thus capable of providing a suitable microenvironment for the growth and differentiation of ESCs.

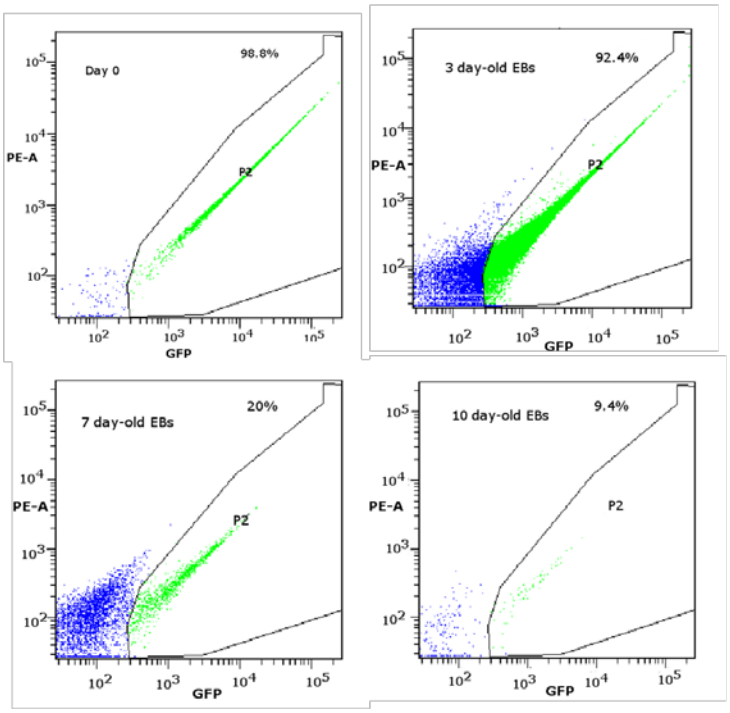


Figure 23 FACS Analysis

GFP expression of liquid marble induced EBs of Oct4B2 cells at day 0 (control), 3, 7 and 10. Numbers indicate the percentage of GFP^+ cells. GFP is indicated on *x*-axis, and auto fluorescence on *y*-axis. GFP^- population is represented by blue dots, while GFP^+ events are represented by green dots.

It has been well documented that during the development of contractile cardiomyocytes, progenitor cells need to first anchor to a substrate followed by cell spreading, withdrawal from the cell cycle, and fusion with nascent myotubes before their ultimate differentiation into cardiomyocytes [284]. In order to promote cardiogenesis of our LM induced EBs, 5-day old EBs were plated onto gelatin-coated 6-wells and allowed to differentiate further. Upon EB adhesion, the cells began to migrate and grew outwards from the periphery of the EB to form a monolayer, as shown in the representative images in **Figure 24 A** and **B**. In the outgrown areas shown in **Figure 24 C**, it was observed that cells exhibited heterogeneous cell morphology. At the start of cardiac differentiation, cells were small and rounded, which upon further differentiation changed to elongated, spindle shaped cells. This observation was in-line with the results of the electrophysiological measurements conducted by Hescheler, et al. 1997 [137] wherein the heterogeneous population of cardiomyocytes undergoes a shift from early stage cardiomyocytes (small and rounded with rarely developed sarcomeres) to terminally differentiated atrial-/ventricular- like (elongated with high content of organized myofibrils) cells.

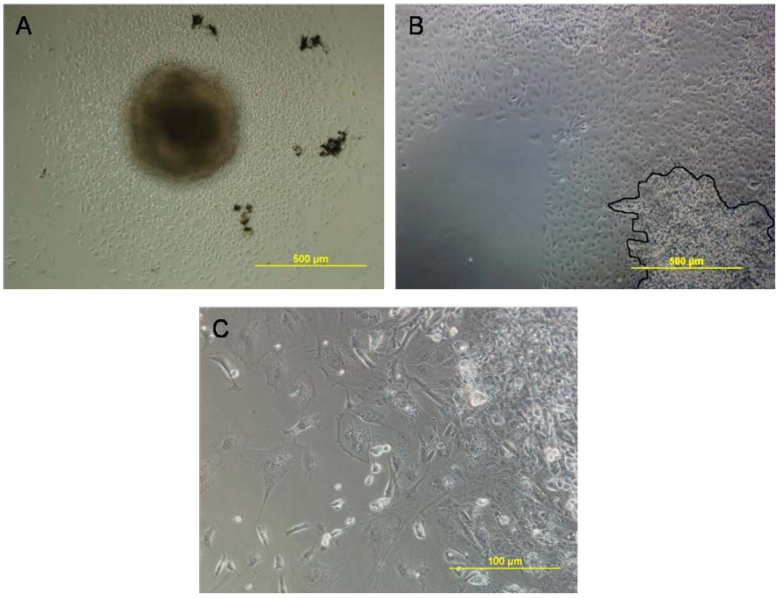


Figure 24 Phase contrast images of a cardiac explant outgrowth

(A) Representative image showing a plated EB attached to a gelatin-coated plate after 1 day, (B) Representative image of plated EB outgrowth at day 4, scale bars represent 500 µm. The black line shows the border of flattened EBs after plating down. (C) Representative image of the plated EB depicting the heterogeneous cell morphology (small and round, spindle shaped cells), at day 8, scale bar represent 100 µm.

4.3.2 Reverse Transcription PCR Analysis of Cardiac Marker Expression during Cardiogenesis

To characterize the differentiation pathway of EBs generated from a liquid marble, the gene expression of EB-derived cells was qualitatively determined using RT-PCR. Cells from a suspended EB obtained from a liquid marble and plated EBs were collected at different time points and were characterized using a series of cardiac markers. Flk-1 expression is found in mesodermal progenitor cells that have the ability to further differentiate into cardiac muscles [285, 286]. Gata4 is a regulator of early cardiogenesis [287] - it is expressed in pre-cardiac mesoderm and subsequently expresses in the endocardial and myocardial tissues of developing heart and heart tube; overexpression of Gata4 is known to up-regulate the expression of transcription factors Nkx2.5 [138]. The cardiac specific transcription factor Nkx-2.5 is the key regulator of cardiac-specific transcription involved in cardiogenesis which is generally observed in the pluripotent stem cell-derived cardiomyocytes [288]. Therefore, Flk-1, Gata4 and Nkx2.5 were all employed as pre-cardiac mesoderm markers in this study. β-actin was used as housekeeping marker gene.

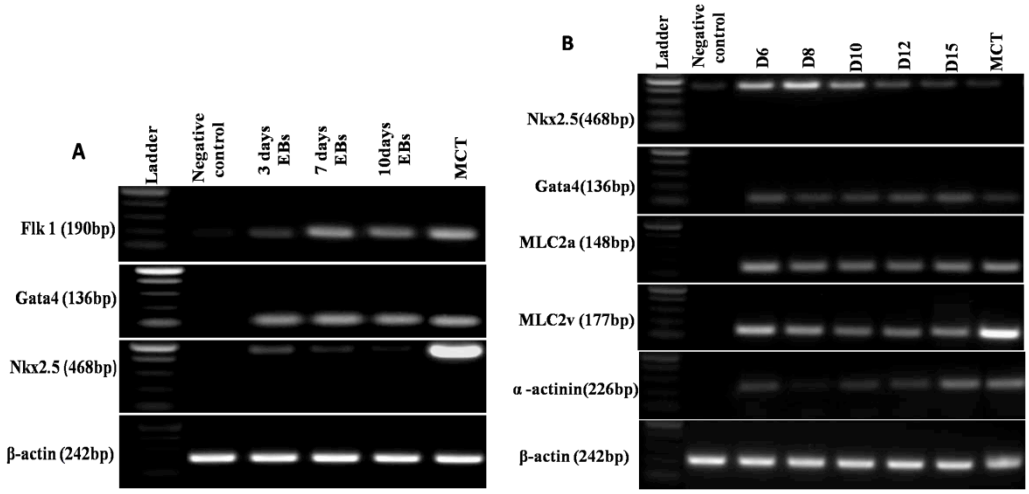


Figure 25 A, B RT-PCR gene expression analysis

(A) Representative RT-PCR analysis for pre-cardiac mesoderm markers expression in cells from EB suspensions obtained from liquid marbles after 3, 7 and 10 days of culture. (B) Representative RT-PCR analysis for pre-cardiac mesoderm and mature cardiomyocytes markers expression from EBs after plating down for 6, 8, 10, 12 and 15 days. β-actin was used as a housekeeping gene. Cells obtained from murine cardiac tissue (MCT) were used as a positive control. Undifferentiated ES cells were used as a negative control.

Atrial myosin light chain 2a (MLC2a) and ventricular myosin light chain (MLC2v) are both cardiac structural proteins, and are often employed as mature cardiac cell markers [289, 290]. Sarcomeric protein α-actinin is a cardiac structural protein that crosslinks actin filaments

within the Z-disc of cardiac muscle [289, 291]. MLC2a, MLC2v and α−actinin were thereby used as mature cardiomyocyte markers in this study. **Figure 25 A** shows that cells from suspended EBs expressed Gata4 from day 3 to day 10. Flk1 and Nkx2.5 expressions could also be detected in suspended EBs from day 3 to day 10.

The positive expression of Flk1, Gata4 and Nkx2.5 suggests that LMs provide a suitable environment to induce cardiac mesoderm differentiation in ESC. **Figure 25 B** shows the RT-PCR analysis of the plated-down EBs. Nkx2.5, Gata4, MLC2a, MLC2v, and α-actinin expressions were detected from day 6 to 15, revealing that these cells had differentiated into pre-cardiac mesoderm and mature cardiomyocytes after plating-down. Similar to the ESC differentiation seen in a hanging drop [292], after removing LIF from the medium in our culture system, signs of ESC differentiation were detected in suspended EBs inside liquid marbles as well as in outgrown cells in plated-down culture. The use of the liquid marble allows the formation of EBs, which in turn enables cell-to-cell interactions. This cell-to-cell interaction is known to stimulate the expression of early cardiac lineage markers [289]. Nevertheless, the LM method permits the use of larger drop volumes compared to the hanging drop method, and is therefore more advantageous for larger scale studies.

4.3.3 Quantitative Real-time PCR Analysis of Cardiac Markers Expression during Cardiogenesis

EBs were allowed to form inside LMs for 5 days, and were plated in gelatin-coated plates with differentiation medium that contained no growth factors. The gene expression of these cells was quantitatively determined using real-time PCR by harvesting cells at days 6, 8, 10, 12. The time dependent expressions of various lineage and cardiac markers during the course of differentiation are shown in **Figure 26**. β-actin was used as a constitutive housekeeping gene for real time PCR and used to normalize changes in specific gene expressions. Octamer-binding transcription factor 4 (Oct4) was used as a marker of undifferentiated cells. Oct4 expression was down regulated from day 6, which suggested that the ESC had lost their pluripotency. The expression of pre-cardiac mesoderm markers Flk-1, Gata4 and Nkx2.5 were up regulated after plating down the EBs for 6 days compared with undifferentiated ES cells. The expression of Flk-1 decreased from day 8 onward, while the expression of Gata4 and Nkx2.5 decreased from day 10 onward. These results together suggested that the ESC had differentiated into cardiac mesoderm and subsequently differentiate into other cell types.

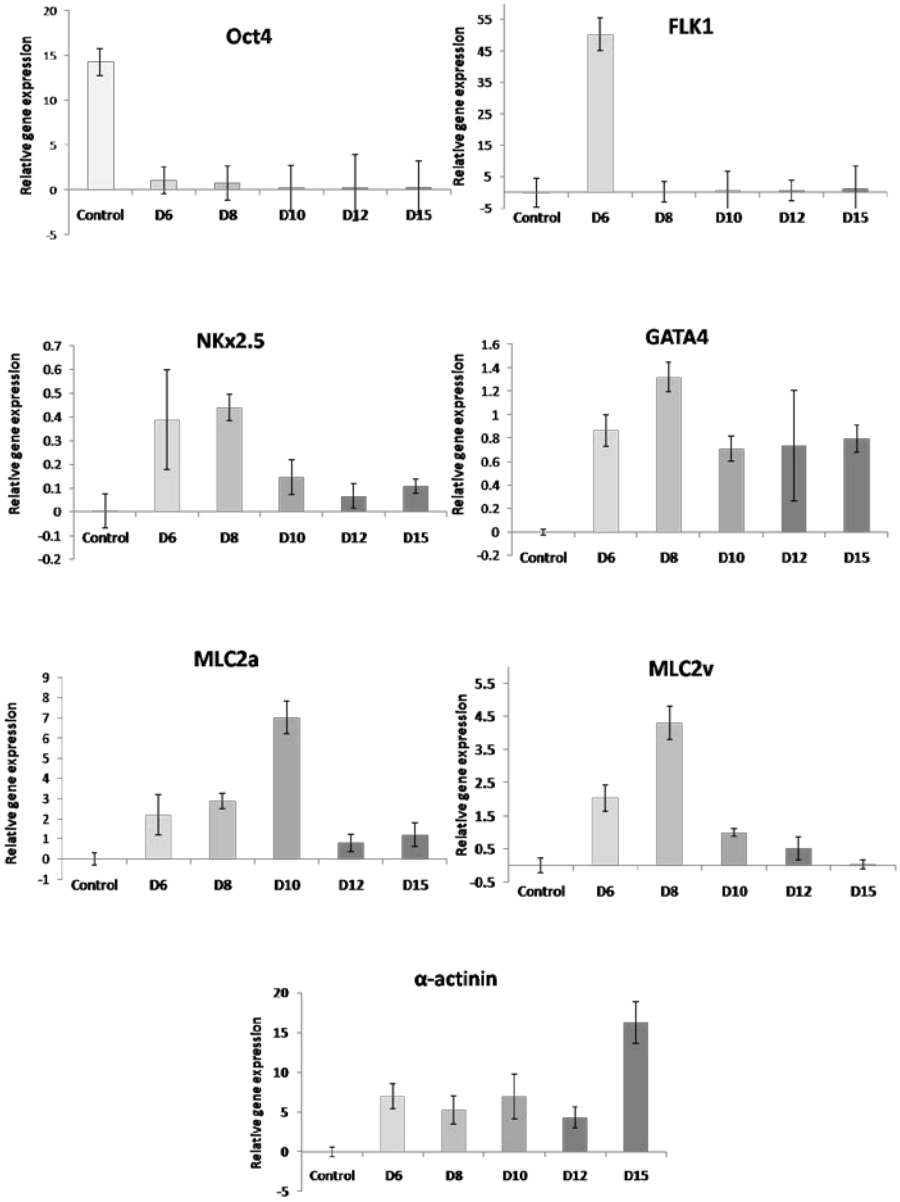


Figure 26 Real-Time PCR analysis of gene expression over 15 days of cardiogenesis

Cardiomyogenesis was characterized by a continuous decrease in pluripotency marker (Oct4) expression over the course of differentiation, followed by an initial increase in the expression of the pre-cardiac mesoderm markers (Flk1, Nkx2.5 and Gata4) expression, which eventually decreased. The increase in the expression of mature cardiomyocytes markers (MLC2v, MLC2a and -actinin) expression as seen resulted in the formation of mature cardiomyocytes.

The formation of cardiac mesoderm is a prerequisite intermediate step for cardiomyocytes differentiation from pluripotent stem cells. Expressions of MLC2a, MLC2v and α–actinin were first detected at day 6. The MLC2a expression increased at day 10, and decreased gradually from day 12. The MLC2v expression increased at day 8, and decreased gradually from day 10. The α–actinin expression remained constant from day 6 to day 12, and increased sharply at day 15, thus suggesting that stable sarcomeric structural protein was formed within

the cell culture. The expression of MLC2a, MLC2v and α−actinin, together indicated that plated EBs had differentiated into cardiomyocytes spontaneously. It is noted that spontaneous beating was observed in EBs after plating down for 4 days. The results are consistent with those reported in other studies, where Nkx2.5, MLC2v and MLC2a are expressed in beating cardiomyocytes that had differentiated from ES cells [138]. Altogether, the results indicate that EBs derived from LMs had differentiated into mesoderm and subsequently into mature cardiomyocytes.

4.3.4 Immunocytochemistry

Five day-old EBs were collected from LM and plated down. Immunostaining was performed on EBs that exhibited spontaneous contraction to detect the presence of the cardiac specific proteins. As shown in representative images in **Figure 27** (top panel), the expression of the cardiac transcription factor Nkx2.5 (AlexaFlour594, stained red) was detected after plating down the EB for 7 days. Nkx2.5 is a transcription factor that is expressed in myocardiogenic progenitor cells during myocardial development; [288, 293] Nkx2.5 is the earliest known marker for cardiogenesis in the veterbrate embryo [294-296]. Representative images in **Figure 27** (bottom panel) show that cells expressed cTnT markers (AlexaFlour594, stained red) after plating down for 12 days. Cardiac troponin T (cTnT) is a marker for mature cardiomyocytes [289]. EB derived cells expressed cardiac specific protein markers, thus further confirming that this LM method can generate functional EBs that can further differentiate into mature cardiomyocytes.

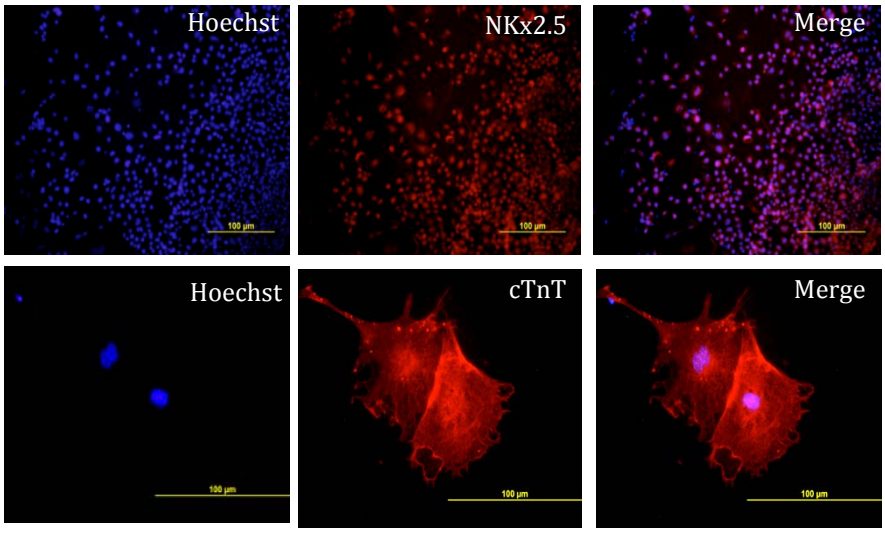


Figure 27 Immunostaining analysis of cardiac proteins

Immunostaining for cardiac progenitor protein Nkx2.5 (after 7 days) and mature cardiomyocyte protein cTNT (after 12 days) of plating down EBs, Nuclei were counterstained with Hoechst (blue). Scale bars represent 100 μm.

4.3.5 Beating Cardiac cells

In order to observe contractile, EBs were kept inside LMs for 7 days. At day 7, EBs were retrieved and suspended in a low-adhesion dish. After 8 hours, it was observed that only ~4.5% of EBs exhibited spontaneous contraction, the contraction only lasted for a few hours, probably because once the ESC had differentiated into lineage-specific progenitor cells, they needed to anchor onto a substrate for optimal differentiation, as reported by Engler et al. [284]. Nevertheless, this observation confirmed the differentiation potential of EBs into generate cardiomyocyte-like cells inside LMs. To examine if EBs generated from liquid marbles can further differentiate into beating cardiac cells, 5 days old EBs were removed from the liquid marble and plated down in a gelatin-coated wells and the activities of the EBs were monitored. These EBs adhered and proliferated well on the substrate. Spontaneous rhythmic beating was detected in the outgrown EBs after plating down for 4 days. Supporting information 1,2 show the videos of beating cells derived from EBs after plating down for 4 and 7 days, respectively. The rhythmic beating was considered as a functional cardiac marker [297]. For brevity, EBs that differentiated into beating cells are denoted as *beating* EBs.

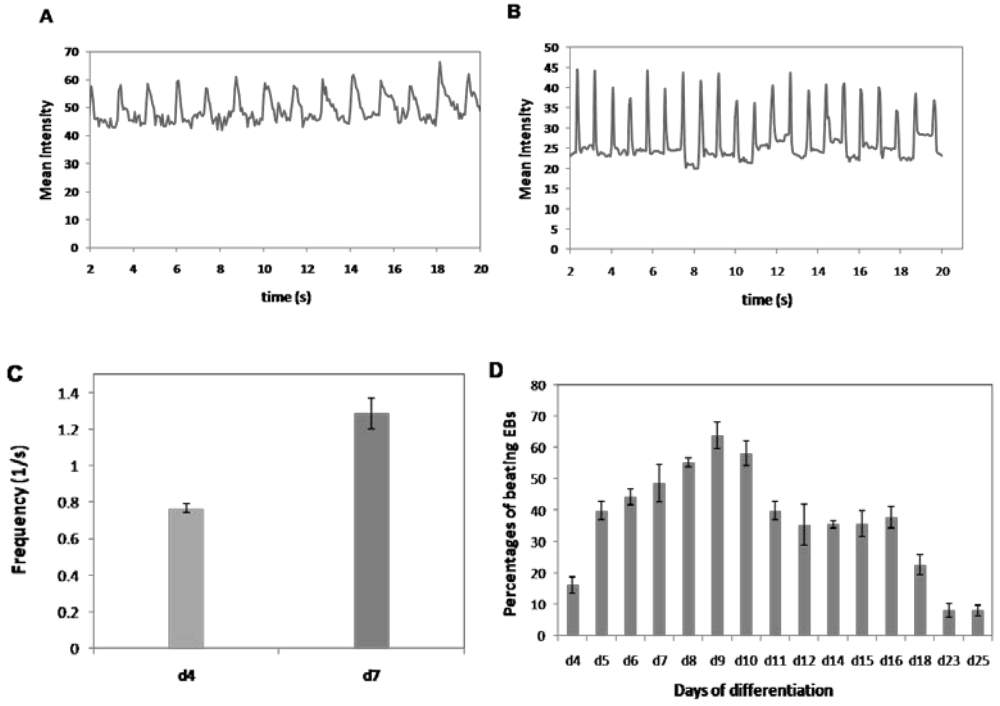


Figure 28 Mean intensity patterns and beating frequency of EBs

Mean intensity patterns obtained from representative areas in contractile EBs after plating for (A) 4 and (B) 7 days. (C) The beating frequency of EBs at day 4 and 7. (D) The percentages of contractile EBs at different time points.

Figure 28 A and **Figure 28 B** show the mean intensity of beating obtained from representative areas of beating foci after plating down the EBs for 4 and 7 days, respectively.

At day 4, the beating was relatively regular with minor arrhythmia. At day 7, the beating became more regular. The beating frequency was calculated by measuring the time interval between two consecutive peaks in the mean intensity patterns. The frequency increased from 0.76 s^{-1} at day 4, to 1.29 s^{-1} at day 7, as shown in **Figure 28 C**. This was probably because the cells were initially beating asynchronously after migrating out from the EB; these cells became more synchronized given the longer culture time.

After plating the EBs for 4 days, approximately 16% of the EBs underwent differentiation into rhythmic beating cells (**Figure 28 D**). The percentage of beating EBs increased to 48% after 7 days. The percentage of beating EBs continued to increase over time reaching a maximum of 64% after 9 days. After 10 days, the rhythmic beating activity declined, possibly due to the overgrowth of cells causing peeling of cells from the plate, although approximately 35% of the EBs still exhibited rhythmic beating activity from day 12 to day 16. Approximately 8% of EBs still exhibited rhythmic beating at day 25. As expected, a larger number of EBs exhibited spontaneous contraction in plated culture compared to suspension culture. This observation is consistent with other studies that found that EBs differentiate extensively after attaching to a substrate [298, 299]. The observation of spontaneous rhythmic beating again confirmed that LM derived EBs differentiated into functional cardiac cells. Taken together, these results indicate that the LM is a promising platform for EB generation, and for facilitating further differentiation into cells with a cardiac lineage.

4.3.6 Summary of Results

This study demonstrated that LM is an efficient micro bioreactor system, which not only allows the culture of ESCs but also supports the differentiation of ESCs to generate cardiomyocytes. As evident by the qRT-PCR results, EBs upon plating lose the expression of pluripotent Oct4 completely by day 15 and an up-regulation of Nkx2.5 by day 5 and α–actinin by day 15 is observed clearly indicating that cells have done down the cardiac differentiation pathway. This demonstrates that liquid marble induced EBs express the meso-endodermal lineage markers, which upon further differentiation express the cardiac progenitor genes and eventually mature cardiac markers. Immunocytochemical results back up the qRT-PCR results in that the cardiac progenitor (Nkx2.5) and mature (cTnT) proteins are expressed by the plated EBs, thereby providing a conclusive proof that mature cardiomyocytes are generated by liquid marble induced EBs. Functional proof of the mature cardiomyocytes is provided by the fact that the spontaneous rhythmic beating of the cells is observed thereby indicating the cardiac nature of the differentiated cells. In summary, this study provides

conclusive proof of LMs being a novel and efficient micro bioreactor to culture pluripotent stem cells.

This study is published as a co first author publication **(Appendix 2)**

Fatemeh Sarvi*, **Kanika Jain***, Tina Arbatan, Paul J. Verma, Kerry Hourigan, Mark C. Thompson, Wei Shen, Peggy P.Y. Chan. *Cardiogenesis of Embryonic Stem Cells with Liquid Marble Micro-Bioreactor*. ***Advanced HealthCare Materials (IF 4.88)*** Article first published online: 12 MAY 2014, DOI: 10.1002/adhm.201400138.

- Equal First Authors.

Section 4.4 Conclusion and future directions

In this study, we investigated the capability of LMs to induce EB formation and subsequent differentiation into cardiomyocytes. LMs were prepared by inoculating ESCs onto a bed of hydrophobic PTFE particles. ESC aggregated to form uniform EBs after inoculation, and subsequently differentiated into cardiac mesoderm cells without the use of growth factors. Further on plating, these EBs further differentiated into contractile cardiomyocytes. The contraction of cardiomyocytes was synchronized with longer time in culture. The liquid marble method was found to be advantageous for EB formation as it is cost effective and simple; it also allows larger-scale EB production compared with the hanging drop method. Overall, this study shows that liquid marbles can serve not only as a novel platform to induce the formation of EBs, but also to facilitate cardiogenesis. The cardiomyocytes generated via this liquid marble strategy could provide a continuous source of donor cardiomyocytes for cell replacement therapy in damaged hearts. Furthermore, this technology would be highly beneficial to provide cardiomyocytes for use in cardiac drug discovery programs and safety testing. Since the quantity of cells required for the above mentioned applications is very high, it becomes imperative to develop defined and efficient *in vitro* protocols, which would then provide the stringent levels of safety and quality control making stem cell transplantation therapy realizable. Our study provides a step up this ladder and gives a new promise and hope in cardiovascular research.

Chapter Five

Standardizing a novel Liquid Marble Bioreactor system for the generation of EBs from iPSCs

Standardizing a novel liquid marble system for the generation of embryoid bodies using induced pluripotent stem cells

Section 5.1 Introduction and aims

Ischemic heart disease, characterized by reduced blood supply to the heart muscle is the primary cause of death throughout the world, including, in most of the low income and middle-income countries [300]. MI, caused by the obstruction of the coronary arteries, is also associated with the death of cardiomyocytes. The division of cardiomyocytes stops in the mammalian heart during early post-natal life [7]. As a result of this pathologic insult, a scar tissue rather than the new cardiac muscle cells replaces the damaged myocardia, leading to an over activation of the sympathetic and renin-angiotensin systems. This eventually leads to an overload on the surviving myocardium and ultimately heart failure [301]. Other causes of heart failure, including high blood pressure, are also characterized by a gradual loss of cardiomyocytes [302].

It is a widely agreed fact that the regenerative capacity of the human myocardium is grossly inadequate to compensate for the severe loss of heart muscle presented by the catastrophic MI or other myocardial diseases [303, 304]. Although recent reports suggest that the mammalian myocardium can regenerate via endogenous stem/progenitor cells, the magnitude of such response appears to be minimal and yet to be fully realised in case of cardiovascular patients [305]. For the end-stage heart failure patients with MI, the only choice is heart transplantation, but this approach suffers from a severe shortage of donor availability [306, 307]. A number of lifesaving drugs and surgical interventions for patients with end stage heart failure have been facilitated with the advent of the new biomedical technologies. For example, devices such as the defibrillators and left-ventricular assist devices can reduce the mortality rates by 20-50% [14]. However, they increase the life expectancy of heart failure patients by only 2-3 years [306]. Thus, the increasing incidence of heart failure and other cardiovascular related deaths around the globe calls for new life saving interventions, which could benefit thousands of patients suffering from heart diseases.

The replacement of the loss of the cardiac mass has been facilitated by a number of strategies appearing on the horizon, a few of which include techniques to restore the cell cycle activity [7], mobilization of myogenic stem cells [308] and direct transplantation of cardiomyocytes [309]. Studies from multiple laboratories have shown that the transplantation of donor cells (fetal and ESC derived cardiomyocytes, skeletal myoblasts, smooth muscle cells and adult stem cells) can improve the function of diseased heart [63]. The general belief is that the

paracrine factors released from the transplanted cells are thought to play key roles in stimulating angiogenesis and improving the function of the engrafted myocardium.

ESCs were first derived in 1981 from the inner cell mass (ICM) of pre-implantation blastocyst by two independent groups [79, 80] and represent a novel source of cardiomyocytes for the treatment of ischemic heart diseases. Further, it has been known that the mouse ESCs can be maintained in an undifferentiated state by growing them on mitomycin-treated MEFs and/or by supplementing cultures with LIF [83, 84]. These cells can proliferate indefinitely and carry a normal karyotype when maintained in an undifferentiated state. ES cells unambiguously fulfil all the requirements of stem cells: clonality, self renewal and multipotentiality [310]. When taken off the undifferentiated culture conditions, ESCs will spontaneously differentiate to form multicellular aggregates known as EBs. Aggregated cells in the EBs can form elements of all the three germ layers: ectoderm, endoderm and mesoderm and also possess the ability to completely regenerate the myocardium [18]. Although ESCs serve to be a reliable source for cell based therapies, clinical use of these stem cells for the treatment of human disease is currently prohibited because of several political, scientific, and ethical reasons [311]. Major efforts have been pursued in this regard to derive ESCs through alternative mechanisms, the most important of that being the genetic conversion of fibroblasts into pluripotent stem cells [19, 20].

A breakthrough was made in 2006, being that ESC-like cells, called iPSCs can been derived from somatic cells by the ectopic expression of the four transcription factors OCT3/4, Sox2, KLF4, and c-Myc [19]. The iPSCs have been found to be very similar to ESCs in morphology, surface marker expression, differentiation into the three germ layers, formation of teratomas, and giving rise to chimeric animals when injected into the blastocysts [293, 311]. Since then, this discovery has been at the forefront of research aimed at simplifying the production of patient-specific stem cells as the patient's somatic cells can be used to generate iPSCs and further differentiate into multiple cell types, including cardiac and neural lineages [293, 312]. Furthermore, iPSCs hold the potential to provide a readily available source of stem cells without the ethical controversies that accompany the use of embryos/ESCs [19, 104]. Therefore, the development of cell reprogramming or iPSC technology opens up a new perspective to the quickly progressing field of cell based therapy.

A further challenge faced by researchers is the development of a reliable and efficient protocol that would be able to help the iPSCs differentiate into the multiple cell types. As mentioned earlier, formation of EBs is the first and foremost step in the spontaneous *in vitro* differentiation of stem cells [313]. For cardiac or neural differentiation of mouse cell lines,

dispersed iPSCs are grown in culture with no LIF and allowed to form EBs. After 3-4 days, EBs are transferred to plastic culture dishes and allowed to attach. Regions of cardiogenesis can be readily identified by the presence of spontaneous contractile activity 8-10 days after EB attachment. Regions of neural differentiation can be easily identified by the appearance of neuronal structures stretching in the periphery of the plated EBs. The neural processes are also easily identified within 5-8 days of plating down the EBs. In order to increase the induction efficiencies, different growth factors have been used during the differentiation processes. These agents include growth factors/hormones (e.g. TGF-β, vascular endothelial growth factor, insulin growth factor-1, BMP-2, oxytocin) and chemical reagents (e.g. hydrogen peroxide (H_2O_2), dimethyl sulfoxide (DMSO), nitric oxide, 5-azacytidine) [125, 156, 314].

The major factor in inducing the formation of EBs is to prevent the stem cells from attaching to the surfaces of the culture vessels and allowing the suspended cells to aggregate. The most standard method to form EBs is via hanging drop and in static suspension culture to allow small-scale formation of aggregates. A balance is maintained between ES cell aggregation essential for EB formation and prevention of EB agglomeration by these culture systems [196, 315]. The method most routinely used to prepare uniform sized EBs is that of hanging drops; however it suffers from the disadvantage of being labor-intensive and that mass production of EBs cannot be carried out via this method, hence hindering the use of differentiated iPSCs for therapeutic applications [316]. Suspending the cells in a static petri-dish can lead to mass EB production - the drawback in this case is that the EBs often fuse together to form large aggregates causing a negative effect on the cell proliferation and differentiation but with increased cell death. Hence, as far as industrial applications are concerned, these methods are restricted because of their complication and difficult manageability [317]. Other bioreactors that facilitate large-scale production of EBs are: (a) stirred suspension culture (spinner flasks), but which cause excessive shear forces enough to damage the cultured cells [318, 319], (b) rotating cell culture system (RCCS), but is characterized by EB immobility due to extremely low fluid shear stress and oxygenation by diffusion [205]. Also, using the suspension culturing methodology using conventional low-adherence vessels limited control over the uniformity and shape of EBs [205]. Even though the preliminary studies are encouraging, further in-depth studies are required in order to fully harness the differentiation capability of the iPSCs in a bioreactor setting to produce clinically relevant cells for cellular therapy.

Liquid marble description was first introduced by Aussillious and Quere [221], wherein a small amount of a liquid rolled along on a bed of very hydrophobic powder. The powder

coats the liquid, separating the liquid core from the supporting surface, while allowing gas exchange between the interior liquid and the surrounding environment [320]. This marble system has proven to be a very effective micro bioreactor system in order to study the culturing and differentiation of ESCs [219, 220]. LM contain chemical and biological reactions, hence, they are being used as miniaturized bioreactors [223, 321]. The chances of contamination is very low since there is no direct contact between the liquid core and the supporting surface, thus proving useful for a variety of therapeutic applications [321]. Unlike the rotation and conventional stirred reactors, this marble reactor system does not induce shear stress on the cells thereby increasing the culture and differentiation efficiency of the ESCs [219].

Given the fact that iPSCs are the new ESCs, as they can be patient specific, they will prove to be a promising resolution for clinical immune responses in cell transplantation [322]. Also, using the LM micro bioreactor can facilitate the generation of larger number of EBs which can be used for generating iPSCs derived EBs, which when given the right conditions will differentiate into cardiac/neural lineages. This chapter will report the use of the novel liquid marble as a micro bioreactor to produce EBs from iPSCs and, for the first time, study its feasibility to further differentiate the EBs into lineage specific cells *in vitro*. Examining their morphological characteristics, gene and protein expression provides an assessment of the differentiation ability of the resulting EBs. This study will demonstrate that a liquid marble micro bioreactor system is a very promising platform to facilitate EB differentiation into cardiac and neural lineages. This is the first study that explored the potential of the liquid marble bioreactor in using iPSCs for the production of EBs and analyzes their differentiation capability.

Aims:

In this chapter we aimed at the following

1) Generation of EBs using murine iPSCs in the liquid marble bioreactor;
2) Explore the differentiation ability (cardiac and neural) of the EBs generated in Aim1;
3) Characterization of the differentiated cells obtained in Aim 2.

Section 5.2 Materials and Method

5.2.1 Tissue Culture

Feeder dependent murine MEF-iPSCs#6 (129/Sv) cell line was used for this work. This cell line was derived from OG2MEFs [236], which possesses an Oct4-GFP transgene under the control of the endogenous Oct4 promoter. For expansion, iPSCs were cultured in Dulbecco's modified eagle medium (Gibco) supplemented with 15% FBS (JRH Biosciences, Australia), 1% non-essential amino acids (NEAA) (Invitrogen), 1% GlutaMAX™ (Invitrogen), 0.5% penicillin-streptomycin (Invitrogen), 0.1 mMβ-mercaptoethanol (Invitrogen), and 1,000 Uml^{-1} ESGRO leukemia inhibitory factor (mLIF, Chemicon, Australia) **(section 4.2.1)**. The medium was filtered through a 0.22 µm filter for sterilization and was stored at 4°C for up to a fortnight. Cells were cultured on mitomycin-C treated MEFs **(Appendix 1)** at 37°C in a humidified 5% CO_2 incubator and were passaged every 2-3 days on feeders. The GFP expression of the cells was monitored using an IX71 Olympus epifluorescence microscope.

5.2.2 Preparation of cell containing liquid marble micro-bioreactor

MEF-iPSCs#6 were cultured in iPSC medium as described in **section 5.2.1,** up to 70-80% confluency, washed with DPBS-/- and trypsinized into single cells using Tryple^{Tm} express (Gibco, Life Technologies, Australia).

Feeder depletion: Care was taken to gently get the iPSC colonies off the feeder layers after Tryple addition. The dispersed cells were then mixed with 10 ml of warm iPSC culture medium and kept in a 10 cm dish in the humidified incubator for 45 minutes to let the feeders settle. Later, the cells in the supernatant were carefully pipetted and used for generating marbles.

Detailed methodology for the formation of marbles is described in **section 4.2.2** and elsewhere [219, 220]. For the purpose of this study, MEF-iPSCs#6 were used. Briefly, to generate LMs, 2×10^4 feeder depleted MEF-iPSCs#6 were suspended in 300 µl of differentiation medium **(section 2.2.3)** consisting of DMEM (Gibco, Life Technologies, Australia) supplemented with 15% FBS (JRH Biosciences, Australia), 1% non-essential amino acids, 1% GlutaMAX™ (Gibco, Life Technologies, Australia), 0.5% penicillin-streptomycin (Gibco, Life Technologies, Australia), 0.1 mM β- mercaptoethanol (Gibco, Life Technologies, Australia) but without mLIF. This drop of cell suspension was placed onto a PTFE (35 µm particle size, Sigma, Australia) powder bed inside a petri dish (60 mm

diameter) using a micropipette. When the drop of cell suspension was rolled on the hydrophobic powder bed, the powder particles wrapped the surface of the drop, thus, forming the LM. The petri dish was then placed inside a larger petri dish (100 mm diameter) containing sterile water to minimise evaporation from the liquid marbles, following which the set of dishes was covered and kept inside a humidified incubator. The iPSCs were allowed to aggregate to form EBs within the liquid marble over a period of 7 days.

5.2.3 EB Morphology and Pluripotency Characterization

iPSCs-containing liquid marbles were prepared and allowed to incubate for a period of 7 days. EB samples were taken from the liquid marbles at day 3, 5 and 7. The morphology and GFP expression of EBs were monitored using optical and epifluorescence microscopy (Olympus IX70 microscope). Both phase-contrast and epifluorescent images were captured. The collected EBs were further dissociated into single cells using Tryple^{Tm} express; the GFP expression of these cells was quantified using fluorescence-activated cell sorting (FACS) at days 0, 3, 5 and 7, respectively.

5.2.4 In Vitro EB differentiation to cardiac and neural lineages

In order to initiate differentiation, 7 days old EBs were removed from the marbles and transferred onto 0.1% gelatin-coated 24-well plates and cultured in MEF medium (**section 2.2.2**) for further analysis. The plated EBs were examined daily for the contractile activity and appearance of neuronal like extensions. Medium was replaced every three days.

5.2.5 Reverse-Transcription (RT) Analysis

The gene expression analysis of the EBs and the cells undergoing differentiation was carried out using the reverse-transcription (RT) PCR. LM suspended EBs were subjected to RT-PCR analysis using various differentiation markers. EBs were allowed to form inside the liquid marbles for 7 days, and were then retrieved from the liquid marble for RT-PCR. The EBs were analysed for differentiation markers at time points D 0, 3, 5, 10 and 13 days. Cells were harvested with Tryple^{TM} express; the resulting cell pellets were snap chilled at -80 °C prior to analysis. For RT-PCR, whole RNA was isolated from cells using the RNeasy kit (Qiagen, Australia) according to the manufacturer's instructions. The isolated RNA was subjected to RQ1 DNase (Ambion, Australia) treatment to remove any contaminating genomic deoxyribonucleic acid (DNA). Complementary DNA (cDNA) was generated using the Superscript III enzyme (Life Technologies, Australia) according to the manufacturer's

protocols. The cDNA samples were amplified using murine cardiac and neuron specific primers. β-actin was used as the internal reference gene. The primer sequences used for the detection of differentiation markers are listed in **Table 6**. The PCR products were size fractionated using 1% agarose gel electrophoresis at 110 V for 1 h.

Table 6 PCR primer sequences used to detect the three germ layer markers in EBs

Gene	Primer Sequence	Annealing Temperature (°C)	NCBI Accession number
Brachyury	F: CAT GTA CTC TTT CTT GCT GG R: GGT CTC GGG AAA GCA GTG GC	55	NM_009309.2
Nestin	F: TCT GGA AGT CAA CAG AGG TGG R: ACG GAG TCT TGT TCA CCT GC	58	NM_016701.3
Nkx2.5	F: ACACCCACGCCTTTCTCAGTCAAA R: CGACAGGTACCGCTGTTGCTTGAA	58	NM_008700.2
Gata4	F: TCT CAC TAT GGG CAC AGC AG R: GCG ATG TCT GAG TGA CAG GA	56	NM_008092
FoxA2	F: TGG TCA CTG GGG ACA AGG GAA R: GCA ACA ACA GCA ATA GAG AAC	58	NM_001291065.1
β-actin	F: CAC CAC ACC TTC TAC AAT GAG C R: TCG TAG ATG GGC ACA GTG TGG G	58	NM_007393.3

5.2.6 Immunocytochemistry

Cells were characterized for protein markers using immunostaining. Incubated cells were first fixed in 4% paraformaldehyde for 30 minutes and washed three times with 1% BSA in DPBS+/+. Fixed cells were incubated with blocking solution (5% goat serum, 1% BSA in DPBS+/+) for 1 hour at room temperature. Cells were then incubated overnight with cardiac Nkx2.5 primary antibody (Santa Cruz, ThermoFisher Scientific, Australia) diluted (1:500), cardiac cTnT primary antibody (Abcam, Sapphire Bioscience, Australia) diluted (1:500) and neuronal Nestin primary antibody (Chemicon) in blocking solution at 4°C.

Next, the cells were washed three times with 1% BSA in DPBS+/+ and then incubated at room temperature for 1 hour with Alexa 594 labelled secondary antibodies (Molecular Probes, Life technologies, Australia) at a concentration of 1:400 in blocking solution. Cells were washed three times with 1% BSA in DPBS+/+ for 10 minutes. Nuclei were counterstained with Hoechst (1 µg/ml, Sigma, Australia) dye. Cells were analysed by epifluorescence microscopy (IX71 Olympus microscope, Australia).

Section 5.3 Results and Discussions

5.3.1 Characterization of MEF iPS#6 cell lines

iPSCs are artificially induced into different states of pluripotency. iPSC induction generally involves the transfection of pMX-based retroviral vectors encoding the mouse DNA sequence of Oct4, Sox2, Klf4 and c-Myc (Addgene) into the packaging Plat-E cells using Fugene 6 transfection reagent [19, 20]. The retroviral vectors are powerful tools to introduce foreign genes into the host chromosomes. However, retroviral transcription is subject to silencing or attenuation in PSCs, including embryonic carcinoma (EC) cells, ESCs and early preimplantation embryos [323]. It is also a well-established fact that the retroviral expression is silenced after direct reprogramming of somatic cells into iPSCs [101-103]. As evident from **Figure 29,** RT-PCR analysis confirmed that the transgenes for Oct4, Sox2, Klf4 and c-Myc were silenced at passage 19 and 20.

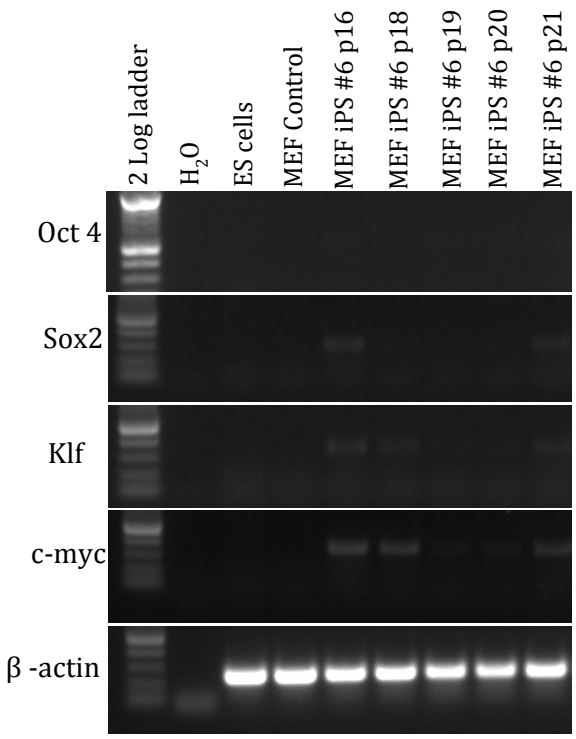


Figure 29 RT-PCR for the expression of transgenes Oct4, Sox2, Klf4 and c-Myc

Expression of Oct4, Sox2, Klf4 and c-Myc transgenes were analysed in the MEF iPSCs#6 at passage 16,18, 19, 20 and 21. β-actin was used as a housekeeping gene while ES cells were used as a negative control. H_2O control was included to eliminate any technical errors. The MEF iPSC #6 are feeder dependent cell lines hence a MEF control was included to rule out any expression of the transgenes in the iPSCs from the feeders. The MEF iPSC#6 at P19 and 20 exhibited complete silencing of all the four transgenes.

The RT-PCR analysis of passage 16 (data shown, **Figure 29**) and earlier (data not shown) showed the presence of transgenes, which upon culturing for further four passages (P18, 19, 20 and 21) led to the silencing of all the transgenes. As, evident from the **Figure 29**, the cells at P21 had been cultured from a different batch. The fact that they are expressing the transgenes simply explains the fact that cells when frozen had their transgenes active. This also points out a very important fact that the expression of the transgenes needs to be checked before the cells are used for differentiation studies. This is important because if the original population of iPSCs, had their transgenes active, the differentiation process will not be efficient or the differentiation might not happen altogether. Furthermore, it holds a significant value that the transgenes in the iPSCs should have been silenced before initiating any experimental work. Okita et al., and Jaenisch and Young, reported that the fully reprogrammed iPSCs consistently exhibited retroviral silencing or attenuation, whereas partially reprogrammed iPSCs express high levels of the exogenous pluripotent transgenes present in the retrovirus [101, 324]. Further work by Nakagawa et al., and Stadtfeld et al., concluded that the expression of exogenous Yamanaka factors is required only for the initial activation of self-renewal genes and that fully reprogrammed iPS cells maintain their pluripotency independent of transgene expression [102, 325]. Thus, based on the retroviral silencing, the MEF iPSC#6 P19 and 20 were confirmed to be fully reprogrammed iPSCs.

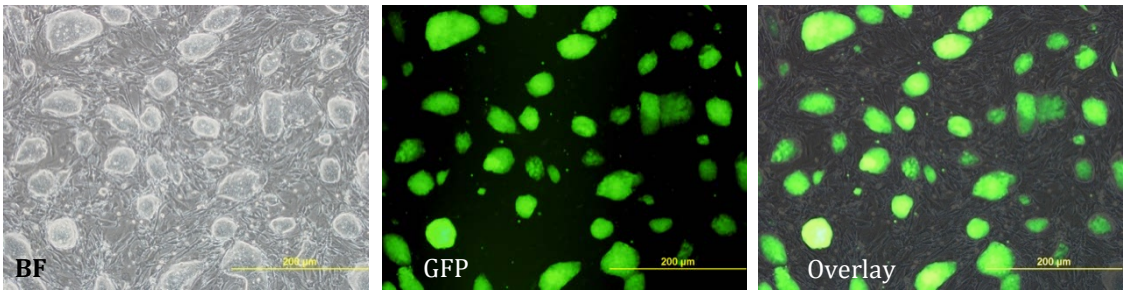


Figure 30 Morphology of MEF iPSC#6 P19

Representative phase-contrast (BF) and fluorescence microscopy (GFP) images showing the morphology of the MEF iPSC# P19 in culture. The morphology of the colonies is round and distinct. The colonies are GFP^{+} indicating the pluripotent nature of the iPSCs in culture. Scale bars represents 200μm

Further, on culturing, the morphology of the MEF iPSC#6 P19 was found to be very similar to that of the ESCs. The cells formed were round and as distinct colonies when plated onto mitomycin-c inactivated feeders. Also, since this cell line has been derived from the OG2 mice, they express GFP when the endogenous Oct4 gene gets activated. Oct 4 is known to be critically involved in the self-renewal of undifferentiated stem cells [326].

Figure 30 shows the phase contrast (BF), fluorescent (GFP) and the overlay images of the cells in culture after 4 days. The FACS analysis (see **Figure 32**) showed that 76.51% of the cells at Day 0, i.e. before setting up the differentiation experiment were GFP^+. The high levels of GFP fluorescence indicated that the cells have the endogenous Oct 4 activated and that they are highly pluripotent. It is important to start with undifferentiated pluripotent stem cells to ensure that their development into EBs is synchronous and to direct their downstream differentiation towards specific cell types.

5.3.2 Embryoid body formation and propagation in liquid marble

In order to realise the therapeutic potential of the iPSCs under study in this chapter, it is imperative to regulate their differentiation in a reproducible manner. One of the most common ways to differentiate iPSCs is via the formation of cell aggregates in non-adherent spheroids called EBs [135, 327]. The various cellular and molecular signals and events within an EB recapitulates numerous aspects of the embryo development and results in the differentiation of cells to the three germ layers; endoderm, mesoderm, and ectoderm, similar to gastrulation of an epiblast-stage embryo *in vivo* [328]. A previous study reports the formation of LM micro-bioreactor by self-assembly of the PTFE powder around a droplet of ESC medium wherein ESCs were cultured inside the droplet and EBs retrieved by day 3 [219]. In another study from our group, we reported the formation of EBs using LM as the micro bioreactor and further studying their cardiogenic ability. The study reported for the first time the generation of cardiomyocytes from the EBs obtained from liquid marble [220].

In this present study, we use LM as micro-bioreactors to generate EBs from MEF iPSC#6 and to investigate, for the first time, the capability of liquid marbles to facilitate differentiation of iPSCs via spontaneous EB formation. The morphology of cells harvested from the liquid marbles was assessed using optical microscopy. The MEF iPSCs#6 contain Oct4 as the pluripotency marker that drives the expression of GFP; hence a positive GFP expression upon culture is a direct indicator of that fact that the cells are pluripotent. Furthermore, as seen from **Figure 31** multiple EBs were obtained from each marble.

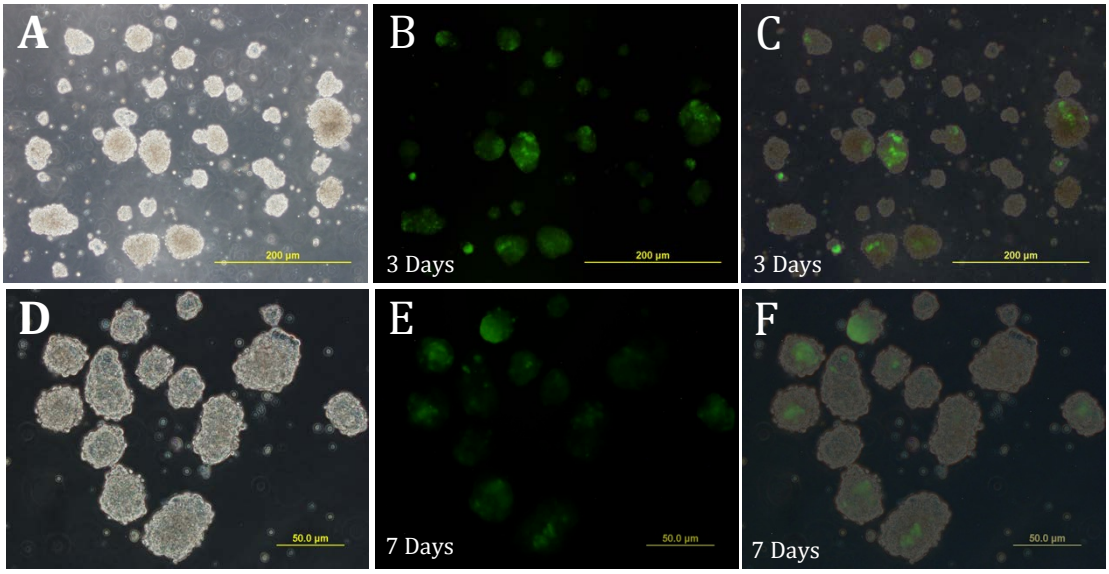


Figure 31 Representative phase contrast and fluorescence microscopy images showing the morphology of EBs obtained from liquid marble

The EBs formed by allowing MEF iPSC#6 to aggregate inside liquid marbles for (A, B, C) 3d (scale bars represent 200 µm) and (D,E,F) 7d (scale bars represent 50µm). B, E) Fluorescence microscopy images show the expression of GFP under the control of Oct4. C,F) Merge images of phase contrast and GFP fluorescence.

Representative images in **Figure 31 (A, D)** show that after cultivating the iPSCs in LM for 3 and 7 days, the cells aggregate and EBs are formed. The EBs generated exhibits a round and compact shape. Also evident from **Figure 31 (B, C)** is that the iPSCs, while aggregating to form clusters, destined to form EBs, do not lose complete GFP fluorescence, indicating that the cells do not lose their pluripotency completely by day 3 even though the EBs have formed. Further, upon allowing the EBs to expand for 7 days **Figure 31 (E, F)**, an increase in the size of EB was observed, suggesting that the LM system is allowing the EBs to expand. Also, a significant loss of GFP was observed (fluorescence), stipulating the loss of pluripotency and that there was an initiation of differentiation of ES cells within the liquid marble. Another significant observation was the increase in the size of the EBs during the course of their formation in the LM. The graph in **Figure 32** depicts the growth of the size of the MEF iPSCs#6 EBs over a period of 5 days in the LM. As evident from the graph, the EBs shows an exponential increase in the size of the EBs over the course of 5 days indicating that the LM allows a suitable environment for the propagation of iPSC EBs.

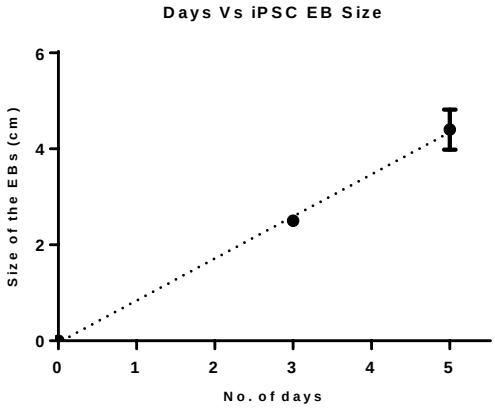


Figure 32 Increase in the size of MEFiPSC#6 EBs in the LM during their course of formation over a period of 5 days.

The LM provides a stable environment for the growth and expansion of the EBs over a period of 5 days. The EBs have almost doubled in size in the 5-day period in the LM.

MEF iPSC#6 were allowed to form EBs in LMs for 7 days; samples were collected from the liquid marbles at day 0 (control), 3, 5 and 7. FACS analysis was performed on the dissociated cells to quantify the number of GFP^+ cells in an EB, in order to examine their pluripotency. The representative FACS analysis profiles in **Figure 33** shows that 76.51% of the cells forming the EBs were GFP^+ at day 0 and the percentage reduced to 39.89% by day 3. Further, loss of GFP expression was observed by days 5 and 7 wherein the GFP percentage of the cells dropped down to 37.82% and 34.16% respectively. The gradual loss of GFP, as suggested by the FACS analysis and the fluorescence data lead us to the conclusion that the LM reactor system has the ability to allow the proliferation and differentiation of iPSCs and that it can be used to generate EBs in larger numbers.

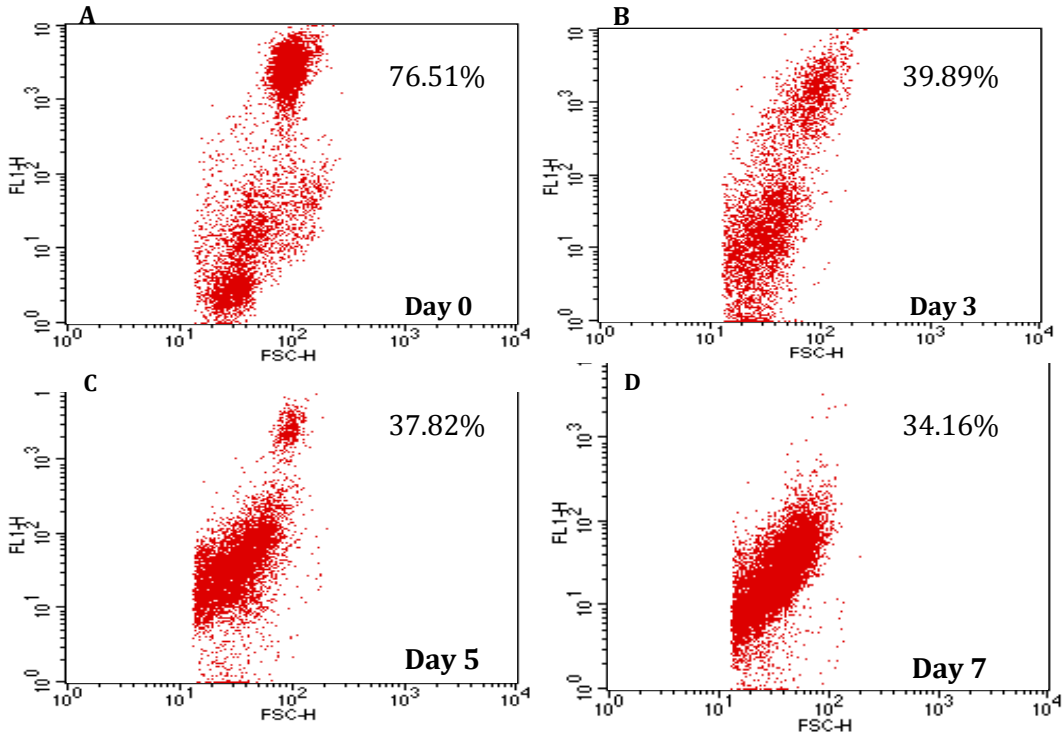


Figure 33 FACS analysis

GFP expression of liquid marble induced EBs of MEF iPSC#6 at Day 0 (control), 3, 5 and 7. Numbers indicate the percentage of GFP^+ cells. GFP is indicated on *x*-axis, and autofluorescence on *y*-axis. The percentage of GFP^+ iPSC cells reduced from 76.51% (see A) on day 0 to approximately 34% by day 7 (see D) in the marble culture system indicating that the cells are losing their pluripotency and starting to differentiate.

In order to promote further differentiation of our iPSC derived EBs to cardiac and neural lineages, the EBs harvested on Day 5 were plated onto 0.1% gelatinised 6-well dishes and cultured in MEF medium. As the EBs proliferated over time, the cells began to migrate and grow from the periphery of the EB. Heterogeneous cells with a different morphology could be seen derived from the attached EBs (**Figure 34**).

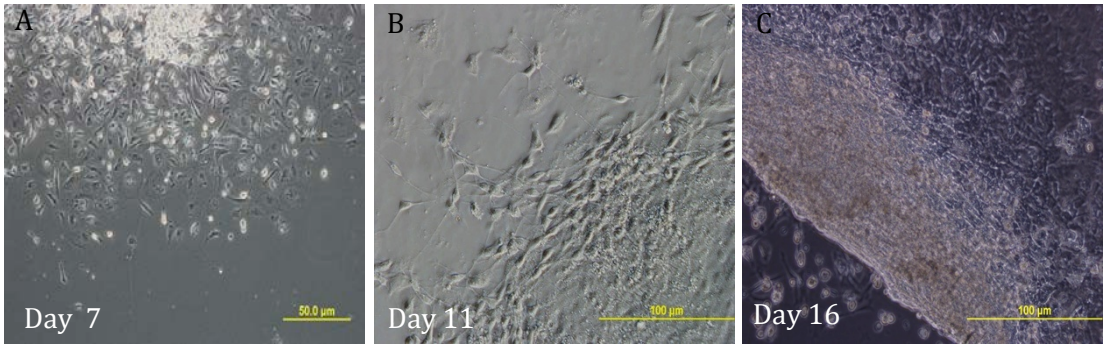


Figure 34 Phase contrast images of the neural and cardiac explant outgrowth

(A) Representative phase contrast image showing a plated EB attached to a gelatin-coated plate at Day 7 (2 days after plating)
(B) Representative phase contrast image of plated EB outgrowth at day 11, scale bars represent 100 µm. The neural processes can be clearly seen in the explant indicating differentiation towards the ectodermal lineage.
(C) Representative phase contrast image of the plated EB depicting the cardiac cluster (elongated, spindle shaped cells), at day 16, scale bar represent 100 µm. This indicates the differentiation progressing towards the mesodermal lineage.

Furthermore, 6 days after EB attachment, cells with extending processes (**Figure 34 B**) were observed in the plated EBs obtained from the LM. The appearance of these extensions are consistent with the previous studies where monolayer differentiation of the stem cells, in the absence of the supplements, produces similar processes indicating that differentiation is proceeding towards ectoderm and subsequently neural lineages [329]. The gene expression and protein analysis of these EBs as reported in **sections 5.3.3 and 5.3.4** give a conclusive proof of the presence of the neuronal progenitor cells. Other sets of the attached EBs were observed forming explants by day 10, which resembled more and more like the cardiac clusters by day 16 **(Figure 34 C)**. Few of those EBs had contractile activity which was a clear indication of the generation of the cardiac lineage. Gene expression and protein analysis of these plated EB revealed the positive expression of cardiac progenitor and mature cardiomyocyte markers **(section 5.3.3 and 5.3.4)**. This directed that the attached EBs were going down the meso-endodermal lineages, eventually forming the cardiomyocytes.

Based on the observations of the formation of EBs in liquid marble, loss of pluripotency over time and differentiation of the plated EBs towards the neural and cardiac lineages, there is strong evidence that the LM micro bioreactor is a facile and an efficient method to culture iPSCs and to study their differentiation ability *in vitro*. The iPS cell line under study in this chapter had a tendency to go towards the ectodermal and the meso-endodermal lineages. Also, as reported by Mauritz et al. [330], the iPSCs are less efficient in generating beating EBs hence, multiple iPSC lines need to be studied in this system in order to understand this observation as each cell line behaves in a different manner. This was beyond the scope of this

study as we were primarily investigating the feasibility of using the liquid marble bioreactor for the culture and differentiation of iPSCs. However, since this study is a stepping-stone for generating higher number of EBs and eventually lineage specific adult cells, it holds tremendous potential to explore the niche of iPS-cell based cellular therapy.

5.3.3 Reverse Transcription PCR analyses showed expression of marker genes typical for mesoderm, endoderm and ectodermal lineages in the differentiating EBs

In order to characterize the differentiation pathway of the EBs generated from a liquid marble, the gene expression of EB derived cells was semi-quantitatively determined using RT-PCR. EBs were allowed to form inside LMs for 3 and 5 days and further plated onto gelatinised dishes post day 5 with differentiation medium that contained no growth factors. iPSCs and iPSC derived EBs were harvested at the following time points: Day 0, 3, 5, 10 (plated), 13 (plated). MEFs were used as a negative control, since the iPSCs were cultured on feeder cells and to rule out any gene expression from MEFs. Also, a no template control was used to exclude any false positives results based on contamination. The time dependent expression of various lineage markers during the course of differentiation is shown in **Figure 35.**

To confirm the pluripotency of the produced EBs, it was important to demonstrate that the EBs are able to generate representatives of all the three developmental germ layers; ectoderm (Nestin), mesoderm (Brachyury, Nkx2.5) and endoderm (Gata4, FoxA2). As a preliminary assay, it was determined whether the EBs expressed germ layer-specific genes and this formed the basis of the choice of our target genes. Nestin and Nkx2.5 were chosen to represent the neural and cardiac lineages, respectively, which arose as a result of the late stage differentiation of the EBs to generate neurons and cardiomyocytes.

To control for the presence of different cellular intermediates and key regulators and to identify neural and cardiac progenitors, a series of marker genes were included in the study. Brachyury is a protein essential for early mesoderm formation [331] and the positive expression of this marker suggests the presence of early mesodermal cells in the EBs. Gata4 is a regulator of early cardiogenesis [287] and it is mostly expressed in pre-cardiac mesoderm; overexpression of Gata4 is known to up-regulate the expression of transcription factor Nkx2.5. Nkx2.5 is known to interact with Gata4 and works in a positive feedback loop with the Gata transcription factors to regulate the formation of cardiomyocytes [244]. FoxA2 is required for notochord formation and is extensively involved in the development of multiple endoderm-derived organ systems such as the liver, pancreas and lungs. Thus, a positive expression of this gene indicates that the differentiating cells are expressing endodermal

lineages [332]. Nestin is a class VI intermediate filament protein that is known to a neural stem/progenitor cell marker [333]. This is an early ectodermal marker and its transient expression in the early differentiation is a critical step in the neural differentiation pathway [334].

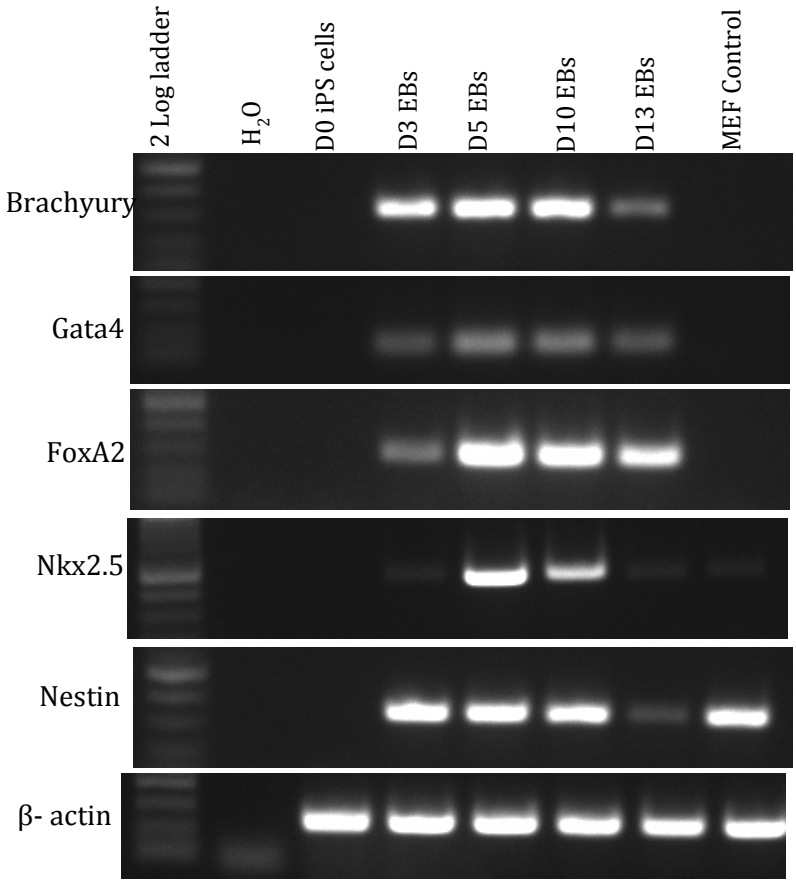


Figure 35 RT-PCR analysis for the differentiation markers indicative of the three germ layers

Differentiation of the MEF iPS#6 cells in liquid marble induced mRNA expression of the markers for ectoderm (Nestin), mesoderm (Brachyury, Nkx2.5) and endoderm (Gata4, FoxA2). RT-PCR was performed on the undifferentiated cells and cells at different points of time after induction of differentiation. To exclude the false positives, MEF control was included and H_2O control was included to rule out any contamination errors. Positive expression of Nkx2.5 and Nestin indicate that the EBs have differentiated towards the cardiac and neural lineages respectively. β- actin was used as the housekeeping gene.

Our RT-PCR analysis **Figure 35** revealed a strong expression of the lineage markers. Even though statements about differences in expression based on semi-quantitative RT-PCR should be judged with caution, our data suggest an up regulation of Brachyury, Gata4 and FoxA2 in day 3 EBs, indicating the appearance of the cells expressing markers of the germ layers. Their expression was maintained through day 5 and day 10 but then down regulated by day 13. Nkx2.5 gene expression was found to increase as the differentiation progressed to day 5 and 10 but then eventually decreased by day13. Positive expression of Nkx2.5 led us to conclude

that the differentiation of the EBs had moved towards generating cardiac progenitor cells [244]. Furthermore, Nestin expression was found to be highly upregulated in day 3 through day 10 EBs and then down regulating by day 13, thus suggesting the formation of neuronal progenitor cells during the course of our differentiation strategy [334]. β- actin was employed as the housekeeping gene.

Our data suggest that the LMs provide a very stable environment for the formation of EBs from iPSCs, which upon further differentiation show the gene expression characteristic of both the neural and cardiac lineages. It is suggested that the liquid marble system provides extensive cell-to-cell interactions during the formation of EBs that help in the stimulation of the early cardiac and neural transcription factors [28].

5.3.4 Immunocytochemistry analyses reveal the appearance of neuronal progenitors, cardiac progenitors and mature cardiomyocytes

EBs were collected from the LMs at day 5 and plated onto gelatinised dishes for further differentiation. Immunostaining was performed on the EBs to check for the expression of the cardiac and neural proteins. As seen in the representative images in **Figure 36** (top and middle panel-Nkx2.5, Nestin), positive expression of Nkx2.5 (AlexaFlour 594) and Nestin (AlexaFlour 594) was detected at day 8 of the differentiation in plated EBs. Nkx2.5 is the earliest known marker for cardiogenesis in the vertebrate embryo and is expressed in the cardiogeneic progenitor cells during myocardial development [28, 137, 335]. Similarly, Nestin is known to be a neural progenitor marker and its early expression indicates the differentiation pathway towards neural lineages [333, 334]. This leads us to conclude that the cells in the iPSC-EBs obtained from the LMs are successfully generating cardiac and neural progenitor cells. This is a very important result as this is giving us a stronger platform to have confidence that the liquid marble is a highly efficient system to be used for the generation of lineage specific cells.

Further immunocytochemical analyses of the day15 plated EBs revealed positive expression of cTnT (AlexaFlour 594) **Figure 36** (bottom panel- cTnT). cTnT is a marker for mature cardiomyocytes [114] thereby confirming the successful generation of mature cardiomyocytes from the iPSC-EBs. Thus, based on the positive expression of Nkx2.5 and cTnT, the generation of cardiac progenitors and mature cardiomyocytes was confirmed. Furthermore, expression of the Nestin protein confirmed the appearance of the neural lineages in the EBs.

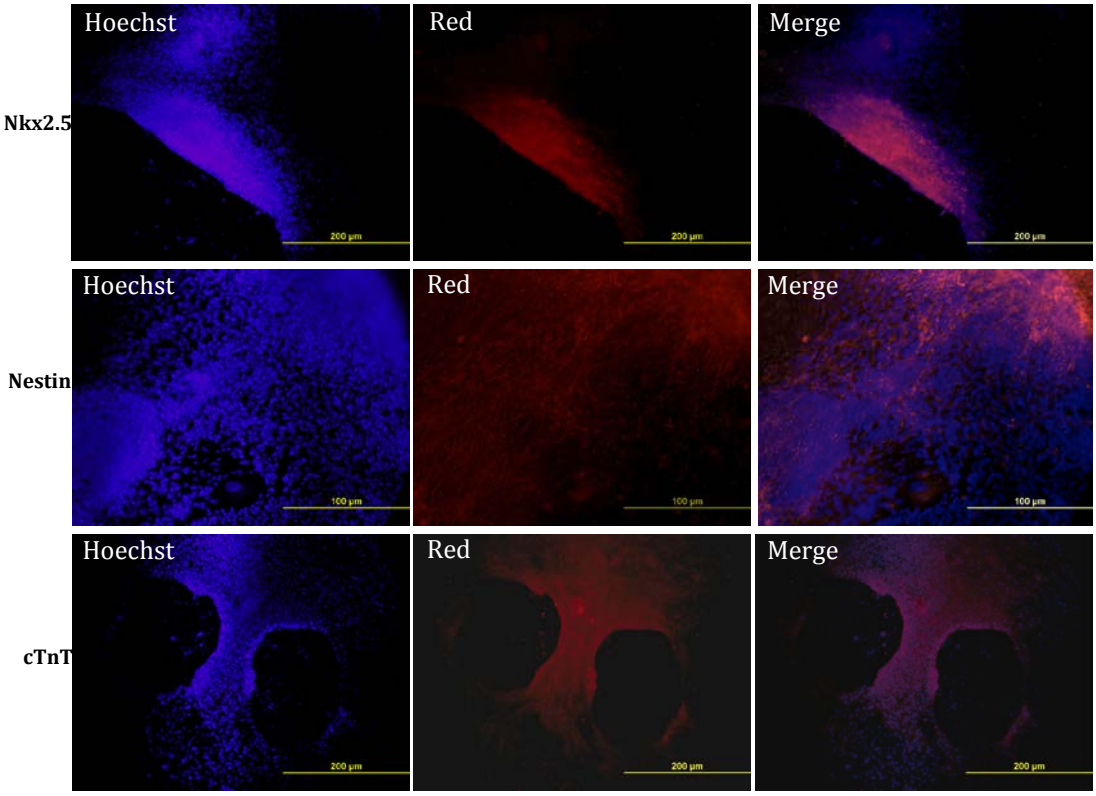


Figure 36 Immunostaining analysis of cardiac and neural proteins

Nkx2.5 (cardiac progenitor) (at day 8), Nestin (neural progenitor) (at day 8) and cTNT (mature cardiomyocytes) (at day 15) of differentiation, Nuclei were counterstained with Hoechst (blue). Scale bars represent 100 μm (Nestin) and 200 μm (Nkx2.5 and cTnT).

Thus, EB derived cells expressed both neural and cardiac proteins, thereby suggesting that functional EBs are generated using this marble reactor system and that this could prove to be a new method to provide continuous source of patient specific lineage cells for cell based therapeutic applications.

5.3.5 Summary of Results

This chapter has successfully explored the possibility of using liquid marbles as a micro bioreactor system in order to produce EBs that can be differentiated further to generate cardiac and neural lineages. This study holds immense therapeutic potential as it can be used to differentiate patient specific iPSCs that can generate either cardiomyocytes or neurons and these cells once introduced into the patients will help to overcome the immune rejection issues. Based on the molecular analysis of the differentiated EBs, this system generates functional EBs expressing the three germ layer markers for ectoderm (Nestin), mesoderm (Brachyury, Nkx2.5) and endoderm (Gata4, FoxA2). This suggested that the LM system is a viable method for generating EBs. Upon further differentiation to the cardiac and neural lineages these cells expressed proteins of cardiac and neural progenitors (Nk2.5 and Nestin)

and also of mature cardiomyocytes (cTnT) thereby giving a conclusive proof that liquid marble micro bioreactor is a facile and an economical method to produce viable EBs and that these EBs hold the capacity to generate cells of multiple lineages.

Section 5.4 Conclusion and future directions

Permanent cell lines of iPSCs, and our increasing ability to direct them into multiple cell types for therapeutic potential, hold enormous promise for future regenerative medicine. Since its birth in 2006, iPSCs have come a long way travelling an arduous path of "patient specific cell therapy". iPSCs offer the advantage of development of cells from any adult individual, which increases the possibility of curing diseases with cell or tissue grafts with a perfect histocompatibility match. This potential calls for efforts to develop new methods of bioprocessing and commercialization of iPS cell and tissue engineering products that can transform breakthroughs from research bench to patient bedside. Laboratory-scale suspension cultures in hanging drops or petri dishes are useful for initial optimization but need to be up-scaled to a significant level to improve high throughput EB production. In this study, we investigated the successful ability of a novel LM micro bioreactor to culture and propagate iPSCs and further induce EB formation and their subsequent differentiation into cardiac and neural lineages. The marbles formed by rolling a cell suspension drop on a hydrophobic powder bed, in our case PTFE, allowed aggregation of the cells in the core of the marble leading to the formation of large number of EBs in one marble. These EBs, when plated and differentiated further were able to give rise to the cells of the three germ layers. Gene expression and protein studies revealed the generation of neural and cardiac lineages post differentiation.

This study holds significant importance, as it is for the **first time that the iPSCs have been used in a liquid marble reactor system to generate lineage specific cells**. This is a very cost effective and simple method to produce higher number of EBs as compared to hanging drops. Since, the system does not hamper the genetic profile of the iPSCs, it will prove to be a promising method in producing higher number of EBs in a lab setting. The lineage specific cells produce via this method can be a continuous source of donor cells for cell replacement therapies in either neural or cardiac diseases. Furthermore, the differentiated cells generated via this technique could be used for drug testing studies, in order to explore the area of understanding the impact of new drugs discovered. In addition, future studies in the area of improving liquid marble micro bioreactor should focus on optimizing the yield of lineage

specific differentiated cells as well as developing a more efficient strategy to increase the production of EBs. This can be achieved by introducing growth factors like retinoic acid that are known to enhance the spontaneous differentiation methodologies. Another aspect that can be considered for prospective studies is to try and work in serum free conditions like using knock out serum that will help in the generation of clinically relevant cells, as they would stand at par with the “Good Clinical Practices (GCP)” requirements. Having said that, the preliminary success in generating functional EBs from iPSCs in the marble bioreactor system has provided us with a highly optimistic mind to sail across the bench work hurdles to the land of ultimate clinical realization.

Chapter Six

Overall Discussions, Conclusions and Future directions

Section 6.1 Overall Discussions and Conclusions

In this thesis, Nkx2.5 TALENs and ObliGaRe donor plasmid, which can be used for the manipulation of ESCs and iPSCs were generated. The plasmids, when transfected into the pluripotent cells, will allow the NHEJ repair mechanism to target the cardiac progenitor Nkx2.5 gene of the cell's genome and allow the insertion of IRES-mCherry-CMV-IRES-hyg3 downstream of the gene. This will allow the expression of mCherry, once the cells start to express Nkx2.5 during differentiation and thus fluorescing red. Generation of TALENs and ObliGaRe donor plasmid presents a powerful tool that allows us to produce knock-in cell lines with minimum off target hits [252, 259]. This is a very efficient strategy that allows for the enrichment of obtaining cardiac progenitors from a heterogeneous pool of differentiated cells post spontaneous differentiation. Cardiac progenitors when sorted and cultured ahead would allow the generation of mature cardiomyocytes. In all, TALENs represents a very robust method for producing targeted cell lines that would aid in the generation of a highly pure population of cardiomyocytes.

This ability was explored in **chapter 3** of this thesis where Nkx2.5 TALENs and the donor plasmid were generated successfully. Nkx2.5 gene, being a cardiac progenitor was selected for targeting, as it will aid in enriching the cardiac population obtained post differentiation. Few studies conducted previously have reported the successful selection of this gene to sort cardiac progenitors [150, 335]. Hence, given the literature, Nkx2.5 became an obvious choice of gene to explore the efficiency of generating TALENs in this study. TALENs provide high target site specificity, enabling researchers to make precise genetic alterations. Also, it was found in our study that the off target activity was very less **(section 3.3.2)**, which makes this a highly reliable method to generate knock in cell lines. This is consistent with the studies conducted by Park et al, where no mutagenic activity was detected at other genome sites homologous to the target site in the iPSC line that was edited [259].

DNA fragments/modules are efficiently and seamlessly assembled in the Golden gate assembly **(section 3.3.3)** where multiple inserts could be inserted into a single vector backbone using only the sequential or simultaneous activities of the type IIS restriction enzyme and T4 DNA ligase [176, 336-338]. This ability allowed us to generate the Nkx2.5-DD and RR plasmids **(section 3.3.3)** that will generate the DSB in the genome of the ESC/iPSCs. Based on the sequencing results obtained, this study was able to generate both the DD and RR TALENs.

In order to insert the reporter cassette (IRES-mCherry-CMV-IRES- hyg3) into the site of DSB, it was imperative to design a delivery vector, which in our case was generated using the ObLiGaRe strategy. Once the DSB is generated by the TALENs at the desired site in the genome, the repair mechanism is initiated to mend the break. Repair is largely accomplished by NHEJ in which the ends are processed and ligated together, allowing for the insertion of nucleotides [339]. We took advantage of the obligated heterodimeric property of the TALENs [247, 340] and designed our donor ObLiGaRe plasmid [248]. Based on the sequencing results obtained, we were successful in the generation of the ObLiGaRe donor plasmid. ObLiGaRe is a strategy that is broadly applicable across different cell types and provides an additional approach for genetic engineering [248].

The TALENs and the ObLiGaRe donor generated in chapter 3 can be used to transfect multiple ESCs and iPSC lines, which when used to study cardiac differentiation will allow the selection of a highly pure population of cardiac progenitors and will thus eventually lead to the generation of a higher number of cardiomyocytes. Before this approach was taken further, it was thus imperative to understand how the ESCs and iPSC lines differentiate *in vitro* conditions. The study carried out in **chapter 4** explored very thoroughly the generation of cardiomyocytes from Oct4B2 ESCs using a novel LM micro bioreactor. This study revealed that the marble system not only sustains the growth of ESCs but also helps in the generation of larger number of EBs when compared to a suspension or hanging drop methodology. Moving a step up in this ladder, **chapter 5** explored the ability of the same micro bioreactor to sustain the growth of iPSCs and also explore its ability to generate functional EBs into the cardiac and neural lineages. Both the studies carried out in chapter 4 and 5 are the first of their kinds in the sense that no earlier reports of the use of LM bioreactors for generating functional EBs has been made.

Thus, it was necessary to understand how the PSCs undergo differentiation. The thesis studied intensely the ability of PSCs to generate cardiac and neural lineages. ESCs and iPSCs possess an outstanding capacity of regeneration due to their potential of infinite expansion and efficient differentiation into most somatic lineages [19, 81]. In order to harness the therapeutic potential of PSCs, it becomes imperative to understand the differentiation pattern followed by these cells. Differentiation of stem cells into cells for regenerative medicine is often initiated by EB formation. EBs recapitulates the early stages of embryonic development and therefore provides a unique system for differentiation. The EBs begin as cell aggregates, which when allowed to spontaneously differentiate transforms into three-dimensional cystic bodies comprising the three germ layers, ectoderm, mesoderm and endoderm. Thus, EBs are used as a model system to study early embryonic development [341, 342].

Many exogenous chemicals have been used to stimulate the increase in the number and efficiency of EBs during aggregation [136, 328, 343]. Addition of various growth factors increases the cost of production thus making it an expensive strategy. This thesis studied a novel and an economical methodology of generating a liquid marble micro bioreactor, wherein in a drop of cell suspension was coated with PTFE powder particles to allow the aggregation of cells to form multiple EBs in one marble. This methodology was tested for use with both ESCs and iPSCs and it was observed that EBs were formed in larger numbers in both the cases. Even though that the formation of EBs is an indication of initiation of differentiation itself, the EBs obtained from the marble micro bioreactor system when differentiated further nonetheless had the potential to form the cardiac and neural lineages. Thus, it was established that the LM micro bioreactor is a very economical and efficient system to generate functional EBs that are differentiated further to produce therapeutically relevant cells.

In-depth studies were carried out in **chapter 4** to produce cardiomyocytes from ESCs. A 20K ESCs/300µl media drop produced the highest number of EBs. The EBs were extracted at day 5 and plated onto the gelatinized dishes, which spontaneously differentiated ahead to produce mature cardiomyocytes by day 15. Various functional, molecular and protein analyses were carried out at various stages of differentiation to obtain a conclusive proof of the generation of the cardiac lineage. EBs plated on day 5 showed regular contractile activity by day 8 and 9. The highest percentage (64%) of beating clusters were observed around day 9 which is highly consistent with the literature where it is known that contractile activity is observed *in vivo* by day 9 [46, 335, 344]. The rhythmic contractile activity is considered as a functional marker during the differentiation, thus implying that the LM generated EBs have the ability to produce functional cardiac cells. Furthermore, gene expression analysis revealed that the down regulation of the pluripotent gene Oct4 and the up regulation of the cardiac progenitor gene Nkx2.5 by day 8. Further, differentiation by day 15 revealed positive expression of the mature cardiomyocyte marker like α-actinin. Expression of Nkx2.5 and α-actinin in our study indicates that the EBs have adopted a cardiac lineage thereby forming cardiac progenitors and mature cardiomyocytes, a fact supported by many studies [38, 126, 150, 167, 345]. These data were supported by the protein analysis where plated EBs were analyzed for cardiac progenitor (Nkx2.5) and mature cardiac marker (cTnT) proteins. The positive expression of the Nkx2.5 and cTnT proteins led to the final conclusion of the successful differentiation strategy wherein ESCs cultured in LMs and allowed to form EBs, can produce functional cardiomyocytes. Together, these results demonstrate that the LM technique is an easily employed, cost effective, and efficient approach to generate EBs and to facilitate their cardiogenesis.

As a continuation of exploring the liquid marble world in generating functional EBs, the use of iPSCs was investigated in **chapter 5**. iPSCs come with an added advantage of being patient specific and carry no ethical baggage [346, 347]. These characteristics make iPSCs an attractive choice and the need of the hour. Promising experiments in mice have suggested that the treatment of genetic disorders is feasible by the use of iPSCs. Specifically, Jaenisch and colleagues [348] demonstrated that iPSCs could be used to counter the effects seen in animal models of sickle cell anaemia.

Keeping this in mind, we hypothesized that by combining the ease of generating liquid marbles and the alluring characteristics of iPSCs, it will be a significant step to obtain functional EBs generated from iPSCs via the LM system. Thus, chapter 5 explores in full detail the generation and characterization of the iPSC–EBs, wherein the EBs showed full potential of generating the cardiac and neural lineages indicating that the marble system sustains the growth of iPSCs; furthermore, these iPSCs retain full ability to differentiate into multiple lineages. This was further confirmed by carrying out the molecular and protein analysis of the plated EBs. LMs were prepared using 20K iPSCs/300µl media drops and rolling them onto a PTFE powder bed. The EBs were retrieved from the marble on day 5 and plated onto gelatinized dishes for further differentiation. Morphologically, neural outgrowths were observed by day 7 in some EBs, whereas contracting cardiac clusters were observed in others by day 9. RT-PCR analysis confirmed the expression of various lineage markers and also that of the cardiac and neural progenitors. There was an up regulation of the cardiac (Nkx2.5) and neural (Nestin) progenitor genes, which were initially not expressed, in the undifferentiated iPSCs. This led to the conclusion that the EBs generated had the potential to form cardiac and neural progenitors. These data was supported by the positive protein expression of the cardiac progenitor (Nkx2.5), mature cardiomyocytes (cTnT) and neural progenitors (Nestin). Given the evidence, chapter 5 led to the conclusion that the marble bioreactor system is an efficient method to generate multiple functional EBs that hold the capability to form cardiac and nerual lineages. This study could prove to be an important contribution to the iPSC field as this process can lead to the generation of various patient specific cell types, especially cardiac and neural, that would hold a relatively high therapeutic value.

In conclusion, this thesis has explored in its scope the generation of TALENs and ObLiGaRe donor plasmid to produce reporter cells lines; this would aid in the purification/enrichment of cardiac progenitors that would further help in the generation of clinically relevant mature cardiomyocytes. Furthermore, the differentiation ability of ESCs/iPSCs was carried out using a novel liquid marble bioreactor system, which generates a higher number of functional EBs,

and, when given the right conditions further generates mature cardiomyocytes. This is a remarkable result as generating a large number of cells for regenerative medicine is an urgent need at present. This study provides evidence of scale up of differentiated cells from EBs, *in vitro* conditions. This study sets a paradigm for future therapeutic application where the specialized cells differentiated from ESCs or iPSCs could then be used for cell therapies or combined with scaffolds to produce tissue construct and transplants for patients

Section 6.2 Future Directions

The thesis research indicates exciting future directions along the path of regenerative medicine. The field is expanding very rapidly and cell therapy has been highly instrumental in understanding human diseases, human biology and developing platforms for drug therapy and predictive toxicology. However, huge challenges remain before these applications can be used globally to develop strategies for treating cardiovascular diseases. Currently, monolayer differentiation produces $2x10^8$-enriched cardiomyocytes per T225 flask. Even though our study has explored the differentiation using a novel liquid marble micro bioreactor *in vitro*, higher scalability could be achieved using the same strategy on a multilayer platform thereby generating larger number of EBs to produce cells differentiated towards cardiac lineage. Other avenues of high throughput EB production *in vitro* could be explored, thus resulting in the generation of higher number of cardiomyocytes post differentiation.

TALENs have proven to an effective tool to enrich a heterogeneous pool of differentiated cells. The current research generated TALENs to target the cardiac progenitor in the genome. This will allow the purification and selection of cardiac progenitors. Further work needs to be carried out in this direction wherein both progenitor and mature cardiac gene could be targeted, thus enabling the selection of a more pure population of mature cardiomyocytes. Another future aspect of this work could be to do a comparison to the recently established method of CRISPR/Cas9 gene targeting system.

This thesis has studied in detail the advantage of using the novel liquid marble bioreactor wherein a huge number of EBs have been generated in a single run, hence increasing the probability of generating higher number of cardiomyocytes. Even though this study has produced remarkable results, much more needs to be understood about the mechanics as to how and why the micro bioreactor system works, and what more needs to be done to improve the efficiency from the present numbers. Since this study is still in its infancy, studying

multiple ESC and iPSC lines would give a huge boost in using this system for multiple cell types. This was beyond the scope of the thesis at this stage but optimization of using murine ESC and iPSCs have been thoroughly established. This study, being the first of its kind, has set a baseline of using the PSCs in a marble bioreactor setting.

Despite numerous technical advances in the area of liquid marble reactor design and study, much more needs to researched in the area of designing advanced and sophisticated monitoring platforms that will allow regular checks at the cellular level of parameters including temperature, pH and oxygen levels. This would allow us to understand the process of exchange of nutrients and gases through the powder coated liquid marbles and will help to improve on the conditions in which the marbles need to be set to produce more efficient EBs.

Another relevant future direction will be to study the effects of the generated cardiomyocytes *in vivo*. When engrafted, the new cardiomyocytes must induce vascularization to keep the graft alive as well as electrically couple with the existing cardiomyocytes and function in synchronization with the existing heart muscle. This would provide valuable cues about the viability and efficiency of the cardiomyocytes generated post differentiation and the results will help to create robust methodologies to generate therapeutically relevant cells.

Although the results in Chapter 5 are clearly promising and the iPSCs can be patient specific and therefore may provide a resolution for clinical immune responses in cell transplantation, the immunogenicity of iPSCs has been reported to be higher than predicted [230]. This has been clearly demonstrated by Zhao et al, hence, reprogramming iPSCs to reduce oncogenic risk and immune response should also be looked into in the future. Furthermore, the cardiac tissue engineering approach is based on the combining pluripotent cells and biological matrix which has shown very promising results, thus, encapsulating therapeutic cells in suitable hydrogels for intra-myocardial injection should also be looked into in the future.

Overall, this thesis has demonstrated that utilizing novel liquid marble bioreactor strategy can prove to be a valuable methodology in order to scale up the existing protocols to produce higher number of lineage specific cells. Also, it has been demonstrated that using gene-targeting strategies can help us to generate reporter cell lines that aid in the enrichment of specific cell types. Although there are many unresolved questions and many hurdles to overcome, the gene targeting and the novel liquid marble bioreactor strategy studied in this thesis has brought new hope for the development of novel regenerative therapies for the treatment of heart failure or other cardiac malfunctions.

Bibliography

1. 2011, A., *Cardiovascular disease: Australian facts 2011.* Cardiovascular disease series, AIHW., AIHW 2011. **35, Cat no. CVD 53. Canberra**.
2. Murry, C.E., H. Reinecke, and L.M. Pabon, *Regeneration gaps: observations on stem cells and cardiac repair.* J Am Coll Cardiol, 2006. **47**(9): p. 1777-85.
3. Kumar, D., T.J. Kamp, and M.M. LeWinter, *Embryonic stem cells: differentiation into cardiomyocytes and potential for heart repair and regeneration.* Coronary Artery Disease, 2005. **16**(2): p. 111-116.
4. Liau, B., D. Zhang, and N. Bursac, *Functional cardiac tissue engineering.* Regen Med, 2012. **7**(2): p. 187-206.
5. Schwarz, F., et al., *Intracoronary thrombolysis in acute myocardial infarction: duration of ischemia as a major determinant of late results after recanalization.* Am J Cardiol, 1982. **50**(5): p. 933-7.
6. Li, F., et al., *Rapid transition of cardiac myocytes from hyperplasia to hypertrophy during postnatal development.* J Mol Cell Cardiol, 1996. **28**(8): p. 1737-46.
7. Pasumarthi, K.B. and L.J. Field, *Cardiomyocyte cell cycle regulation.* Circ Res, 2002. **90**(10): p. 1044-54.
8. Clubb, F.J., Jr. and S.P. Bishop, *Formation of binucleated myocardial cells in the neonatal rat. An index for growth hypertrophy.* Lab Invest, 1984. **50**(5): p. 571-7.
9. Sdek, P., et al., *Rb and p130 control cell cycle gene silencing to maintain the postmitotic phenotype in cardiac myocytes.* J Cell Biol, 2011. **194**(3): p. 407-23.
10. Cooper, D.K., et al., *Report of the Xenotransplantation Advisory Committee of the International Society for Heart and Lung Transplantation: the present status of xenotransplantation and its potential role in the treatment of end-stage cardiac and pulmonary diseases.* J Heart Lung Transplant, 2000. **19**(12): p. 1125-65.
11. Braunwald, E. and E.M. Antman, *Evidence-Based Coronary Care.* Annals of Internal Medicine, 1997. **126**(7): p. 551-553.
12. McCarthy, P.M. and W.A. Smith, *Mechanical circulatory support--a long and winding road.* Science, 2002. **295**(5557): p. 998-9.
13. Rose, E.A., et al., *Long-term use of a left ventricular assist device for end-stage heart failure.* N Engl J Med, 2001. **345**(20): p. 1435-43.
14. Bardy, G.H., et al., *Amiodarone or an implantable cardioverter-defibrillator for congestive heart failure.* N Engl J Med, 2005. **352**(3): p. 225-37.
15. Dowell, J.D., L.J. Field, and K.B. Pasumarthi, *Cell cycle regulation to repair the infarcted myocardium.* Heart Fail Rev, 2003. **8**(3): p. 293-303.
16. Hansson, E.M., M.E. Lindsay, and K.R. Chien, *Regeneration next: toward heart stem cell therapeutics.* Cell Stem Cell, 2009. **5**(4): p. 364-77.
17. Gnecchi, M., et al., *Paracrine action accounts for marked protection of ischemic heart by Akt-modified mesenchymal stem cells.* Nat Med, 2005. **11**(4): p. 367-8.

18. Laflamme, M.A. and C.E. Murry, *Regenerating the heart.* Nat Biotechnol, 2005. **23**(7): p. 845-56.
19. Takahashi, K. and S. Yamanaka, *Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors.* Cell, 2006. **126**(4): p. 663-76.
20. Takahashi, K., et al., *Induction of pluripotent stem cells from adult human fibroblasts by defined factors.* Cell, 2007. **131**(5): p. 861-72.
21. Kehat, I., et al., *High-resolution electrophysiological assessment of human embryonic stem cell-derived cardiomyocytes: a novel in vitro model for the study of conduction.* Circ Res, 2002. **91**(8): p. 659-61.
22. Wobus, A.M., G. Wallukat, and J. Hescheler, *Pluripotent mouse embryonic stem cells are able to differentiate into cardiomyocytes expressing chronotropic responses to adrenergic and cholinergic agents and Ca2+ channel blockers.* Differentiation, 1991. **48**(3): p. 173-82.
23. Narazaki, G., et al., *Directed and Systematic Differentiation of Cardiovascular Cells From Mouse Induced Pluripotent Stem Cells.* Circulation, 2008. **118**(5): p. 498-506.
24. Mummery, C., et al., *Cardiomyocyte differentiation of mouse and human embryonic stem cells*.* Journal of Anatomy, 2002. **200**(3): p. 233-242.
25. Lee, M.Y., et al., *High density cultures of embryoid bodies enhanced cardiac differentiation of murine embryonic stem cells.* Biochemical and Biophysical Research Communications, 2011. **416**(1–2): p. 51-57.
26. Ou, D.B., et al., *The long-term differentiation of embryonic stem cells into cardiomyocytes: an indirect co-culture model.* PLoS ONE, 2013. **8**(1): p. e55233.
27. Mummery, C., et al., *Differentiation of human embryonic stem cells to cardiomyocytes: role of coculture with visceral endoderm-like cells.* Circulation, 2003. **107**(21): p. 2733-40.
28. Rajala, K., et al., *Cardiac Differentiation of Pluripotent Stem Cells.* Stem Cells International, 2011. **2011**.
29. Murry, C.E., et al., *Skeletal myoblast transplantation for repair of myocardial necrosis.* J Clin Invest, 1996. **98**(11): p. 2512-23.
30. Strauer, B.E., et al., *Repair of infarcted myocardium by autologous intracoronary mononuclear bone marrow cell transplantation in humans.* Circulation, 2002. **106**(15): p. 1913-8.
31. Kuethe, F., et al., *Lack of regeneration of myocardium by autologous intracoronary mononuclear bone marrow cell transplantation in humans with large anterior myocardial infarctions.* Int J Cardiol, 2004. **97**(1): p. 123-7.
32. Leistner, D.M., et al., *Transplantation of progenitor cells and regeneration enhancement in acute myocardial infarction (TOPCARE-AMI): final 5-year results suggest long-term safety and efficacy.* Clin Res Cardiol, 2011. **100**(10): p. 925-34.
33. Siminiak, T., et al., *Percutaneous trans-coronary-venous transplantation of autologous skeletal myoblasts in the treatment of post-infarction myocardial contractility impairment: the POZNAN trial.* Eur Heart J, 2005. **26**(12): p. 1188-95.

34. Perin, E.C., et al., *Transendocardial, autologous bone marrow cell transplantation for severe, chronic ischemic heart failure.* Circulation, 2003. **107**(18): p. 2294-302.
35. Assmus, B., et al., *Clinical outcome 2 years after intracoronary administration of bone marrow-derived progenitor cells in acute myocardial infarction.* Circ Heart Fail, 2010. **3**(1): p. 89-96.
36. Kehat, I., et al., *Human embryonic stem cells can differentiate into myocytes with structural and functional properties of cardiomyocytes.* J Clin Invest, 2001. **108**(3): p. 407-14.
37. Caspi, O., et al., *Transplantation of human embryonic stem cell-derived cardiomyocytes improves myocardial performance in infarcted rat hearts.* J Am Coll Cardiol, 2007. **50**(19): p. 1884-93.
38. Xu, C., et al., *Characterization and enrichment of cardiomyocytes derived from human embryonic stem cells.* Circ Res, 2002. **91**(6): p. 501-8.
39. Laflamme, M.A., et al., *Cardiomyocytes derived from human embryonic stem cells in pro-survival factors enhance function of infarcted rat hearts.* Nat Biotech, 2007. **25**(9): p. 1015-1024.
40. Laflamme, M.A., et al., *Formation of human myocardium in the rat heart from human embryonic stem cells.* Am J Pathol, 2005. **167**(3): p. 663-71.
41. Xu, C., et al., *Cardiac bodies: a novel culture method for enrichment of cardiomyocytes derived from human embryonic stem cells.* Stem Cells Dev, 2006. **15**(5): p. 631-9.
42. Kolossov, E., et al., *Identification and characterization of embryonic stem cell-derived pacemaker and atrial cardiomyocytes.* FASEB J, 2005. **19**(6): p. 577-9.
43. Shiba, Y., K.D. Hauch, and M.A. Laflamme, *Cardiac applications for human pluripotent stem cells.* Curr Pharm Des, 2009. **15**(24): p. 2791-806.
44. Kita-Matsuo, H., et al., *Lentiviral vectors and protocols for creation of stable hESC lines for fluorescent tracking and drug resistance selection of cardiomyocytes.* PLoS ONE, 2009. **4**(4): p. e5046.
45. Zhu, W.Z., et al., *Neuregulin/ErbB signaling regulates cardiac subtype specification in differentiating human embryonic stem cells.* Circ Res, 2010. **107**(6): p. 776-86.
46. Burridge, P.W., et al., *Production of de novo cardiomyocytes: human pluripotent stem cell differentiation and direct reprogramming.* Cell Stem Cell, 2012. **10**(1): p. 16-28.
47. Srivastava, D. and K.N. Ivey, *Potential of stem-cell-based therapies for heart disease.* Nature, 2006. **441**(7097): p. 1097-9.
48. Tam, P.P. and D.A. Loebel, *Gene function in mouse embryogenesis: get set for gastrulation.* Nat Rev Genet, 2007. **8**(5): p. 368-81.
49. Buckingham, M., S. Meilhac, and S. Zaffran, *Building the mammalian heart from two sources of myocardial cells.* Nat Rev Genet, 2005. **6**(11): p. 826-35.
50. David, R., et al., *Induction of MesP1 by Brachyury(T) generates the common multipotent cardiovascular stem cell.* Cardiovasc Res, 2011. **92**(1): p. 115-22.
51. Costello, I., et al., *The T-box transcription factor Eomesodermin acts upstream of Mesp1 to specify cardiac mesoderm during mouse gastrulation.* Nat Cell Biol, 2011. **13**(9): p. 1084-91.

52. Bondue, A., et al., *Mesp1 acts as a master regulator of multipotent cardiovascular progenitor specification.* Cell Stem Cell, 2008. **3**(1): p. 69-84.
53. Saga, Y., S. Kitajima, and S. Miyagawa-Tomita, *Mesp1 expression is the earliest sign of cardiovascular development.* Trends Cardiovasc Med, 2000. **10**(8): p. 345-52.
54. Kataoka, H., et al., *Expressions of PDGF receptor alpha, c-Kit and Flk1 genes clustering in mouse chromosome 5 define distinct subsets of nascent mesodermal cells.* Dev Growth Differ, 1997. **39**(6): p. 729-40.
55. David, R., et al., *MesP1 drives vertebrate cardiovascular differentiation through Dkk-1-mediated blockade of Wnt-signalling.* Nat Cell Biol, 2008. **10**(3): p. 338-45.
56. Bondue, A. and C. Blanpain, *Mesp1: a key regulator of cardiovascular lineage commitment.* Circ Res, 2010. **107**(12): p. 1414-27.
57. Naito, A.T., et al., *Developmental stage-specific biphasic roles of Wnt/beta-catenin signaling in cardiomyogenesis and hematopoiesis.* Proc Natl Acad Sci U S A, 2006. **103**(52): p. 19812-7.
58. Kwon, C., et al., *A regulatory pathway involving Notch1/beta-catenin/Isl1 determines cardiac progenitor cell fate.* Nat Cell Biol, 2009. **11**(8): p. 951-7.
59. Qyang, Y., et al., *The renewal and differentiation of Isl1+ cardiovascular progenitors are controlled by a Wnt/beta-catenin pathway.* Cell Stem Cell, 2007. **1**(2): p. 165-79.
60. Solloway, M.J. and R.P. Harvey, *Molecular pathways in myocardial development: a stem cell perspective.* Cardiovasc Res, 2003. **58**(2): p. 264-77.
61. Grego-Bessa, J., et al., *Notch signaling is essential for ventricular chamber development.* Dev Cell, 2007. **12**(3): p. 415-29.
62. Lavine, K.J., et al., *Endocardial and epicardial derived FGF signals regulate myocardial proliferation and differentiation in vivo.* Dev Cell, 2005. **8**(1): p. 85-95.
63. McMullen, N.M. and K.B. Pasumarthi, *Donor cell transplantation for myocardial disease: does it complement current pharmacological therapies?* Can J Physiol Pharmacol, 2007. **85**(1): p. 1-15.
64. Reinecke, H., V. Poppa, and C.E. Murry, *Skeletal muscle stem cells do not transdifferentiate into cardiomyocytes after cardiac grafting.* J Mol Cell Cardiol, 2002. **34**(2): p. 241-9.
65. Rubart, M., et al., *Spontaneous and evoked intracellular calcium transients in donor-derived myocytes following intracardiac myoblast transplantation.* J Clin Invest, 2004. **114**(6): p. 775-83.
66. Taylor, D.A., et al., *Regenerating functional myocardium: improved performance after skeletal myoblast transplantation.* Nat Med, 1998. **4**(8): p. 929-33.
67. Ghostine, S., et al., *Long-term efficacy of myoblast transplantation on regional structure and function after myocardial infarction.* Circulation, 2002. **106**(12 Suppl 1): p. I131-6.
68. Wang, Q.-D. and P.-O. Sjöquist, *Myocardial regeneration with stem cells: Pharmacological possibilities for efficacy enhancement.* Pharmacological Research, 2006. **53**(4): p. 331-340.

69. Dixon, J.A. and F.G. Spinale, *Large animal models of heart failure: a critical link in the translation of basic science to clinical practice.* Circ Heart Fail, 2009. **2**(3): p. 262-71.
70. Yoon, Y.S., et al., *Clonally expanded novel multipotent stem cells from human bone marrow regenerate myocardium after myocardial infarction.* J Clin Invest, 2005. **115**(2): p. 326-38.
71. Balsam, L.B., et al., *Haematopoietic stem cells adopt mature haematopoietic fates in ischaemic myocardium.* Nature, 2004. **428**(6983): p. 668-73.
72. Bittner, R.E., et al., *Recruitment of bone-marrow-derived cells by skeletal and cardiac muscle in adult dystrophic mdx mice.* Anat Embryol (Berl), 1999. **199**(5): p. 391-6.
73. Haider, H. and M. Ashraf, *Bone marrow cell transplantation in clinical perspective.* J Mol Cell Cardiol, 2005. **38**(2): p. 225-35.
74. Morrison, S.J., *Stem cell potential: can anything make anything?* Curr Biol, 2001. **11**(1): p. R7-9.
75. Terada, N., et al., *Bone marrow cells adopt the phenotype of other cells by spontaneous cell fusion.* Nature, 2002. **416**(6880): p. 542-5.
76. Alvarez-Dolado, M., et al., *Fusion of bone-marrow-derived cells with Purkinje neurons, cardiomyocytes and hepatocytes.* Nature, 2003. **425**(6961): p. 968-73.
77. Nygren, J.M., et al., *Bone marrow-derived hematopoietic cells generate cardiomyocytes at a low frequency through cell fusion, but not transdifferentiation.* Nat Med, 2004. **10**(5): p. 494-501.
78. Laflamme, M.A. and C.E. Murry, *Heart regeneration.* Nature, 2011. **473**(7347): p. 326-335.
79. Evans, M.J., Kaufman, M.H., *Establishment in culture of pluripotential cells from mouse embryos.* Nature 1981. **292**(5819): p. 154-156.
80. Martin, G.R., *Isolation of a pluripotent cell line from early mouse embryos cultured in medium conditioned by teratocarcinoma stem cells.* Proc Natl Acad Sci U S A, 1981. **78**(12): p. 7634-8.
81. Thomson, J.A., et al., *Embryonic stem cell lines derived from human blastocysts.* Science, 1998. **282**(5391): p. 1145-7.
82. Trounson, A., *The production and directed differentiation of human embryonic stem cells.* Endocr Rev, 2006. **27**(2): p. 208-19.
83. Pasumarthi, K.B. and L.J. Field, *Cardiomyocyte enrichment in differentiating ES cell cultures: strategies and applications.* Methods Mol Biol, 2002. **185**: p. 157-68.
84. Nichols, J., E.P. Evans, and A.G. Smith, *Establishment of germ-line-competent embryonic stem (ES) cells using differentiation inhibiting activity.* Development, 1990. **110**(4): p. 1341-8.
85. Matsuda, T., et al., *STAT3 activation is sufficient to maintain an undifferentiated state of mouse embryonic stem cells.* EMBO J, 1999. **18**(15): p. 4261-9.
86. Ying, Q.L., et al., *BMP induction of Id proteins suppresses differentiation and sustains embryonic stem cell self-renewal in collaboration with STAT3.* Cell, 2003. **115**(3): p. 281-92.
87. Ginis, I., et al., *Differences between human and mouse embryonic stem cells.* Dev Biol, 2004. **269**(2): p. 360-80.

88. Kawase, E., et al., *Strain difference in establishment of mouse embryonic stem (ES) cell lines.* Int J Dev Biol, 1994. **38**(2): p. 385-90.
89. Draper, J.S., et al., *Surface antigens of human embryonic stem cells: changes upon differentiation in culture.* J Anat, 2002. **200**(Pt 3): p. 249-58.
90. Henderson, J.K., et al., *Preimplantation human embryos and embryonic stem cells show comparable expression of stage-specific embryonic antigens.* Stem Cells, 2002. **20**(4): p. 329-37.
91. Niwa, H., *Molecular mechanism to maintain stem cell renewal of ES cells.* Cell Struct Funct, 2001. **26**(3): p. 137-48.
92. Rossant, J., *Stem cells from the Mammalian blastocyst.* Stem Cells, 2001. **19**(6): p. 477-82.
93. Odorico, J.S., D.S. Kaufman, and J.A. Thomson, *Multilineage differentiation from human embryonic stem cell lines.* Stem Cells, 2001. **19**(3): p. 193-204.
94. Hayashi, Y., et al., *BMP4 induction of trophoblast from mouse embryonic stem cells in defined culture conditions on laminin.* In Vitro Cell Dev Biol Anim, 2010. **46**(5): p. 416-30.
95. Reubinoff, B.E., et al., *Embryonic stem cell lines from human blastocysts: somatic differentiation in vitro.* Nat Biotechnol, 2000. **18**(4): p. 399-404.
96. Bendall, S.C., et al., *IGF and FGF cooperatively establish the regulatory stem cell niche of pluripotent human cells in vitro.* Nature, 2007. **448**(7157): p. 1015-21.
97. Min, J.Y., et al., *Transplantation of embryonic stem cells improves cardiac function in postinfarcted rats.* J Appl Physiol, 2002. **92**(1): p. 288-96.
98. Min, J.Y., et al., *Long-term improvement of cardiac function in rats after infarction by transplantation of embryonic stem cells.* J Thorac Cardiovasc Surg, 2003. **125**(2): p. 361-9.
99. Hodgson, D.M., et al., *Stable benefit of embryonic stem cell therapy in myocardial infarction.* Am J Physiol Heart Circ Physiol, 2004. **287**(2): p. H471-9.
100. Menard, C., et al., *Transplantation of cardiac-committed mouse embryonic stem cells to infarcted sheep myocardium: a preclinical study.* Lancet, 2005. **366**(9490): p. 1005-12.
101. Okita, K., T. Ichisaka, and S. Yamanaka, *Generation of germline-competent induced pluripotent stem cells.* Nature, 2007. **448**(7151): p. 313-7.
102. Maherali, N., et al., *Directly reprogrammed fibroblasts show global epigenetic remodeling and widespread tissue contribution.* Cell Stem Cell, 2007. **1**(1): p. 55-70.
103. Wernig, M., et al., *In vitro reprogramming of fibroblasts into a pluripotent ES-cell-like state.* Nature, 2007. **448**(7151): p. 318-24.
104. Yu, J., et al., *Induced pluripotent stem cell lines derived from human somatic cells.* Science, 2007. **318**(5858): p. 1917-20.
105. Woltjen, K., et al., *piggyBac transposition reprograms fibroblasts to induced pluripotent stem cells.* Nature, 2009. **458**(7239): p. 766-70.
106. Kaji, K., et al., *Virus-free induction of pluripotency and subsequent excision of reprogramming factors.* Nature, 2009. **458**(7239): p. 771-5.
107. Yu, J., et al., *Human induced pluripotent stem cells free of vector and transgene sequences.* Science, 2009. **324**(5928): p. 797-801.
108. Kim, D., et al., *Generation of human induced pluripotent stem cells by direct delivery of reprogramming proteins.* Cell Stem Cell, 2009. **4**(6): p. 472-6.

109. Rangappa, S., et al., *Transformation of adult mesenchymal stem cells isolated from the fatty tissue into cardiomyocytes.* Ann Thorac Surg, 2003. **75**(3): p. 775-9.
110. Koninckx, R., et al., *Mesenchymal stem cells or cardiac progenitors for cardiac repair? A comparative study.* Cell Mol Life Sci, 2011. **68**(12): p. 2141-56.
111. Wobus, A.M., G. Wallukat, and J. Hescheler, *Pluripotent mouse embryonic stem cells are able to differentiate into cardiomyocytes expressing chronotropic responses to adrenergic and cholinergic agents and Ca2+ channel blockers.* Differentiation, 1991. **48**(3): p. 173-182.
112. Zhang, J., et al., *Functional cardiomyocytes derived from human induced pluripotent stem cells.* Circ Res, 2009. **104**(4): p. e30-41.
113. He, J.Q., et al., *Human embryonic stem cells develop into multiple types of cardiac myocytes: action potential characterization.* Circ Res, 2003. **93**(1): p. 32-9.
114. Ieda, M., et al., *Direct reprogramming of fibroblasts into functional cardiomyocytes by defined factors.* Cell, 2010. **142**(3): p. 375-86.
115. Sartiani, L., et al., *Developmental changes in cardiomyocytes differentiated from human embryonic stem cells: a molecular and electrophysiological approach.* Stem Cells, 2007. **25**(5): p. 1136-44.
116. Zhu, W.Z., L.F. Santana, and M.A. Laflamme, *Local control of excitation-contraction coupling in human embryonic stem cell-derived cardiomyocytes.* PLoS ONE, 2009. **4**(4): p. e5407.
117. Kehat, I., et al., *Electromechanical integration of cardiomyocytes derived from human embryonic stem cells.* Nat Biotechnol, 2004. **22**(10): p. 1282-9.
118. Yang, L., et al., *Human cardiovascular progenitor cells develop from a KDR+ embryonic-stem-cell-derived population.* Nature, 2008. **453**(7194): p. 524-8.
119. Ziman, A.P., et al., *Excitation-contraction coupling changes during postnatal cardiac development.* J Mol Cell Cardiol, 2010. **48**(2): p. 379-86.
120. Marvin, M.J., et al., *Inhibition of Wnt activity induces heart formation from posterior mesoderm.* Genes Dev, 2001. **15**(3): p. 316-27.
121. Mima, T., et al., *Fibroblast growth factor receptor is required for in vivo cardiac myocyte proliferation at early embryonic stages of heart development.* Proc Natl Acad Sci U S A, 1995. **92**(2): p. 467-71.
122. Winnier, G., et al., *Bone morphogenetic protein-4 is required for mesoderm formation and patterning in the mouse.* Genes Dev, 1995. **9**(17): p. 2105-16.
123. Zhang, H. and A. Bradley, *Mice deficient for BMP2 are nonviable and have defects in amnion/chorion and cardiac development.* Development, 1996. **122**(10): p. 2977-86.
124. Murry, C.E. and G. Keller, *Differentiation of embryonic stem cells to clinically relevant populations: lessons from embryonic development.* Cell, 2008. **132**(4): p. 661-80.
125. Boheler, K.R., et al., *Differentiation of Pluripotent Embryonic Stem Cells Into Cardiomyocytes.* Circulation Research, 2002. **91**(3): p. 189-201.

126. Christoforou, N., et al., *Mouse ES cell-derived cardiac precursor cells are multipotent and facilitate identification of novel cardiac genes.* J Clin Invest, 2008. **118**(3): p. 894-903.
127. Moretti, A., et al., *Multipotent embryonic isl1+ progenitor cells lead to cardiac, smooth muscle, and endothelial cell diversification.* Cell, 2006. **127**(6): p. 1151-65.
128. Lindsley, R.C., et al., *Mesp1 coordinately regulates cardiovascular fate restriction and epithelial-mesenchymal transition in differentiating ESCs.* Cell Stem Cell, 2008. **3**(1): p. 55-68.
129. Gadue, P., et al., *Wnt and TGF-beta signaling are required for the induction of an in vitro model of primitive streak formation using embryonic stem cells.* Proc Natl Acad Sci U S A, 2006. **103**(45): p. 16806-11.
130. Foley, A.C. and M. Mercola, *Heart induction by Wnt antagonists depends on the homeodomain transcription factor Hex.* Genes Dev, 2005. **19**(3): p. 387-96.
131. Foley, A.C., et al., *Multiple functions of Cerberus cooperate to induce heart downstream of Nodal.* Dev Biol, 2007. **303**(1): p. 57-65.
132. Schneider, V.A. and M. Mercola, *Wnt antagonism initiates cardiogenesis in Xenopus laevis.* Genes Dev, 2001. **15**(3): p. 304-15.
133. Ueno, S., et al., *Biphasic role for Wnt/beta-catenin signaling in cardiac specification in zebrafish and embryonic stem cells.* Proc Natl Acad Sci U S A, 2007. **104**(23): p. 9685-90.
134. Chen, V.C., et al., *Notch signaling respecifies the hemangioblast to a cardiac fate.* Nat Biotechnol, 2008. **26**(10): p. 1169-78.
135. Doetschman, T.C., et al., *The in vitro development of blastocyst-derived embryonic stem cell lines: formation of visceral yolk sac, blood islands and myocardium.* J Embryol Exp Morphol, 1985. **87**: p. 27-45.
136. Wobus, A.M., et al., *Embryonic stem cells as a model to study cardiac, skeletal muscle, and vascular smooth muscle cell differentiation.* Methods Mol Biol, 2002. **185**: p. 127-56.
137. Hescheler, J., et al., *Embryonic stem cells: a model to study structural and functional properties in cardiomyogenesis.* Cardiovasc Res, 1997. **36**(2): p. 149-62.
138. Boheler, K.R., et al., *Differentiation of Pluripotent Embryonic Stem Cells Into Cardiomyocytes.* Circulation Research 2002. **91**: p. 189-201.
139. Westfall, M.V., et al., *Ultrastructure and cell-cell coupling of cardiac myocytes differentiating in embryonic stem cell cultures.* Cell Motil Cytoskeleton, 1997. **36**(1): p. 43-54.
140. Fassler, R., et al., *Differentiation and integrity of cardiac muscle cells are impaired in the absence of beta 1 integrin.* J Cell Sci, 1996. **109 (Pt 13)**: p. 2989-99.
141. Robbins, J., et al., *Mouse embryonic stem cells express the cardiac myosin heavy chain genes during development in vitro.* J Biol Chem, 1990. **265**(20): p. 11905-9.
142. Klug, M.G., et al., *Genetically selected cardiomyocytes from differentiating embronic stem cells form stable intracardiac grafts.* J Clin Invest, 1996. **98**(1): p. 216-24.

143. Zwi-Dantsis, L., et al., *Derivation and cardiomyocyte differentiation of induced pluripotent stem cells from heart failure patients.* European Heart Journal, 2012.
144. Takei, S., et al., *Bone morphogenetic protein-4 promotes induction of cardiomyocytes from human embryonic stem cells in serum-based embryoid body development.* Am J Physiol Heart Circ Physiol, 2009. **296**(6): p. H1793-803.
145. Tran, T.H., et al., *Wnt3a-induced mesoderm formation and cardiomyogenesis in human embryonic stem cells.* Stem Cells, 2009. **27**(8): p. 1869-78.
146. Bu, L., et al., *Human ISL1 heart progenitors generate diverse multipotent cardiovascular cell lineages.* Nature, 2009. **460**(7251): p. 113-117.
147. Cao, N., et al., *Ascorbic acid enhances the cardiac differentiation of induced pluripotent stem cells through promoting the proliferation of cardiac progenitor cells.* Cell Res, 2012. **22**(1): p. 219-36.
148. Rust, W., T. Balakrishnan, and R. Zweigerdt, *Cardiomyocyte enrichment from human embryonic stem cell cultures by selection of ALCAM surface expression.* Regen Med, 2009. **4**(2): p. 225-37.
149. Laflamme, M.A., et al., *Cardiomyocytes derived from human embryonic stem cells in pro-survival factors enhance function of infarcted rat hearts.* Nat Biotechnol, 2007. **25**(9): p. 1015-24.
150. Elliott, D.A., et al., *NKX2-5eGFP/w hESCs for isolation of human cardiac progenitors and cardiomyocytes.* Nat Meth, 2011. **advance online publication**.
151. Xu, X.Q., et al., *Global expression profile of highly enriched cardiomyocytes derived from human embryonic stem cells.* Stem Cells, 2009. **27**(9): p. 2163-74.
152. Yoon, B.S., et al., *Enhanced differentiation of human embryonic stem cells into cardiomyocytes by combining hanging drop culture and 5-azacytidine treatment.* Differentiation, 2006. **74**(4): p. 149-59.
153. Norstrom, A., et al., *Molecular and pharmacological properties of human embryonic stem cell-derived cardiomyocytes.* Exp Biol Med (Maywood), 2006. **231**(11): p. 1753-62.
154. Zhang, F. and K.B.S. Pasumarthi, *Embryonic Stem Cell Transplantation: Promise and Progress in the Treatment of Heart Disease.* BioDrugs, 2008. **22**(6): p. 361-374 10.2165/0063030-200822060-00003.
155. Maltsev, V.A., et al., *Embryonic stem cells differentiate in vitro into cardiomyocytes representing sinusnodal, atrial and ventricular cell types.* Mechanisms of Development, 1993. **44**(1): p. 41-50.
156. Maltsev, V.A., et al., *Cardiomyocytes differentiated in vitro from embryonic stem cells developmentally express cardiac-specific genes and ionic currents.* Circ Res, 1994. **75**(2): p. 233-44.
157. Trautwein, W. and J. Hescheler, *Regulation of cardiac L-type calcium current by phosphorylation and G proteins.* Annu Rev Physiol, 1990. **52**: p. 257-74.
158. DiFrancesco, D., *Pacemaker mechanisms in cardiac tissue.* Annu Rev Physiol, 1993. **55**: p. 455-72.

159. Pelzer, D., A. Cavalie, and W. Trautwein, *Cardiac Ca channel currents at the level of single cells and single channels.* Basic Res Cardiol, 1985. **80 Suppl 2**: p. 65-9.
160. Schultz, G., et al., *Role of G proteins in calcium channel modulation.* Annu Rev Physiol, 1990. **52**: p. 275-92.
161. Dolnikov, K., et al., *Functional properties of human embryonic stem cell-derived cardiomyocytes: intracellular Ca2+ handling and the role of sarcoplasmic reticulum in the contraction.* Stem Cells, 2006. **24**(2): p. 236-45.
162. Hattori, F., et al., *Nongenetic method for purifying stem cell-derived cardiomyocytes.* Nat Methods, 2010. **7**(1): p. 61-6.
163. Dubois, N.C., et al., *SIRPA is a specific cell-surface marker for isolating cardiomyocytes derived from human pluripotent stem cells.* Nat Biotechnol, 2011. **29**(11): p. 1011-8.
164. Kattman, S.J., et al., *Stage-specific optimization of activin/nodal and BMP signaling promotes cardiac differentiation of mouse and human pluripotent stem cell lines.* Cell Stem Cell, 2011. **8**(2): p. 228-40.
165. Wobus, A.M. and K. Guan, *Embryonic Stem Cell-Derived Cardiac Differentiation: Modulation of Differentiation and "Loss-of-Function" Analysis In Vitro.* Trends Cardiovasc Med, 1998. **8**(2): p. 64-74.
166. Anderson, D., et al., *Transgenic enrichment of cardiomyocytes from human embryonic stem cells.* Mol Ther, 2007. **15**(11): p. 2027-36.
167. Huber, I., et al., *Identification and selection of cardiomyocytes during human embryonic stem cell differentiation.* FASEB J, 2007. **21**(10): p. 2551-63.
168. Urnov, F.D., et al., *Genome editing with engineered zinc finger nucleases.* Nat Rev Genet, 2010. **11**(9): p. 636-46.
169. Soldner, F., et al., *Generation of isogenic pluripotent stem cells differing exclusively at two early onset Parkinson point mutations.* Cell, 2011. **146**(2): p. 318-31.
170. de Souza, N., *Primer: genome editing with engineered nucleases: a brief description of tools for targeted cleavage and tailored modification of genomes is presented.* Nature Methods, 2012. **9**: p. 27.
171. Bogdanove, A.J., S. Schornack, and T. Lahaye, *TAL effectors: finding plant genes for disease and defense.* Curr Opin Plant Biol, 2010. **13**(4): p. 394-401.
172. Scholze, H. and J. Boch, *TAL effectors are remote controls for gene activation.* Curr Opin Microbiol, 2011. **14**(1): p. 47-53.
173. Boch, J. and U. Bonas, *Xanthomonas AvrBs3 family-type III effectors: discovery and function.* Annu Rev Phytopathol, 2010. **48**: p. 419-36.
174. Mussolino, C. and T. Cathomen, *TALE nucleases: tailored genome engineering made easy.* Current Opinion in Biotechnology, (0).
175. Cermak, T., et al., *Efficient design and assembly of custom TALEN and other TAL effector-based constructs for DNA targeting.* Nucleic Acids Res, 2011. **39**(12): p. e82.
176. Engler, C., R. Kandzia, and S. Marillonnet, *A one pot, one step, precision cloning method with high throughput capability.* PLoS ONE, 2008. **3**(11): p. e3647.

177. Geissler, R., et al., *Transcriptional activators of human genes with programmable DNA-specificity.* PLoS ONE, 2011. **6**(5): p. e19509.
178. Morbitzer, R., et al., *Assembly of custom TALE-type DNA binding domains by modular cloning.* Nucleic Acids Res, 2011. **39**(13): p. 5790-9.
179. Boch, J., et al., *Breaking the code of DNA binding specificity of TAL-type III effectors.* Science, 2009. **326**(5959): p. 1509-12.
180. Smith, J., et al., *Requirements for double-strand cleavage by chimeric restriction enzymes with zinc finger DNA-recognition domains.* Nucleic Acids Res, 2000. **28**(17): p. 3361-9.
181. Lombardo, A., et al., *Gene editing in human stem cells using zinc finger nucleases and integrase-defective lentiviral vector delivery.* Nat Biotechnol, 2007. **25**(11): p. 1298-306.
182. Dreyer, A.K. and T. Cathomen, *Zinc-finger nucleases-based genome engineering to generate isogenic human cell lines.* Methods Mol Biol, 2012. **813**: p. 145-56.
183. Ran, F.A., et al., *Genome engineering using the CRISPR-Cas9 system.* Nat. Protocols, 2013. **8**(11): p. 2281-2308.
184. Mali, P., et al., *RNA-guided human genome engineering via Cas9.* Science, 2013. **339**(6121): p. 823-6.
185. Cong, L., et al., *Multiplex genome engineering using CRISPR/Cas systems.* Science, 2013. **339**(6121): p. 819-23.
186. Doudna, J.A. and E. Charpentier, *The new frontier of genome engineering with CRISPR-Cas9.* Science, 2014. **346**(6213).
187. Doudna, J.A. and E. Charpentier, *Genome editing. The new frontier of genome engineering with CRISPR-Cas9.* Science, 2014. **346**(6213): p. 1258096.
188. Wang, H., et al., *One-step generation of mice carrying mutations in multiple genes by CRISPR/Cas-mediated genome engineering.* Cell, 2013. **153**(4): p. 910-8.
189. Shen, B., et al., *Generation of gene-modified mice via Cas9/RNA-mediated gene targeting.* Cell Res, 2013. **23**(5): p. 720-3.
190. Gaj, T., C.A. Gersbach, and C.F. Barbas, III, *ZFN, TALEN, and CRISPR/Cas-based methods for genome engineering.* Trends in Biotechnology. **31**(7): p. 397-405.
191. Kim, Y., et al., *A library of TAL effector nucleases spanning the human genome.* Nat Biotechnol, 2013. **31**(3): p. 251-8.
192. Wei, C., et al., *TALEN or Cas9 - rapid, efficient and specific choices for genome modifications.* J Genet Genomics, 2013. **40**(6): p. 281-9.
193. Bacman, S.R., et al., *Specific elimination of mutant mitochondrial genomes in patient-derived cells by mitoTALENs.* Nat Med, 2013. **19**(9): p. 1111-3.
194. Wu, K., et al., *Application of stem cells for cardiovascular grafts tissue engineering.* Transpl Immunol, 2006. **16**(1): p. 1-7.
195. Placzek, M.R., et al., *Stem cell bioprocessing: fundamentals and principles.* J R Soc Interface, 2009. **6**(32): p. 209-32.
196. Rungarunlert, S., et al., *Embryoid body formation from embryonic and induced pluripotent stem cells: Benefits of bioreactors.* World Journal of Stem Cells, 2009. **1**(1): p. 11-21.

197. Zweigerdt, R., et al., *Generation of confluent cardiomyocyte monolayers derived from embryonic stem cells in suspension: a cell source for new therapies and screening strategies.* Cytotherapy, 2003. **5**(5): p. 399-413.
198. Fong, W.J., et al., *Perfusion cultures of human embryonic stem cells.* Bioprocess Biosyst Eng, 2005. **27**(6): p. 381-7.
199. Bratt-Leal, A.M., R.L. Carpenedo, and T.C. McDevitt, *Engineering the embryoid body microenvironment to direct embryonic stem cell differentiation.* Biotechnol Prog, 2009. **25**(1): p. 43-51.
200. Krawetz, R., et al., *Large-scale expansion of pluripotent human embryonic stem cells in stirred-suspension bioreactors.* Tissue Eng Part C Methods, 2010. **16**(4): p. 573-82.
201. zur Nieden, N.I., et al., *Embryonic stem cells remain highly pluripotent following long term expansion as aggregates in suspension bioreactors.* J Biotechnol, 2007. **129**(3): p. 421-32.
202. Cormier, J.T., et al., *Expansion of undifferentiated murine embryonic stem cells as aggregates in suspension culture bioreactors.* Tissue Eng, 2006. **12**(11): p. 3233-45.
203. Lock, L.T. and E.S. Tzanakakis, *Expansion and differentiation of human embryonic stem cells to endoderm progeny in a microcarrier stirred-suspension culture.* Tissue Eng Part A, 2009. **15**(8): p. 2051-63.
204. Barron, V., et al., *Bioreactors for cardiovascular cell and tissue growth: a review.* Ann Biomed Eng, 2003. **31**(9): p. 1017-30.
205. Cameron, C.M., W.S. Hu, and D.S. Kaufman, *Improved development of human embryonic stem cell-derived embryoid bodies by stirred vessel cultivation.* Biotechnol Bioeng, 2006. **94**(5): p. 938-48.
206. King, J.A. and W.M. Miller, *Bioreactor development for stem cell expansion and controlled differentiation.* Curr Opin Chem Biol, 2007. **11**(4): p. 394-8.
207. Serra, M., et al., *Integrating human stem cell expansion and neuronal differentiation in bioreactors.* BMC Biotechnol, 2009. **9**: p. 82.
208. Zandstra, P.W., et al., *Scalable production of embryonic stem cell-derived cardiomyocytes.* Tissue Eng, 2003. **9**(4): p. 767-78.
209. Chisti, Y., *Hydrodynamic damage to animal cells.* Crit Rev Biotechnol, 2001. **21**(2): p. 67-110.
210. Fok, E.Y. and P.W. Zandstra, *Shear-controlled single-step mouse embryonic stem cell expansion and embryoid body-based differentiation.* Stem Cells, 2005. **23**(9): p. 1333-42.
211. Gerecht-Nir, S., S. Cohen, and J. Itskovitz-Eldor, *Bioreactor cultivation enhances the efficiency of human embryoid body (hEB) formation and differentiation.* Biotechnol Bioeng, 2004. **86**(5): p. 493-502.
212. Lu, S., et al., *Bioreactor cultivation enhances NTEB formation and differentiation of NTES cells into cardiomyocytes.* Cloning Stem Cells, 2008. **10**(3): p. 363-70.
213. Magyar, J.P., et al., *Mass production of embryoid bodies in microbeads.* Ann N Y Acad Sci, 2001. **944**: p. 135-43.
214. Dang, S.M., et al., *Controlled, scalable embryonic stem cell differentiation culture.* Stem Cells, 2004. **22**(3): p. 275-82.
215. Levenberg, S., et al., *Differentiation of human embryonic stem cells on three-dimensional polymer scaffolds.* Proc Natl Acad Sci U S A, 2003. **100**(22): p. 12741-6.

216. Nonaka, J., et al., *CoCl(2) inhibits neural differentiation of retinoic acid-treated embryoid bodies.* J Biosci Bioeng, 2008. **106**(2): p. 141-7.
217. Hwang, Y.S., et al., *The use of murine embryonic stem cells, alginate encapsulation, and rotary microgravity bioreactor in bone tissue engineering.* Biomaterials, 2009. **30**(4): p. 499-507.
218. Zhang, J., et al., *The incorporation of 70s bioactive glass to the osteogenic differentiation of murine embryonic stem cells in 3D bioreactors.* J Tissue Eng Regen Med, 2009. **3**(1): p. 63-71.
219. Sarvi, F., et al., *A novel technique for the formation of embryoid bodies inside liquid marbles.* RSC Advances, 2013. **3**(34): p. 14501-14508.
220. Sarvi, F., et al., *Cardiogenesis of embryonic stem cells with liquid marble micro-bioreactor.* Adv Healthc Mater, 2015. **4**(1): p. 77-86.
221. Aussillous, P. and D. Quere, *Liquid marbles.* Nature, 2001. **411**(6840): p. 924-7.
222. Tian, J., et al., *Liquid marble for gas sensing.* Chem Commun (Camb), 2010. **46**(26): p. 4734-6.
223. Arbatan, T., et al., *Microreactors: Liquid Marbles as Micro-bioreactors for Rapid Blood Typing (Adv. Healthcare Mater. 1/2012).*
224. Aussillous, P. and D. Quere, *Liquid marbles.* Nature, 2001. **411**(6840): p. 924-927.
225. Bormashenko, E., G. Chaniel, and O. Gendelman, *Hydrophilization and hydrophobic recovery in polymers obtained by casting of polymer solutions on water surface.* J Colloid Interface Sci, 2014. **435**: p. 192-7.
226. Paunov, V.N., et al., *Emulsions stabilised by food colloid particles: role of particle adsorption and wettability at the liquid interface.* J Colloid Interface Sci, 2007. **312**(2): p. 381-9.
227. Arbatan, T., et al., *Tumor inside a pearl drop.* Adv Healthc Mater, 2012. **1**(4): p. 467-9.
228. Arbatan, T., et al., *Liquid marbles as micro-bioreactors for rapid blood typing.* Adv Healthc Mater, 2012. **1**(1): p. 80-3.
229. Zhao, Y., et al., *Magnetic liquid marbles: manipulation of liquid droplets using highly hydrophobic Fe3O4 nanoparticles.* Adv Mater, 2010. **22**(6): p. 707-10.
230. Zhao, T., et al., *Immunogenicity of induced pluripotent stem cells.* Nature, 2011. **474**(7350): p. 212-5.
231. Braam, S.R., et al., *Prediction of drug-induced cardiotoxicity using human embryonic stem cell-derived cardiomyocytes.* Stem Cell Res, 2010. **4**(2): p. 107-16.
232. Davis, R.P., et al., *Pluripotent stem cell models of cardiac disease and their implication for drug discovery and development.* Trends Mol Med, 2011. **17**(9): p. 475-84.
233. Josowitz, R., et al., *Induced pluripotent stem cell-derived cardiomyocytes as models for genetic cardiovascular disorders.* Curr Opin Cardiol, 2011. **26**(3): p. 223-9.
234. Ma, J., et al., *High purity human-induced pluripotent stem cell-derived cardiomyocytes: electrophysiological properties of action potentials and ionic currents.* Am J Physiol Heart Circ Physiol, 2011. **301**(5): p. H2006-17.

235. Willems, E., P.J. Bushway, and M. Mercola, *Natural and synthetic regulators of embryonic stem cell cardiogenesis.* Pediatr Cardiol, 2009. **30**(5): p. 635-42.
236. Yeom, Y.I., et al., *Germline regulatory element of Oct-4 specific for the totipotent cycle of embryonal cells.* Development, 1996. **122**(3): p. 881-94.
237. Altschul, S.F., et al., *Basic local alignment search tool.* J Mol Biol, 1990. **215**(3): p. 403-10.
238. Larkin, M.A., et al., *Clustal W and Clustal X version 2.0.* Bioinformatics, 2007. **23**(21): p. 2947-8.
239. Metzger, J.M., et al., *Embryonic stem cell cardiogenesis applications for cardiovascular research.* Trends Cardiovasc Med, 1997. **7**(2): p. 63-8.
240. Yutzey, K.E. and D. Bader, *Diversification of cardiomyogenic cell lineages during early heart development.* Circ Res, 1995. **77**(2): p. 216-9.
241. Srivastava, D. and E.N. Olson, *A genetic blueprint for cardiac development.* Nature, 2000. **407**(6801): p. 221-6.
242. Lints, T.J., et al., *Nkx-2.5: a novel murine homeobox gene expressed in early heart progenitor cells and their myogenic descendants.* Development, 1993. **119**(2): p. 419-31.
243. Komuro, I. and S. Izumo, *Csx: a murine homeobox-containing gene specifically expressed in the developing heart.* Proc Natl Acad Sci U S A, 1993. **90**(17): p. 8145-9.
244. Durocher, D., et al., *The cardiac transcription factors Nkx2-5 and GATA-4 are mutual cofactors.* The EMBO Journal, 1997. **16**(18): p. 5687-5696.
245. Moscou, M.J. and A.J. Bogdanove, *A simple cipher governs DNA recognition by TAL effectors.* Science, 2009. **326**(5959): p. 1501.
246. Bogdanove, A.J. and D.F. Voytas, *TAL effectors: customizable proteins for DNA targeting.* Science, 2011. **333**(6051): p. 1843-6.
247. Miller, J.C., et al., *A TALE nuclease architecture for efficient genome editing.* Nat Biotechnol, 2011. **29**(2): p. 143-8.
248. Maresca, M., et al., *Obligate ligation-gated recombination (ObLiGaRe): custom-designed nuclease-mediated targeted integration through nonhomologous end joining.* Genome Res, 2013. **23**(3): p. 539-46.
249. Doyle, E.L., et al., *TAL Effector-Nucleotide Targeter (TALE-NT) 2.0: tools for TAL effector design and target prediction.* Nucleic acids research, 2012. **40**(W1): p. W117-W122.
250. Doyle, E.L., et al., *TAL Effector-Nucleotide Targeter (TALE-NT) 2.0: tools for TAL effector design and target prediction.* Nucleic Acids Res, 2012. **40**(Web Server issue): p. W117-22.
251. Gaj, T., C.A. Gersbach, and C.F. Barbas, 3rd, *ZFN, TALEN, and CRISPR/Cas-based methods for genome engineering.* Trends Biotechnol, 2013. **31**(7): p. 397-405.
252. Hockemeyer, D., et al., *Genetic engineering of human pluripotent cells using TALE nucleases.* Nat Biotechnol, 2011. **29**(8): p. 731-4.
253. Osborn, M.J., et al., *TALEN-based gene correction for epidermolysis bullosa.* Mol Ther, 2013. **21**(6): p. 1151-9.
254. Carlson, D.F., et al., *Efficient TALEN-mediated gene knockout in livestock.* Proc Natl Acad Sci U S A, 2012. **109**(43): p. 17382-7.

255. Frank, S., B.V. Skryabin, and B. Greber, *A modified TALEN-based system for robust generation of knock-out human pluripotent stem cell lines and disease models.* BMC Genomics, 2013. **14**: p. 773.
256. Sakuma, T., et al., *Efficient TALEN construction and evaluation methods for human cell and animal applications.* Genes Cells, 2013. **18**(4): p. 315-26.
257. Perez-Pinera, P., et al., *Synergistic and tunable human gene activation by combinations of synthetic transcription factors.* Nat Methods, 2013. **10**(3): p. 239-42.
258. Bedell, V.M., et al., *In vivo genome editing using a high-efficiency TALEN system.* Nature, 2012. **491**(7422): p. 114-8.
259. Park, C.Y., et al., *Targeted inversion and reversion of the blood coagulation factor 8 gene in human iPS cells using TALENs.* Proc Natl Acad Sci U S A, 2014. **111**(25): p. 9253-8.
260. Taylor, D.A., et al., *Regenerating functional myocardium: Improved performance after skeletal myoblast transplantation.* Nature Medicine, 1998. **4**(8): p. 929-933.
261. Orlic, D., et al., *Transplanted adult bone marrow cells repair myocardial infarcts in mice*, in *Hematopoietic Stem Cells 2000 Basic and Clinical Sciences*, D. Orlic, et al., Editors. 2001. p. 221-230.
262. Evans, M.J. and M.H. Kaufman, *Establishment in culture of pluripotential cells from mouse embryos* Nature, 1981. **292**(5819): p. 154-156.
263. Koike, M., et al., *Characterization of embryoid bodies of mouse embryonic stem cells formed under various culture conditions and estimation of differentiation status of such bodies.* Journal of Bioscience and Bioengineering, 2007. **104**(4): p. 294-299.
264. Maltsev, V.A., et al., *Embryonic stem-cell differentiate in vitro into cardiomyocytes representing sinusnodal, atrial and ventricular cell types.* Mechanisms of Development, 1993. **44**(1): p. 41-50.
265. Ungrin, M.D., et al., *Reproducible, ultra high-throughput formation of multicellular organization from single cell suspension-derived human embryonic stem cell aggregates.* Plos One, 2008. **3**(2).
266. Doetschman, T.C., H. Eistetter, and M. Katz, *The in vitro development of blastocyst-derived embryonic stem cell lines: formation of visceral yolk sac, blood islands and myocardium.* J. Embryol. exp. Morph., 1985. **87**: p. 27-45.
267. Kurosawa, H., *Methods for inducing embryoid body formation: In vitro differentiation system of embryonic stem cells.* Journal of Bioscience and Bioengineering, 2007. **103**(5): p. 389-398.
268. Keller, G.M., *In vitro differentiation of embryonic stem cells.* Current Opinion in Cell Biology, 1995. **7**(6): p. 862-869.
269. Smith, A.G., *Embryo-derived stem cells: Of mice and men.* Annual Review of Cell and Developmental Biology, 2001. **17**: p. 435-462.
270. Niebruegge, S., et al., *Cardiomyocyte production in mass suspension culture: embryonic stem cells as a source for great amounts of functional Cardiomyocytes.* Tissue Engineering Part A, 2008. **14**(10): p. 1591-1601.
271. Burridge, P.W., et al., *Improved human embryonic stem cell embryoid body homogeneity and cardiomyocyte differentiation from a novel V-96 plate aggregation system highlights interline variability.* Stem Cells, 2007. **25**(4): p. 929-938.

272. Bormashenko, E., R. Balter, and D. Aurbach, *Micropump based on liquid marbles.* Applied Physics Letters, 2010. **97**(9).
273. Mahadevan, L. and Y. Pomeau, *Rolling droplets.* Physics of Fluids, 1999. **11**(9): p. 2449-2453.
274. Bormashenko, E., et al., *On the Mechanism of Floating and Sliding of Liquid Marbles.* Chemphyschem, 2009. **10**(4): p. 654-656.
275. Tian, J., et al., *Liquid marble for gas sensing.* Chemical Communications, 2010. **46**(26): p. 4734-4736.
276. Xue, Y., et al., *Magnetic Liquid Marbles: A "Precise" Miniature Reactor.* Advanced Materials, 2010. **22**(43): p. 4814-+.
277. McHale, G. and M.I. Newton, *Liquid marbles: principles and applications.* Soft Matter, 2011. **7**(12): p. 5473-5481.
278. Arbatan, T., et al., *Tumor Inside a Pearl Drop.* Advanced Healthcare Materials, 2012. **1**(4): p. 467-469.
279. Arbatan, T., et al., *Microreactors: Liquid marbles as micro-bioreactors for rapid blood typing (Adv. Healthcare Mater. 1/2012).* Advanced Healthcare Materials, 2012. **1**(1): p. 79-79.
280. Livak, K.J. and T.D. Schmittgen, *Analysis of relative gene expression data using real-time quantitative PCR and the 2(T)(-Delta Delta C) method.* Methods, 2001. **25**(4): p. 402-408.
281. Arshi, A., et al., *Rigid microenvironments promote cardiac differentiation of mouse and human embryonic stem cells.* Science and Technology of Advanced Materials, 2013. **14**(2).
282. O'Shea, K.S., *Self-renewal vs. differentiation of mouse embryonic stem cells.* Biology of Reproduction, 2004. **71**(6): p. 1755-1765.
283. Sarvi, F., et al., *Surface-functionalization of PDMS for potential micro-bioreactor and embryonic stem cell culture applications.* Journal of Materials Chemistry B, 2013. **1**(7): p. 987-996.
284. Engler, A.J., et al., *Myotubes differentiate optimally on substrates with tissue-like stiffness: pathological implications for soft or stiff microenvironments.* Journal of Cell Biology, 2004. **166**(6): p. 877-887.
285. Ema, M., S. Takahashi, and J. Rossant, *Deletion of the selection cassette, but not cis-acting elements, in targeted Flk1-lacZ allele reveals Flk1 expression in multipotent mesodermal progenitors.* Blood, 2006. **107**(1): p. 111.
286. Motoike, T., et al., *Evidence for novel fate of Flk1(+) progenitor: Contribution to muscle lineage.* Genesis, 2003. **35**(3): p. 153-159.
287. Svensson, E.C., et al., *Molecular cloning of FOG-2: A modulator of transcription factor GATA-4 in cardiomyocytes.* Proceedings of the National Academy of Sciences of the United States of America, 1999. **96**(3): p. 956-961.
288. Lints, T.J., et al., *NKX-2.5 - A novel murine homeobox gene expressed in early progenitor cells and their myogenic descendants* Development, 1993. **119**(2): p. 419-431.
289. Rajala, K., M. Pekkanen-Mattila, and K. Aalto-Setala, *Cardiac Differentiation of Pluripotent Stem Cells.* Stem Cells International, 2011: p. 1-12.
290. Baba, S., et al., *Generation of cardiac and endothelial cells from neonatal mouse testis-derived multipotent germline stem cells.* Stem Cells, 2007. **25**(6): p. 1375-1383.

291. Samarel, A.M., *Costameres, focal adhesions, and cardiomyocyte mechanotransduction.* Am J Physiol-Heart C, 2005. **289**(6): p. H2291-H2301.
292. O'Shea, K.S., *Embryonic stem cell models of development.* Anatomical Record, 1999. **257**(1): p. 32-41.
293. Mauritz, C., et al., *Generation of Functional Murine Cardiac Myocytes From Induced Pluripotent Stem Cells.* Circulation, 2008. **118**(5): p. 507-517.
294. Biben, C. and R.P. Harvey, *Homeodomain factor Nkx2-5 controls left/right asymmetric expression of bHLH gene eHand during murine heart development.* Genes & Development, 1997. **11**(11): p. 1357-1369.
295. Lyons, I., et al., *Myogenic and morphonenetic deffects in the heart of murine embryos lacking the homeo box gene Nkx2.5.* Genes & Development, 1995. **9**(13): p. 1654-1666.
296. Azpiazu, N. and M. Frasch, *TINMAN AND BAGPIPE - 2 HOMEO BOX GENES THAT DETERMINE CELL FATES IN THE DORSAL MESODERM OF DROSOPHILA.* Genes & Development, 1993. **7**(7B): p. 1325-1340.
297. Zhu, M.-X., J.-Y. Zhao, and G.-A. Chen, *Mesoderm is committed to hemato-endothelial and cardiac lineages in human embryoid bodies by sequential exposure to cytokines.* Experimental Cell Research, 2013. **319**(1): p. 21-34.
298. Martin, G.R. and M.J. Evans, *Differentiation of clonal lines of teratocarcinoma cells-formation of embyoid bodies in vitro* Proceedings of the National Academy of Sciences of the United States of America, 1975. **72**(4): p. 1441-1445.
299. Gajovic, S. and P. Gruss, *Differentiation of the mouse embryoid bodies grafted on the chorioallantoic membrane of the chick embryo.* International Journal of Developmental Biology, 1998. **42**(2): p. 225-228.
300. Lopez, A.D., et al., *Global and regional burden of disease and risk factors, 2001: systematic analysis of population health data.* (1474-547X (Electronic)).
301. Segers, V.F. and R.T. Lee, *Stem-cell therapy for cardiac disease.* Nature, 2008. **451**(7181): p. 937-42.
302. Diwan, A., et al., *Decompensation of cardiac hypertrophy: cellular mechanisms and novel therapeutic targets*

Inhibition of ischemic cardiomyocyte apoptosis through targeted ablation of Bnip3 restrains postinfarction remodeling in mice. (1548-9213 (Print)).

303. Wagers, A.J. and I.M. Conboy, *Cellular and molecular signatures of muscle regeneration: current concepts and controversies in adult myogenesis.* Cell, 2005. **122**(5): p. 659-67.
304. Shi, X. and D.J. Garry, *Muscle stem cells in development, regeneration, and disease.* Genes Dev, 2006. **20**(13): p. 1692-708.
305. Nadal-Ginard, B., G.M. Ellison, and D. Torella, *The cardiac stem cell compartment is indispensable for myocardial cell homeostasis, repair and regeneration in the adult.* Stem Cell Research, 2014. **13**(3, Part B): p. 615-630.
306. Lloyd-Jones, D.M., *The risk of congestive heart failure: sobering lessons from the Framingham Heart Study.* Curr Cardiol Rep, 2001. **3**(3): p. 184-90.
307. Patel, J. and J.A. Kobashigawa, *Cardiac transplantation: the alternate list and expansion of the donor pool.* Curr Opin Cardiol, 2004. **19**(2): p. 162-5.

308. Anversa, P. and B. Nadal-Ginard, *Myocyte renewal and ventricular remodelling.* Nature, 2002. **415**(6868): p. 240-3.
309. Reinlib, L. and L. Field, *Cell transplantation as future therapy for cardiovascular disease?: A workshop of the National Heart, Lung, and Blood Institute.* Circulation, 2000. **101**(18): p. E182-7.
310. Garry, D.J. and E.N. Olson, *A common progenitor at the heart of development.* Cell, 2006. **127**(6): p. 1101-4.
311. Das, S., et al., *Generation of embryonic stem cells: limitations of and alternatives to inner cell mass harvest.* Neurosurg Focus, 2008. **24**(3-4): p. E4.
312. Zhang, J., et al., *Functional Cardiomyocytes Derived From Human Induced Pluripotent Stem Cells.* Circulation Research, 2009. **104**(4): p. e30-e41.
313. Ungrin, M.D., et al., *Reproducible, ultra high-throughput formation of multicellular organization from single cell suspension-derived human embryonic stem cell aggregates.* PLoS One, 2008. **3**(2): p. e1565.
314. Siu, C.W., J.C. Moore, and R.A. Li, *Human embryonic stem cell-derived cardiomyocytes for heart therapies.* Cardiovasc Hematol Disord Drug Targets, 2007. **7**(2): p. 145-52.
315. Keller, G.M., *In vitro differentiation of embryonic stem cells.* Curr Opin Cell Biol, 1995. **7**(6): p. 862-9.
316. Kurosawa, H., *Methods for inducing embryoid body formation: in vitro differentiation system of embryonic stem cells.* J Biosci Bioeng, 2007. **103**(5): p. 389-98.
317. Dang, S.M. and P.W. Zandstra, *Scalable production of embryonic stem cell-derived cells.* Methods Mol Biol, 2005. **290**: p. 353-64.
318. Kehoe, D.E., et al., *Propagation of embryonic stem cells in stirred suspension without serum.* Biotechnol Prog, 2008. **24**(6): p. 1342-52.
319. Kehoe, D.E., et al., *Scalable stirred-suspension bioreactor culture of human pluripotent stem cells.* Tissue Eng Part A, 2010. **16**(2): p. 405-21.
320. Bormashenko, E., R. Balter, and D. Aurbach, *Formation of liquid marbles and wetting transitions.* J Colloid Interface Sci, 2012. **384**(1): p. 157-61.
321. Bormashenko, E., *New insights into liquid marbles.* Soft Matter, 2012. **8**(43): p. 11018-11021.
322. Asgari, S., et al., *Induced pluripotent stem cells: a new era for hepatology.* (1600-0641 (Electronic)).
323. Pannell, D. and J. Ellis, *Silencing of gene expression: implications for design of retrovirus vectors.* Rev Med Virol, 2001. **11**(4): p. 205-17.
324. Jaenisch, R. and R. Young, *Stem cells, the molecular circuitry of pluripotency and nuclear reprogramming.* Cell, 2008. **132**(4): p. 567-82.
325. Nakagawa, M., et al., *Generation of induced pluripotent stem cells without Myc from mouse and human fibroblasts.* Nat Biotechnol, 2008. **26**(1): p. 101-6.
326. Stadtfeld, M., et al., *Defining molecular cornerstones during fibroblast to iPS cell reprogramming in mouse.* Cell Stem Cell, 2008. **2**(3): p. 230-40.
327. Hopfl, G., M. Gassmann, and I. Desbaillets, *Differentiating embryonic stem cells into embryoid bodies.* Methods Mol Biol, 2004. **254**: p. 79-98.
328. Itskovitz-Eldor, J., et al., *Differentiation of human embryonic stem cells into embryoid bodies compromising the three embryonic germ layers.* Mol Med, 2000. **6**(2): p. 88-95.

329. Ying, Q.L. and A.G. Smith, *Defined conditions for neural commitment and differentiation.* Methods Enzymol, 2003. **365**: p. 327-41.
330. Mauritz, C., et al., *Generation of functional murine cardiac myocytes from induced pluripotent stem cells.* Circulation, 2008. **118**(5): p. 507-17.
331. Beddington, R.S., P. Rashbass, and V. Wilson, *Brachyury--a gene affecting mouse gastrulation and early organogenesis.* Dev Suppl, 1992: p. 157-65.
332. Maier, J.A., Y. Lo, and B.D. Harfe, *Foxa1 and Foxa2 are required for formation of the intervertebral discs.* PLoS One, 2013. **8**(1): p. e55528.
333. Lendahl, U., L.B. Zimmerman, and R.D. McKay, *CNS stem cells express a new class of intermediate filament protein.* Cell, 1990. **60**(4): p. 585-95.
334. Mansergh, F.C., et al., *Gene expression profiles during early differentiation of mouse embryonic stem cells.* BMC Dev Biol, 2009. **9**: p. 5.
335. Hidaka, K., et al., *Chamber-specific differentiation of Nkx2.5-positive cardiac precursor cells from murine embryonic stem cells.* The FASEB Journal, 2003.
336. Engler, C., et al., *Golden gate shuffling: a one-pot DNA shuffling method based on type IIs restriction enzymes.* PLoS One, 2009. **4**(5): p. e5553.
337. Werner, S., et al., *Fast track assembly of multigene constructs using Golden Gate cloning and the MoClo system.* Bioeng Bugs, 2012. **3**(1): p. 38-43.
338. Lee, J.H., et al., *Sequential amplification of cloned DNA as tandem multimers using class-IIS restriction enzymes.* Genet Anal, 1996. **13**(6): p. 139-45.
339. Rouet, P., F. Smih, and M. Jasin, *Introduction of double-strand breaks into the genome of mouse cells by expression of a rare-cutting endonuclease.* Mol Cell Biol, 1994. **14**(12): p. 8096-106.
340. Ramalingam, S., et al., *Creating designed zinc-finger nucleases with minimal cytotoxicity.* J Mol Biol, 2011. **405**(3): p. 630-41.
341. Coucouvanis, E. and G.R. Martin, *BMP signaling plays a role in visceral endoderm differentiation and cavitation in the early mouse embryo.* Development, 1999. **126**(3): p. 535-46.
342. Vallier, L. and R.A. Pedersen, *Human embryonic stem cells: an in vitro model to study mechanisms controlling pluripotency in early mammalian development.* Stem Cell Rev, 2005. **1**(2): p. 119-30.
343. Takahashi, T., et al., *Ascorbic acid enhances differentiation of embryonic stem cells into cardiac myocytes.* Circulation, 2003. **107**(14): p. 1912-6.
344. Brand, T., *Heart development: molecular insights into cardiac specification and early morphogenesis.* Developmental Biology, 2003. **258**(1): p. 1-19.
345. Craft, A.M., et al., *SIRPA is a specific cell-surface marker for isolating cardiomyocytes derived from human pluripotent stem cells.* Nature Biotechnology, 2011. **29**(11): p. 1011+.
346. Okano, H., et al., *Steps Toward Safe Cell Therapy Using Induced Pluripotent Stem Cells.* Circulation Research, 2013. **112**(3): p. 523-533.
347. Zhao, T., et al., *Immunogenicity of induced pluripotent stem cells.* Nature, 2011. **474**(7350): p. 212-215.
348. Hanna, J., et al., *Treatment of sickle cell anemia mouse model with iPS cells generated from autologous skin.* Science, 2007. **318**(5858): p. 1920-3.

Appendix 1

Isolation and Handling of Mouse Embryonic Fibroblasts

Chapter 13

Isolation and Handling of Mouse Embryonic Fibroblasts

Kanika Jain, Paul J. Verma, and Jun Liu

Abstract

Primary mouse embryonic fibroblasts (MEFs) are the most commonly used feeder layers that help to support growth and maintain pluripotency of embryonic stem cells (ESC) in long-term culture. Feeders provide substrates/nutrients that are essential to maintain pluripotency and prevent spontaneous differentiation of ESC. Since embryonic fibroblasts stop dividing after a few passages, care must be taken to isolate them freshly. Here, we provide a protocol to derive MEFs and describe the method to inactivate the cells using mitomycin C treatment. The protocol also describes freezing, thawing, and passaging of MEFs. This basic protocol works well in our laboratory. However, it can be modified and adapted according to any user's particular requirement.

Key words Mouse embryonic fibroblasts, Feeder layers, Mitomycin C, Cell culture

1 Introduction

MEFs are the primary cell lines that are generally derived from 12.5- to 13.5-day fetuses from pregnant female mice [1]. MEF isolation involves dissecting the uterus and embryonic sac to release the fetuses, discarding the head and visceral tissue, mincing the remaining tissue, and culturing the minced tissue in MEF medium. All newly isolated MEF cultures should be tested for mycoplasma before serving as feeder layers for ES cell culture [2].

The use of MEFs as feeder layers for the culture of pluripotent stem cells (PSC) has been well established since the first mouse ESC were derived in 1981 [3, 4]. Many ES cell lines have been established on MEFs and it is recommended to maintain each of these cell lines on the feeder type on which it was originally established. Induced pluripotent stem cells (iPSC), like ESC, should be grown on monolayers of MEF feeders for long-term culture and maintenance [5, 6]. To generate the feeder cells, MEFs are mitotically inactivated by treatment with mitomycin C or γ irradiation, which inhibits their cell division but keeps the cells metabolically active [7, 8]. Feeders are known to provide a complex but somewhat

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unknown mixture of nutrients and factors that helps to maintain the pluripotency of PSC during their long-term growth and proliferation [9]. Both, MEFs and inactivated feeder MEFs can be frozen and stored in liquid nitrogen indefinitely.

Care must be taken while culturing and maintaining MEFs, since being a primary cell line, they have a limited life-span in culture [10]. If these cells begin to elongate or increase the doubling time, they are going beyond their normal and useful state. Our recommendation is passaging cells no more than five times, which necessitates new stocks being made on a regular basis. The most important factor while culturing MEFs is to keep them in a healthy and proliferative state.

Appropriate institutional animal ethics approvals must be obtained before any work is commenced.

2 Materials

All culture medium must be filtered with a 0.22 μm filter unit. Store all media at 2–6 °C for up to 2 weeks.

2.1 Mice

1. Naturally mated or superovulated day-12.5–13.5 post-coitus (*dpc*) pregnant CD1 mice (*see* **Note 1**).

2.2 Media

1. MEF culture medium: Add 10 % fetal bovine serum (FBS) to 90 % Dulbecco's minimal essential medium (DMEM) with high glucose, supplemented with penicillin/streptomycin (0.5 mL/100 mL). Store at 4 °C.
2. 2× Freezing medium: 20 % FBS, 20 % DMSO, and 60 % DMEM. Pour all components, with DMSO last, into a 0.22 μm filter container and filter (*see* **Note 2**).
3. Mitomycin C solution: 8 μg/mL Mitomycin C. Weigh 2 mg mitomycin C (Sigma) and add to 250 mL DMEM. Filter through a 0.22 μm filter unit and aliquot 7 or 14 mL per Falcon tube. Store at −20 °C.

3 Methods

3.1 MEF Stocks

1. Sacrifice the pregnant mice when embryos are about 12.5–13.5 *dpc* by exposure to CO_2 or cervical dislocation. Sterilize the abdomen with 70 % ethanol and dissect the abdominal cavity to expose the uterine horns.
2. Transfer the uterine horns into 100 mm Petri dishes containing PBS. Use two pairs of watchmakers' forceps to dissect the embryos away from the uterus and all the membranes. Transfer the embryos into a new dish containing PBS. Count the number of the embryos.

3. Wash retrieved embryos three times with PBS. Remove heads and visceral organs (liver, heart, kidney, lung, and intestine) with the same instruments.
4. Wash three times with PBS to remove as much blood and cell debris as possible.
5. Mince the embryos using sharp scissors.
6. Add 10 mL trypsin-EDTA (0.5 %, 10×), transfer into a 50 mL Falcon tube, and incubate for at least 20 min at 37 °C with occasional shaking.
7. Add 10 mL MEF medium to neutralize trypsin (to stop trypsin activity), pipetting the cell suspension up and down thoroughly.
8. Distribute the cell suspension evenly into T75 culture flasks (two embryos per flask), and add additional MEF medium in flasks to a total volume of 20 mL.
9. Culture the cells in a 5 % CO_2 incubator until confluent, and change medium when it is yellowish (do not vacuum the lumps).
10. After 2 to 3 days of culture, the MEF should form a confluent monolayer. Trypsinize each flask and re-plate further into five T75 flasks.
11. When the flasks are confluent (usually after 2 or 3 days), freeze the cells.

3.2 MEF Freezing

Check flasks to see if the cells cover the whole surface of the flask. If there are still some spaces between cells, wait for another day before freezing. If confluent, then freeze cells.

1. Remove medium and all lumps possible from the flasks.
2. Add 3 mL trypsin-EDTA (0.25 %, 10×) to cover the entire culture-flask surface. Incubate for at least 10 min at 37 °C.
3. Tap the side of the flask to loosen the cells, add 6 mL MEF medium to neutralize the trypsin, and wash the flask wall with the medium.
4. Collect cell suspension into a 50 mL Falcon tube; cell suspensions in other flasks from the same mouse could be collected together in the same 50 mL tube (*see* **Note 3**).
5. Centrifuge for 5 min at 200×*g*.
6. Remove supernatant, resuspend in desired volume of MEF medium, and pipette in order to fracture the pellet.
7. Add equivalent volume of freezing medium drop by drop and mix gently (*see* **Note 4**).
8. Distribute to each freezing vial with 1 mL of cell suspension (*see* **Note 5**).
9. Freeze vials overnight at −70 °C in Nalgene freezing boxes (containing Iso-Propanol) and transfer freezing vials to liquid nitrogen container.

3.3 MEF Thawing

1. Remove vials from liquid nitrogen and thaw briefly in a 37 °C water bath (*see* **Note 6**).
2. When a small ice pellet of frozen cell remains, clean the vial using 70 % ethanol.
3. Pipette the contents of the vial once, and transfer cells into a 15 mL Falcon tube.
4. Drop by drop add 2 ml MEF medium, and mix gently (*see* **Note 7**).
5. Centrifuge for 5 min at 200 × *g*.
6. Resuspend the pellet in 5 ml MEF medium, pipetting up and down twice.
7. Transfer the cell suspension into a TC75 flask and add additional 10 ml MEF medium (*see* **Note 8**).

3.4 MEF Splitting

1. Remove the conditioned medium from the flask and add 3 mL trypsin-EDTA (0.25 %) to cover the entire culture-flask surface.
2. Incubate for at least 10 min at 37 °C.
3. Tap the side of the flask to loosen the wells, and add 6 mL MEF medium.
4. Wash the flask wall to remove cells completely and then transfer the cell suspension to a 50 mL Falcon tube. Cell suspension from the same batch can be collected in the same Falcon tube.
5. Centrifuge for 5 min at 200 × *g*.
6. Remove supernatant and resuspend the cell pellet in desired volume of MEF medium and pipetting up and down to fracture the pellet.
7. Distribute cell suspension to desired number of culture flasks (*see* **Note 9**).
8. Add MEF medium to final volume of 10–15 ml per flask.

3.5 Preparation of MEF Feeder Layers

1. Remove conditioned medium from the confluent T75 flask.
2. Add 7 mL mitomycin C solution (8 µg/mL) per flask and incubate cells at 37 °C, 5 % CO_2, for 2–3 h.
3. Remove mitomycin C solution and wash twice with 10 mL PBS.
4. Add 3 mL trypsin-EDTA (0.25 %) for 10 min at 37 °C.
5. Add 6 mL MEF medium to neutralize the trypsin and break any cell aggregates by pipetting.
6. Transfer cell suspension into a conical tube.
7. Centrifuge for 5 min at 200 × *g*.
8. Remove supernatant and resuspend the cell pellet in 10 mL MEF medium.

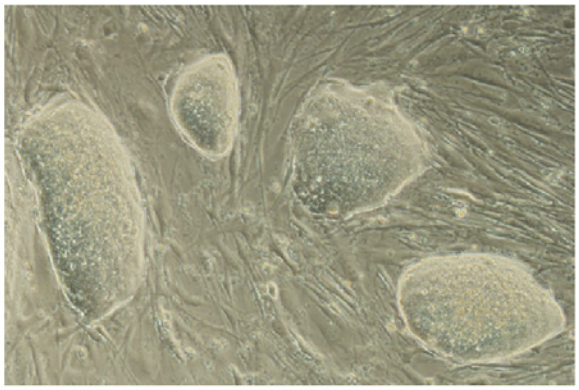

Fig. 1 Morphology of MEFs supporting growth of mouse iPSC colonies. Image of mitomycin C-inactivated MEFs displaying typical morphology and seeding density, supporting mouse iPSC colony growth

9. Titrate the pellet up and down to dissociate.
10. Count the cells and resuspend in desired medium volume.
11. Plate the cells immediately onto tissue culture plates containing MEF medium. For 6-well plate, 400,000 cells/well and 2 ml per well, and for 4-well plate, 100,000 cells/well and 0.5 ml per well (*see* **Note 10**).
12. Allow feeders to attach at least 2 h, but preferably overnight, before seeding ESC or iPSC.
13. Change the medium to ESC medium immediately before adding ESC or iPSC. Mitomycin C-treated MEF feeders can be used for up to 5 days with medium changes every 3 days (Fig. 1).

4 Notes

1. We have not found obvious difference between MEF made from CD1 or Swiss outbred, C57BL/6j inbred, or MTKneo transgenic mice expressing the bacterial neomycin phosphotransferase gene (neo). Superovulated or natural estrus female mice can be used for mating with fertile stud males to generate pregnancies. The superovulation regime entails administration of 5 IU equine chorionic gonadotrophin (eCG; Folligon, Intervet) per prepubertal (~3–4 weeks old) female mouse followed by 5 IU human chorionic gonadotrophin (hCG: Chorulon, Intervet) 48 h later. The females are placed with stud male mice immediately following the hCG injection.

2. We find that it is best to prepare this fresh each time.
3. Collect all flasks from the same mouse and mark them with a batch number (e.g., the date of freezing), due to possible variations between different mice.
4. Adding the freezing medium drop by drop is crucial for cell recovery.
5. It is recommended to freeze four vials from one confluent T75 flask.
6. Normally two different batches of MEF are thawed and cultured parallelly, in case one is contaminated or of bad quality.
7. Adding the medium drop by drop is crucial for cell recovery.
8. It is recommended to thaw one or two vials into one T75 flask.
9. Normally one confluent flask cell is split to three or four flasks.
10. Cell number can also be calculated as $3–4 \times 10^4$ cells per cm^2.

Acknowledgement

This work was supported by Victoria Government's Infrastructure Operational Program.

References

1. Nagy A, Gertsenstein M, Vintersten K, Behringer R (2006) Preparing mouse embryo fibroblasts. CSH Protoc. doi:10.1101/pdb.prot4398
2. E Michalska A (2007) Isolation and propagation of mouse embryonic fibroblasts and preparation of mouse embryonic feeder layer cells. Curr Protoc Stem Cell Biol Chapter 1: Unit1C 3
3. Martin GR (1981) Isolation of a pluripotent cell line from early mouse embryos cultured in medium conditioned by teratocarcinoma stem cells. Proc Natl Acad Sci U S A 78:7634–7638
4. Evans MJ, Kaufman MH (1981) Establishment in culture of pluripotential cells from mouse embryos. Nature 292:154–156
5. Takahashi K, Yamanaka S (2006) Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. Cell 126:663–676
6. Okita K, Ichisaka T, Yamanaka S (2007) Generation of germline-competent induced pluripotent stem cells. Nature 448:313–317
7. Nagy A, Gertsenstein M, Vintersten K, Behringer R (2006) Preparing feeder cell layers from STO or mouse embryo fibroblast (MEF) cells: treatment with mitomycin C. CSH Protoc. doi:10.1101/pdb.prot4399
8. Nagy A, Gertsenstein M, Vintersten K, Behringer R (2006) Preparing feeder cell layers from STO or mouse embryo fibroblast (MEF) cells: treatment with gamma-irradiation. CSH Protoc. doi:10.1101/pdb.prot4400
9. Robertson EJ (1997) Derivation and maintenance of embryonic stem cell cultures. Methods Mol Biol 75:173–184
10. Wesselschmidt RL, Schwartz PH (2011) The stem cell laboratory: design, equipment, and oversight. Methods Mol Biol 767:3–13

Appendix 2

Cardiogenesis of Embryonic Stem Cells with Liquid Marble Micro-Bioreactor

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Cardiogenesis of Embryonic Stem Cells with Liquid Marble Micro-Bioreactor

Fatemeh Sarvi, Kanika Jain, Tina Arbatan, Paul J. Verma, Kerry Hourigan, Mark C. Thompson, Wei Shen, and Peggy P. Y. Chan**

A liquid marble micro-bioreactor is prepared by placing a drop of murine embryonic stem cell (ESC) (Oct4B2-ESC) suspension onto a polytetrafluoroethylene (PTFE) particle bed. The Oct4B2-ESC aggregates to form embryoid bodies (EBs) with relatively uniform size and shape in a liquid marble within 3 d. For the first time, the feasibility of differentiating ESC into cardiac lineages within liquid marbles is being investigated. Without the addition of growth factors, suspended EBs from liquid marbles express various precardiac mesoderm markers including Flk-1, Gata4, and Nkx2.5. Some of the suspended EBs exhibit spontaneous contraction. These results indicate that the liquid marble provides a suitable microenvironment to induce EB formation and spontaneous cardiac mesoderm differentiation. Some of the EBs are subsequently plated onto gelatin-coated tissue culture dishes. Plated EBs express mature cardiac markers atrial myosin light chain 2a (MLC2a) and ventricular myosin light chain (MLC2v), and the cardiac structural marker α-actinin. More than 60% of the plated EBs exhibit spontaneous contraction and express mature cardiomyocyte marker cardiac troponin T (cTnT), indicating that these EBs have differentiated into functional cardiomyocytes. Together, these results demonstrate that the liquid-marble technique is an easily employed, cost effective, and efficient approach to generate EBs and facilitating their cardiogenesis.

1. Introduction

Adult hearts have a very limited capacity for self-regeneration after myocardial infarction (MI; heart attack). Transplanted stem cells or progenitor cells have the capacity to repair infarcted myocardium.[1,2] Pluripotent embryonic stem cell (ESC), isolated from the inner cell mass of a developing blastocyst,[3] possess the ability to self-renew, and have the potential to differentiate into various cell lineages, including all three germ layers[4] and cardiomyocytes.[5] The ability to engineer these ESC genetically, together with their ability to differentiate into cardiomyocytes in vitro, makes them valuable and promising cell sources for cell therapy, tissue engineering, and regenerative medicine.[6] Forming 3D embryo-like cell aggregates, known as embryoid bodies (EBs), is a key step for the in vitro differentiation of ESC.[7] Indeed, an EB consists of three germ layers (ectodermal, mesodermal, and endodermal tissues) that emulate the features of a developing embryo,[4,8] thereby providing a valuable tool for various embryogenesis studies.[9,10]

Several methods have been employed to form EBs from ESC and to subsequently differentiate them into cardiomyocytes. These include hanging-drop culture,[9] spinner flask,[11] centrifuge-forced aggregation,[12] and suspension culture in a low-adherence vessel.[4,7] The hanging drop method is the most commonly used technique for EB formation, in which an ESC suspension was placed on the inner surface of a Petri dish lid. EBs can be formed after inverting the lid due to the balance of gravitational and surface tension forces. Changing the droplet volume and seeding density can tune the size of the EBs. However, the hanging drop method is labor intensive and time consuming. It is also practically impossible to perform medium exchange using this method. In addition, the drop volume is limited to less than 50 µL, thereby making it incapable of supporting large-scale production.[8] Rotation-based methods, such as the spinner flask and centrifuge-forced aggregation methods, can facilitate the large-scale production. However, these methods require costly equipment; moreover, the shear stress induced by the rotation may reduce cell viability, and disrupt cell–cell signaling and the subsequent cell differentiation. The method based on suspension culturing using conventional low-adherence vessels has limited control over size, shape, and uniformity of EBs.[13]

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The liquid marble was first described by[14] and consists of a drop of liquid encapsulated by hydrophobic powder particles. These particles adhere to the surface of the liquid drop, isolating the liquid core from the supporting surface, while allowing gas exchange between the interior liquid and the surrounding environment.[15] A liquid marble can be rolled around similar to a droplet of mercury.[16] As the shells of liquid marbles are made from discrete particles, the shells can be opened, allowing materials such as reagents and products to be introduced into or extracted from the liquid marble; this unique property therefore facilitates chemical and biochemical reactions to be controlled within liquid marbles.

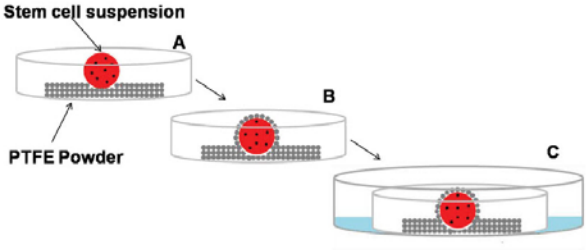

Figure 1. Schematic illustration of the steps involved in preparing a liquid marble bioreactor. A) 300 mL of cell suspension is placed onto a hydrophobic PTFE powder bed, B) The Petri dish was then rolled gently to allow the PTFE particles to cover the cell suspension to form the liquid marble. C) Placing the marble dish inside a bigger Petri dish with sterilized water to prevent evaporation

In addition, reagent consumption can be reduced due to the small size of a liquid marble. The chance of contamination is low as a result of the indirect contact between the liquid core and the supporting surface, thus providing an advantage for a variety of applications.[15,17–19] The use of liquid marbles as miniaturized bioreactors is particularly attractive because of the capability to contain chemical and biological reactions.[18,20–23] Our previous studies reported the production of 3D cancer-cell spheroids using liquid marble micro-bioreactors.[20] This liquid-marble method is advantageous for spheroid production, as it allows the production of spheroids with homogeneous size and shape at a larger scale compared with the hanging drop method, as well as facilitating medium exchange. Unlike rotation-based methods, the liquid-marble method does not induce shear stress on the spheroids, thereby producing viable spheroids.[13]

Herein, we report the use of the liquid marble as a micro-bioreactor to produce EBs from ESC and, for the first time, we study the feasibility to further differentiate the EBs into lineage-specific cells. The in vitro cardiac differentiation ability of the resulting EBs was assessed by examining gene expression, protein expression, and contraction characteristics. We demonstrate that liquid marbles provide a promising platform to facilitate EB differentiation into cardiac lineages.

2. Result and Discussion

2.1. Pluripotency and Propagation of EB

Formation of 3D aggregates called EBs is an important step that precedes the initiation of in vitro differentiation of ESC into various cell types.[24] Under the in vitro conditions, an EB is known to simulate the events of a developing embryo. In a previous study, we reported the possibility of using liquid marbles (LMs) as a facile and efficient micro-bioreactor for in vitro EB formation, the viability of cells in EB obtained using LM was much higher than those obtained using conventional suspension culture method.[13] In that study, liquid marble micro-bioreactor was formed by self-assembled polytetrafluoroethylene (PTFE) powder around a droplet of ESC suspension as illustrated in **Figure 1**, ESC were cultivated inside liquid marbles and cell aggregates were obtained from day 3. All three germ layers developed spontaneously within the cell aggregates, indicating that the ESC formed EBs successfully inside the liquid marbles.[13] In our present study, we use liquid marbles as micro-bioreactors to generate EBs from Oct4B2-ESC and investigate, for the first time, the capability of liquid marble to facilitate cardiac differentiation. **Figure 2** illustrates the steps involved in cardiac differentiation. The morphology of cells harvested from the liquid marbles was assessed using optical microscopy. The Oct4B2-ESC contain a pluripotency marker that drives the expression of GFP, hence GFP expression is a direct indicator of cell pluripotency.[25]

The pluripotency of cells forming the EBs was monitored by examining their GFP expression. Representative images in **Figure 3** show that after cultivating Oct4B2-ESC in liquid marbles for 3 d, these cells aggregated to form EBs, and that these EBs exhibited a compact and round shape with relatively uniform size.

As evident from Figure 3A,B, the ES cells, while aggregating to form clusters destined to form EBs, retained their GFP expression, indicating that the cells do not lose their pluripotency. The day 10 analysis of the EBs revealed an increase in size suggesting the proliferation of cells within the LM. There was a decrease in the GFP flourescence observed, stipulating the loss of pluripotency and that there was an initiation of differentiation of ES cells within the LM (Figure 3D).

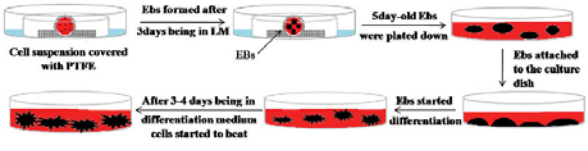

Figure 2. Schematic steps leading to cardiac differentiation.

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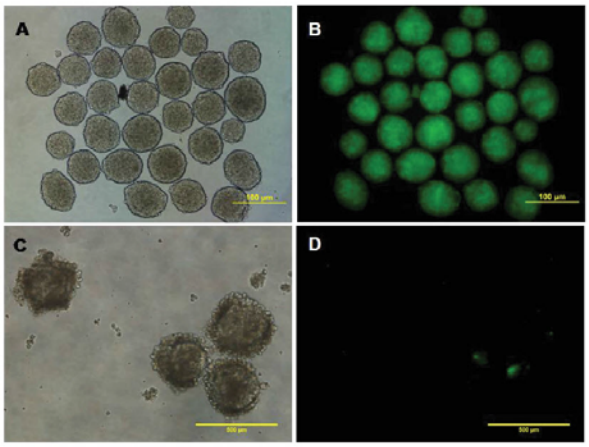

Figure 3. Representative phase-contrast and fluorescence microscopy images showing the morphology of EBs. These EBs were formed by allowing Oct4B2-ESC to aggregate inside liquid marbles for A,B) 3 d (scale bars represent 100 μm) and C,D) 10 d (scale bars represent 500 μm). B,D) Fluorescence microscopy images show the expression of GFP under the control of Oct4.

Oct4B2 cells were allowed to form EBs in liquid marbles for 10 d; samples were collected from the liquid marbles at days 0 (control), 3, 7, and 10. Fluorescence-activated cell sorting (FACS) analysis was performed on the dissociated cells to quantify the number of GFP^+ cells in an EB in order to examine their pluripotency. Representative FACS analysis profiles in **Figure 4** show that 98.8% and 92.4% of cells expressed GFP at days 0 and 3, respectively. The GFP expression gradually reduced to 20% and 9.4% at days 7 and 10, respectively. Thus, the loss of GFP expression as suggested from the FACS analysis and the flourescence data implies the ability of LM to allow proliferation and differentiation of Oct4B2 cells. This novel system is thus capable of providing a suitable microenvironment for the growth and differentiation of ESCs.

It has been well documented that during the development of contractile cardiomyocytes, progenitor cells need to first anchor to a substrate followed by cell spreading, withdrawal from the cell cycle, and fusion with nascent myotubes before their ultimate differentiation into cardiomyocytes.[26] In order to promote cardiogenesis of our LM-induced EBs, 5-day-old EBs were plated onto gelatin-coated six wells and allowed to differentiate further. Upon EB adhesion, the cells began to migrate and grew outwards from the periphery of the EB to form a monolayer, as shown in the representative images in **Figure 5**A,B. In the outgrown areas shown in Figure 5C, it was observed that cells exhibited heterogeneous cell morphology. At the start of cardiac differentiation, cells were small and rounded, which upon further differentiation changed to elongated, spindle-shaped cells. This observation was in-line with the results of the electrophysiological measurements conducted by Hescheler et al.[27] wherein the heterogeneous population of cardiomyocytes undergoes a shift from early stage cardiomyocytes (small and rounded with rarely developed sarcomeres) to terminally differentiated atrial-/ventricular-like (elongated with high content of organized myofibrils) cells.

It is worth mentioning that a double Petri dishes set-up was used to minimize evaporation of liquid marble in this study. With this set-up, very little change in liquid marble size was observed over the period of cell culture study. For future work, the influence of evaporation on the liquid marble size, thus its volume, can be monitored using a video camera and image-processing technique. A calibration curve of volume change versus liquid marble size can be established, such that any volume loss due to evaporation can be quantified using images of the liquid marble and the calibration curve, and equivalent amount of fresh medium can be added to replace any volume loss. However, this is beyond the scope of the current study, as this work aims to demonstrate the feasibility of generating cardiomyocytes in liquid marble micro-bioreactor.

2.2. Reverse Transcription PCR Analysis of Cardiac Marker Expression During Cardiogenesis

To characterize the differentiation pathway of EBs generated from a liquid marble, the gene expression of EB-derived cells was qualitatively determined using reverse-transcription-polymerase chain reaction (RT-PCR). Cells from a suspended EB obtained from a liquid marble and plated EBs were collected at different time points and were characterized using a series of cardiac markers. Flk-1 expression is found in mesodermal progenitor cells that have the ability to further differentiate into cardiac muscles.[28,29] Gata4 is a regulator of early cardiogenesis,[30] it expresses in precardiac mesoderm and subsequently expresses in the endocardial and myocardial tissues of developing heart and heart tube; overexpression of Gata4 is known to up-regulate the expression of transcription factors Nkx2.5.[31] The cardiac specific transcription factor Nkx-2.5 is the key regulator of cardiac-specific transcription involved in cardiogenesis, which is generally observed in the pluripotent stem cell-derived cardiomyocytes.[32] Therefore, Flk-1, Gata4, and Nkx2.5 were all employed as precardiac mesoderm markers in this study. β-actin was used as housekeeping marker gene. Atrial myosin light chain 2a (MLC2a) and ventricular myosin light chain (MLC2v) are both cardiac structural proteins, and are often employed as mature cardiac cell markers.[33,34] Sarcomeric protein α-actinin is a cardiac structural protein that crosslinks actin filaments within the Z-disc of cardiac muscle.[34,35] MLC2a, MLC2v, and α–actinin were thereby used as mature cardiomyocyte markers in this study. **Figure 6**A shows that cells from suspended EBs expressed Gata4 from days 3 to 10. Flk1 and Nkx2.5 expressions could also be detected in suspended EBs from days 3 to 10. The positive expression of Flk1, Gata4, and

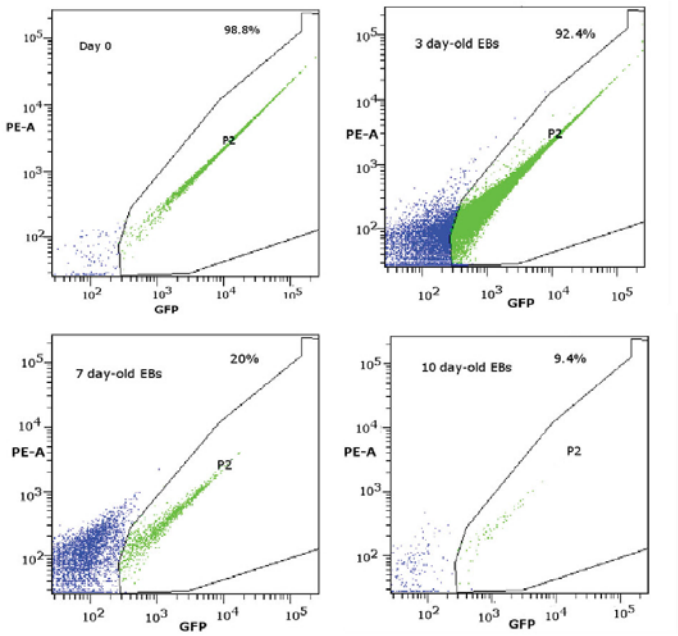

Figure 4. FACS analysis: GFP expression of LM induced EBs of Oct4B2 at days 0 (control), 3, 7, and 10. Numbers indicate the percentage of GFP$^+$ cells. GFP is indicated on x-axis, and autofluorescence on y-axis. GFP$^-$ population is represented by blue dots, while GFP$^+$ events are represented by green dots.

Nkx2.5 suggests that liquid marbles provide a suitable environment to induce cardiac mesoderm differentiation in ESC. Figure 6B shows the RT-PCR analysis of the plated-down EBs. Nkx2.5, Gata4, MLC2a, MLC2v, and α-actinin expressions were detected from days 6 to 15, revealing that these cells had differentiated into precardiac mesoderm and mature cardiomyocytes after plating-down. Similar to the ESC differentiation seen in a hanging drop,[36] removing leukemia inhibitory factor (LIF) from the medium in our culture system, signs of ESC differentiation were detected in suspended EBs inside liquid marbles as well as in outgrown cells in plated-down culture. The use of the liquid marble allows the formation of EBs, which, in turn, enables cell-to-cell interactions. This cell-to-cell interaction is known to stimulate the expression of early cardiac lineage markers.[34] Nevertheless, the liquid-marble method permits the use of larger drop volumes compared to the hanging drop method and is therefore more advantageous for larger scale studies.

2.3. Quantitative Real-Time PCR Analysis of Cardiac Markers Expression During Cardiogenesis

EBs were allowed to form inside liquid marbles for 5 d, and were plated in gelatin-coated plates with differentiation medium that contained no growth factors. The gene expression of these cells was quantitatively determined using real-time PCR by harvesting cells at days 6, 8, 10, 12. The time-dependent

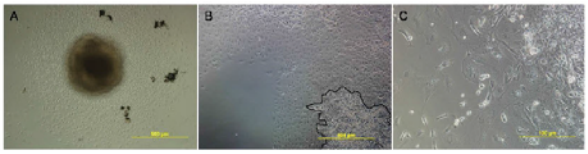

Figure 5. Phase-contrast images of a cardiac explants outgrowth. A) Representative image showing a plated EB attached to a gelatin-coated plate after 1 d, B) representative image of plated EB outgrowth at day 4, scale bars represent 500 μm. The black line shows the border of flattened EBs after plating down. C) Representative image of the plated EB depicting the heterogeneous cell morphology (small and round, spindle shaped cells), at day 8, scale bar represent 100 μm.

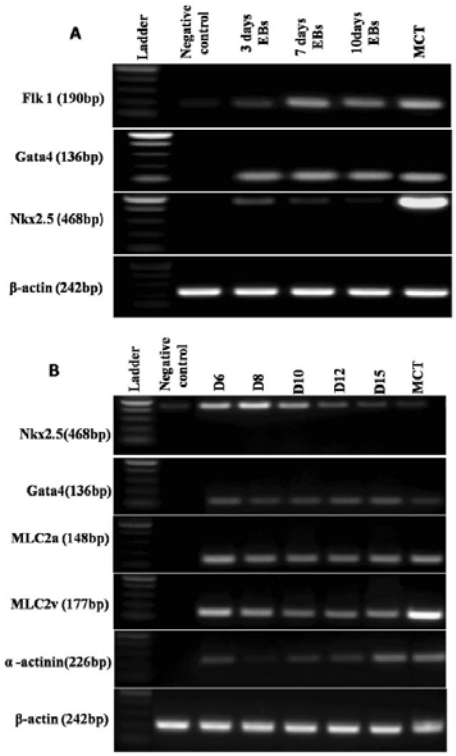

Figure 6. A) Representative RT-PCR analysis for precardiac mesoderm markers expression in cells from EB suspensions obtained from liquid marbles after 3, 7, and 10 d of culture. B) Representative RT-PCR analysis for precardiac mesoderm and mature cardiomyocytes markers expression from EBs after plating down for 6, 8, 10, 12, and 15 d. β-actin was used as a housekeeping gene. Cells obtained from murine cardiac tissue (MCT) were used as a positive control. Undifferentiated ES cells were used as a negative control.

expressions of various lineage and cardiac markers during the course of differentiation are shown in **Figure 7**. β-actin was used as a constitutive housekeeping gene for real-time PCR and used to normalize changes in specific gene expressions. Octamer-binding transcription factor 4 (Oct4) was used as both a positive control for pluripotent cells and a negative control for cardiac cells. The pluripotent or undifferentiated ESCs express high level of Oct4 gene, whereas cells that are undertaking cardiac differentiation express negligible levels of Oct4 gene. Oct4 expression was down regulated from day 6, which suggested that the ESC had lost their pluripotency. The expression of precardiac mesoderm markers Flk-1, Gata4, and Nkx2.5 were up regulated after plating down the EBs for 6 d compared with undifferentiated ES cells. The expression of Flk-1 decreased from day 8 onward, while the expression of Gata4 and Nkx2.5 decreased from day 10 onward. These results together suggested that the ESC had differentiated into cardiac mesoderm and subsequently differentiate into other cell types. The formation of cardiac mesoderm is a prerequisite intermediate step for cardiomyocytes differentiation from pluripotent stem cells. Expressions of MLC2a, MLC2v, and α–actinin were first detected at day 6. The MLC2a expression increased at day 10, and decreased gradually from day 12. The MLC2v expression increased at day 8, and decreased gradually from day 10. The α–actinin expression remained constant from days 6 to 12, and increased sharply at day 15, thus suggesting that stable sarcomeric structural protein was formed within the cell culture. The expression of MLC2a, MLC2v, and α–actinin, together indicated that plated EBs had differentiated into cardiomyocytes spontaneously. It is noted that spontaneous beating was observed in EBs after plating down for 4 d. The results are consistent with those reported in other studies, where Nkx2.5, MLC2v, and MLC2a are expressed in beating cardiomyocytes that had differentiated from ES cells.[31] Altogether, the results indicate that EBs derived from liquid marbles had differentiated into mesoderm and subsequently into mature cardiomyocytes.

2.4. Immunocytochemistry

Five-day-old EBs were collected from liquid marbles and plated down. Immunostaining was performed on EBs that exhibited spontaneous contraction to detect the presence of the cardiac-specific proteins. As shown in representative images in **Figure 8** (top panel), the expression of the cardiac transcription factor Nkx2.5 (AlexaFlour594, stained red) was detected after plating down the EB for 7 d. Nkx2.5 is a transcription factor that is expressed in myocardiogenic progenitor cells during myocardial development;[32,37] Nkx2.5 is the earliest known marker for cardiogenesis in the veterbrate embryo.[38–40] Representative images in Figure 8 (bottom panel) show that cells expressed cardiac troponin T (cTnT) markers (AlexaFlour594, stained red) after plating down for 12 d. cTnT is a marker for mature cardiomyocytes,[34] EB-derived cells expressed cardiac-specific protein markers, thus further confirming that this liquid marble method can generate functional EBs that can further differentiate into mature cardiomyocytes.

2.5. Beating Cardiac Cells

In order to observe contractile, EBs were kept inside liquid marbles for 7 d. At day 7, EBs were retrieved and suspended in a low-adhesion dish. After 8 h, it was observed that only ≈4.5% of EBs exhibited spontaneous contraction (Supporting Information 1), the contraction only lasted for a few hours, probably because once the ESC had differentiated into lineage-specific progenitor cells, they needed to anchor onto a substrate for optimal differentiation, as reported by Engler et al.[26] Nevertheless, this observation confirmed the differentiation potential of EBs into generate cardiomyocyte-like cells inside liquid marbles. To examine if EBs generated from liquid marbles can further differentiate into beating cardiac cells, 5-day-old EBs

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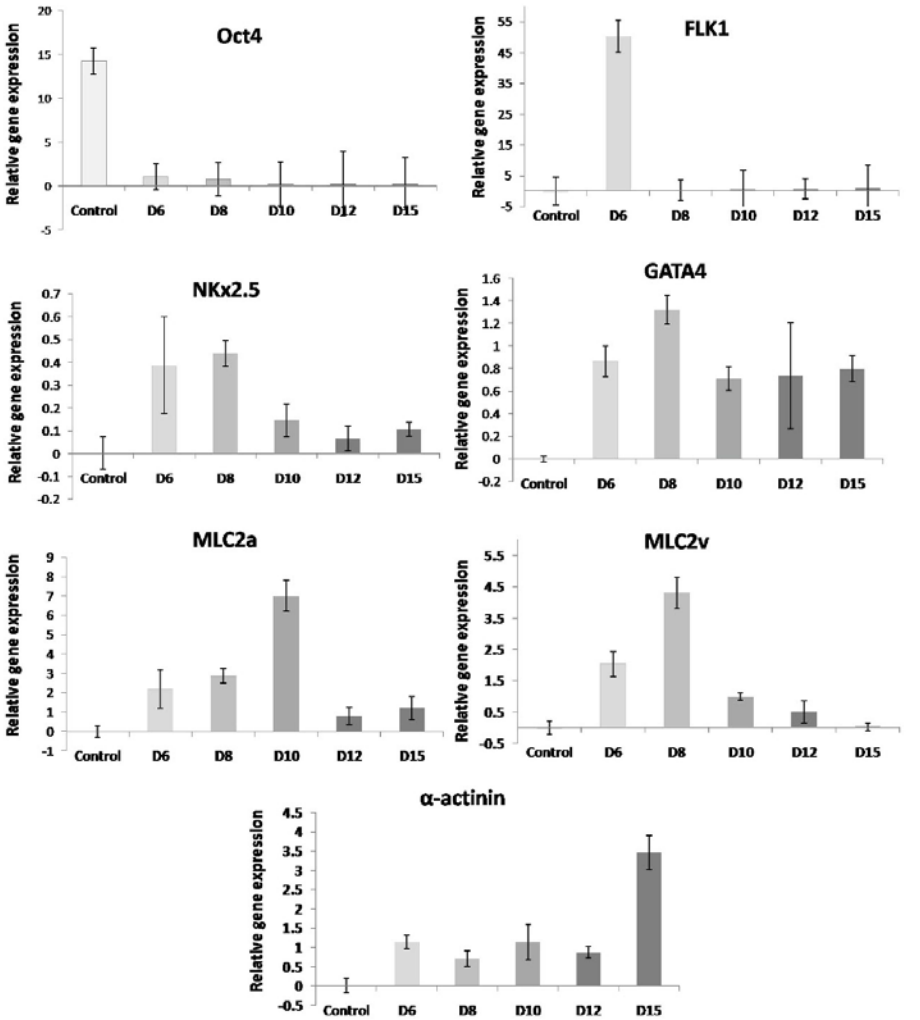

Figure 7. Real-time PCR analysis of gene expression over 15 d of cardiomyogenesis after plating down 5-d old EBs derived from liquid marbles. Cardiomyogenesis was characterized by a continuous decrease in pluripotency marker (Oct4) expression over the course of differentiation, followed by an initial increase in the expression of the precardiac mesoderm markers (Flk1, Nkx2.5, and Gata4) expression, which eventually decreased. The increase in the expression of mature cardiomyocytes markers (MLC2v, MLC2a, and α-actinin) expression as seen resulted in the formation of mature cardiomyocytes.

were removed from the liquid marble and plated down in a gelatin-coated wells and the activities of the EBs were monitored. These EBs adhered and proliferated well on the substrate. Spontaneous rhythmic beating was detected in the outgrown EBs after plating down for 4 d. Supporting Information 2,3 show the videos of beating cells derived from EBs after plating

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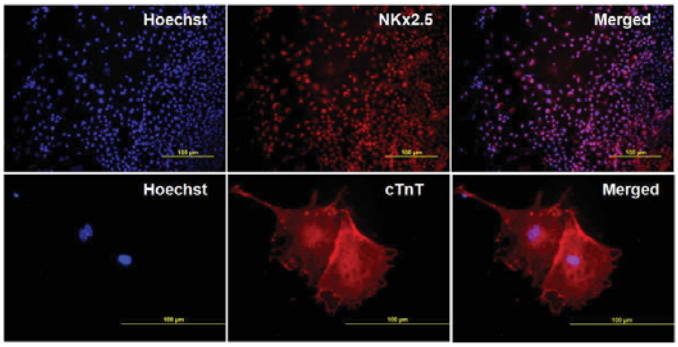

Figure 8. Immunostaining for Nkx2.5 (after 7 d) and cTNT (after 12 d) of plated down cells. Nuclei were counterstained with Hoechst (blue). Scale bars represent 100 μm.

down for 4 and 7 d, respectively. The rhythmic beating was considered as a functional cardiac marker.[41] For brevity, EBs that differentiated into beating cells are denoted as beating EBs. **Figure 9** A,B show the mean intensity of beating obtained from representative areas of beating foci after plating down the EBs for 4 and 7 d, respectively. At day 4, the beating was relatively regular with minor arrhythmia. At day 7, the beating became more regular. The beating frequency was calculated by measuring the time interval between two consecutive peaks in the mean intensity patterns. The frequency increased from 0.76 s^{-1} at day 4, to 1.29 s^{-1} at day 7, as shown in Figure 9C. This was probably because the cells were initially beating asynchronously after migrating out from the EB; these cells became more synchronized given the longer culture time.

After plating the EBs for 4 d, approximately 16% of the EBs underwent differentiation into rhythmic beating cells (Figure 9D). The percentage of beating EBs increased to 48% after 7 d. The percentage of beating EBs continued to increase

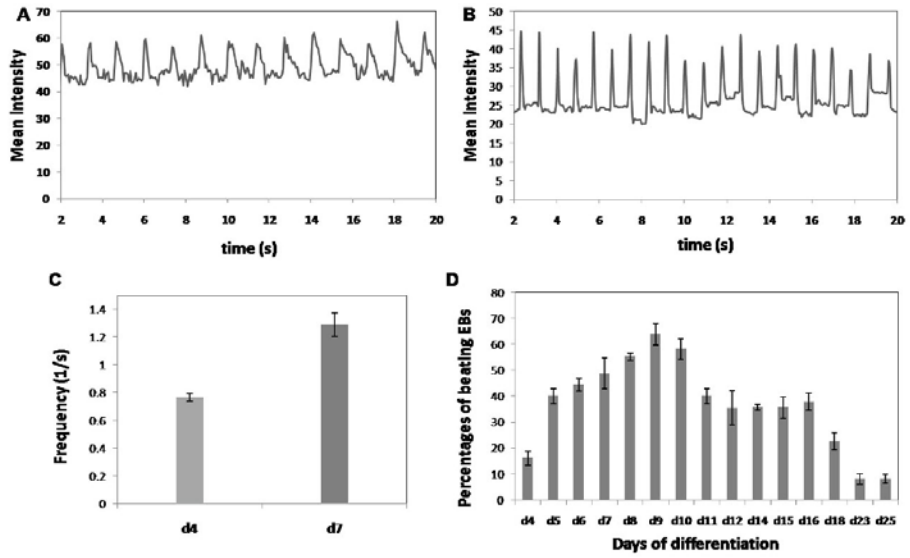

Figure 9. Mean intensity patterns obtained from representative areas in contractile EBs after plating for A) 4 and B) 7 d. C) The beating frequency of EBs at days 4 and 7. D) The percentages of contractile EBs at different time points.

over time reaching a maximum of 64% after 9 d. After 10 d, the rhythmic beating activity declined, possibly due to the overgrowth of cells causing peeling of cells from the plate, although approximately 35% of the EBs still exhibited rhythmic beating activity from days 12 to 16. Approximately 8% of EBs still exhibited rhythmic beating at day 25. As expected, a larger number of EBs exhibited spontaneous contraction in plated culture compared to suspension culture. This observation is consistent with other studies that found that EBs differentiate extensively after attaching to a substrate.[42,43] The observation of spontaneous rhythmic beating again confirmed that liquid-marble-derived EBs differentiated into functional cardiac cells. Taken together, these results indicate that the liquid marble is a promising platform for EB generation, and for facilitating further differentiation into cells with a cardiac lineage.

3. Conclusions

In this study, we investigated the capability of liquid marbles to induce EB formation and subsequent differentiation into cardiomyocytes. Liquid marbles were prepared by inoculating ESC onto a bed of hydrophobic PTFE particles. ESC aggregated to form uniform EBs after inoculation, and subsequently differentiated into cardiac mesoderm cells without the use of growth factors. Further on plating, these EBs further differentiated into contractile cardiomyocytes. The contraction of cardiomyocytes was synchronized with longer time in culture. The liquid-marble method was found to be advantageous for EB formation as it is cost effective and simple; it also allows larger scale EB production compared with the hanging drop method. Overall, this study shows that liquid marbles can serve not only as a novel platform to induce the formation of EBs but also to facilitate cardiogenesis. The cardiomyocytes generated via this liquid marble strategy could provide a continuous source of donor cardiomyocytes for cell replacement therapy in damaged hearts. Furthermore, this technology would be highly beneficial to provide cardiomyocytes for use in cardiac drug discovery programs and safety testing. Since the quantity of cells required for the above-mentioned applications is very high, it becomes imperative to develop defined and efficient in vitro protocols, which would then provide the stringent levels of safety and quality control making stem cell transplantation therapy realizable. Our study provides a step up this ladder and gives a new promise and hope in cardiovascular research

4. Experimental Section

Tissue Culture: Feeder free murine Oct4B2-ESC (129/Sv) containing the Oct4-GFP-IRES-puromycin and hygromycin resistance cassettes were used for this work. For expansion, ESC were cultured in Dulbecco's modified Eagle medium (cat#11995; Gibco) supplemented with 10% FBS (JRH Biosciences, Australia), 1% non-essential amino acids (NEAA) (cat. #11140-050; Invitrogen), 1% GlutaMAX (cat#35050061; Invitrogen), 0.5% penicillin–streptomycin (cat#15070–063; Invitrogen), 0.1×10^{-3}M β-mercaptoethanol (cat. #21985-023; Invitrogen), and 1000 U mL^{-1} ESGRO leukemia inhibitory factor (mLIF, Chemicon, Australia). The medium was filtered through a 0.22-μm filter for sterilization and was stored at 4 °C for up to a fortnight. Cells were cultured on 0.1% gelatin-coated six-well plates (BD Falcon) at 37 °C in a humidified 5% CO_2 incubator and were passaged every 2–3 d. The GFP expression of the cells was monitored using an IX71 Olympus epifluorescence microscope.

Preparation of Cell Containing Liquid Marble Micro-Bioreactor: ESC were cultured to 70%–80% confluency; cells were then washed with Dulbecco's phosphate buffered saline (DPBS; Sigma) and dispersed into single cells using Tryple express (Gibco, Life Technologies, Australia). To form liquid marbles, 2×10^4 ESCs were suspended in 300 μL of differentiation medium. The differentiation medium consisted of high-glucose DMEM (Gibco, Life Technologies, Australia) supplemented with 10% FBS (JRH Biosciences, Australia), 1% non-essential amino acids, 1% GlutaMAX (Gibco, Life Technologies, Australia), 0.5% penicillin–streptomycin (Gibco, Life Technologies, Australia), 0.1×10^{-3}M β-mercaptoethanol (Gibco, Life Technologies, Australia) and without mLIF. This drop of cell suspension was placed onto a PTFE (35 μm particle size; Sigma, Australia) powder bed inside a Petri dish (60 mm diameter) using a micropipette. When the drop of cell suspension was rolled on the hydrophobic powder bed, the powder particles wrapped the surface of the drop, thus leading to the formation of a liquid marble. The strong hydrophobic and chemically inert properties of PTFE powder are the desirable properties for making liquid marble biological reactors for this work. Two critical requirements to the reactor shell, apart from air permeability, are that the shell should resist cell adhesion and the marble shell should be mechanically strong and maintain its integrity for the duration of the experiment. Our recent work[13,20] showed that PTFE powder marble shell satisfies both requirements. The selection of 35 μm PTFE powder was based on the balanced liquid marble shell mechanical strength and its ability to resist cell adhesion so as to encourage the formation of stem cell EBs.[13] Arbatan et al. characterized the mechanical strength of the liquid marble by measuring the force by which a glass cover slip cut through the powder-covered water surface.[44] They found that liquid marble shell formed by 35 μm powder has a sufficient mechanical strength to sustain the reactor integrity, while also produces the most uniform stem cell EBs.[13] The Petri dish containing the liquid marble was then placed inside a larger Petri dish (100 mm diameter) containing sterile water to minimize evaporation from the liquid marbles, following which the set of dishes was covered and kept inside a humidified incubator. Periodically, the size of the liquid marble was being checked visually. The ESCs were allowed to aggregate to form EBs within the liquid marble over a period of 5 d. Figure 1 illustrates the process of forming the liquid marbles. The color of the medium in liquid marble was monitored on a daily basis, no color change was observed over the first 5 d. From day 5 onward, whenever a color change was observed in the liquid marble, 50–100 μL of spent medium was removed and replaced by the same amount of fresh medium using a micropipette.

EB Morphology and Pluripotency Characterization: ESC-containing liquid marbles were prepared and allowed to incubate for a period of 10 d. EB samples were taken from the liquid marbles at days 3, 7, and 10. The morphology and GFP expression of EBs were monitored using optical and epifluorescence microscopy (Olympus 1×70 microscope). Both phase-contrast and epifluorescent images were captured. The collected EBs were further dissociated into single cells using Tryple express; the GFP expression of these cells was quantified using FACS at different time points during EB formation.

In Vitro Cardiac Differentiation: For cardiac differentiation, 5-day-old EBs were removed from the marbles and transferred to 0.1% gelatin-coated 24-well plates and cultured in differentiation medium for further analysis. The plated EBs were examined daily for contractile activity based on videos captured at 15 fps using a camera through an optical microscope. The detailed description of the protocol is illustrated in Figure 2.

Reverse-Transcription and Real-Time Polymerase Chain Reaction: The gene expression analysis of the EBs and the cells undergoing differentiation were carried out quantitatively using the RT-PCR. Liquid-marble-suspended EBs and plated-down EBs were both subjected to RT-PCR analysis using various differentiation markers. EBs were

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Table 1. PCR primer sequences and the NCBI accession numbers for the corresponding genes.

Gene name	Forward/reverse (5′ 3′)	NCBI accession no.
Flk-1	GCCCGTCGTCACAGTATCTT/ CTCCGTCATCTACACCATCC	NM_010612.2
Nkx2.5	ACACCCACCCCTTTCTCACTCAAA/ CCACAGCTACCCCTCTTCCTTCAA	NM_008700.2
Gata4	TCTCACTATCCCCACACCAC/ CCGATGTCTGACTGACACGA	NM_008092
MLC2a	TCACCTCCATTGACCACAAC/ AACACCGTCAACTTCATCGG	NM_022879.2
MLC2v	AAAGAGCCTCCAGGTCCAAT/ CCTCTCTCCTTCTGTCGTCA	NM_010861.3
α-actinin	ATCAGCATTCCCTCCTTT/ TCTTCCACCCCGTCTTC	NM_013456
β-actin	CACCACACCTTCTACAATCACC/ TCCTACATCCCCACACTCTCCC	NM_007393.3
Oct4	GTTCAGCCACACCACCATCT/ CCAGGGTCTCCCATTTCCAT	NM_013633

allowed to form inside the liquid marbles for 3, 7, and 10 d, and were then retrieved from the LM for RT-PCR. To allow further differentiation, some EBs were retrieved from liquid marble after 5 d and plated down on gelatin-coated wells for another 15 d. The plated EBs were analysed for differentiation markers at time points D6, 8, 10, 12, and 15 d. Cells were harvested with Tryple express, the resulting cell pellets were snap chilled at −80 °C prior to analysis. For RT-PCR, ribonucleic acid (RNA) was isolated from cells using the RNeasy kit (Qiagen, Australia) according to the manufacturer's instructions. RNA quality and concentration were measured using a NanoDrop ND-1000 (NanoDrop Technologies, Australia). The isolated RNA was subjected to RQ1 DNase (Ambion, Australia) treatment to remove any contaminating genomic deoxyribonucleic acid (DNA). Complementary DNA (cDNA) was generated using the Superscript III enzyme (Life Technologies, Australia) according to the manufacturer's protocols. The cDNA samples were subjected to PCR amplification with mouse cardiac-specific primers. The primer sequences were obtained from the online NCBI Primer-Blast databank and are listed in **Table** 1. The PCR products were size fractionated using 1% agarose gel electrophoresis at 110 V for 1 h. For quantification, real-time PCR was performed. Real-time PCR analysis was performed using a 7900HT fast real-time PCR system (Applied Biosystems, Australia) at standard reaction conditions using the Power SYBR Green PCR Master Mix (Applied Biosystems, Australia). Briefly, after 2 min denaturation at 95 °C, 35 PCR cycles were carried out at 95 °C for 15 s, 58 °C for 30 s, and 72 °C for 30 s, followed by a dissociation stage. Relative mRNA levels were calculated using the ΔΔCT method[45] and were analysed using SDS Version 2.4.1 software, β-actin was used as internal reference gene. The results were reported as fold change values compared to murine cardiac tissue (MCT). The experiment was replicated three times.

Immunocytochemistry: The protein markers on the cells were characterized using immunostaining. Incubated cells were first fixed in 4% paraformaldehyde for 30 min and washed three times with 1% bovine serum albumin (BSA) in +/+DPBS. Fixed cells were incubated with blocking solution (5% goat serum, 1% BSA in +/+DPBS) for 1 h at room temperature. Cells were then incubated overnight with NKx2.5 primary antibody (Santa Cruz, ThermoFisher Scientific, Australia) and cTnT primary antibody (Abcam, Sapphire Bioscience, Australia) diluted (1:500) in blocking solution at 4 °C. Next, the cells were washed three times with 1% BSA in +/+DPBS and then incubated at room temperature for 1 h with Alexa 594-labeled secondary antibodies (Molecular Probes, Life technologies, Australia) at a concentration of 1:400 in blocking solution. Cells were washed three times with 1% BSA in DPBS for 10 min. Nuclei were counterstained with Hoechst (1 μg mL^{-1}; Sigma) dye. Cells were analyzed by epifluorescence microscopy (IX71 Olympus microscope, Australia).

Image Analysis: After plating 5-day-old EBs for another 4 d in gelatin-coated wells, beating foci appeared in the outgrowing EBs. Videos of the beating foci were captured using the microscope camera system; these videos were converted into image sequences using NIS viewer elements software (Nikon, USA). The images were converted to gray scale. The contraction rhythm of the EBs was evaluated using a modified image processing method described in Arshi et al.[46] As the EB underwent rhythmic beating, the cell cluster color changed from dark to light. Ten different areas were selected from each video, the contracting motion through changes in gray scale intensity in each area was analyzed using ImageJ software (NIH, USA), and the resulting mean intensity was plotted against time.

Supporting Information

Supporting Information is available from the Wiley Online Library or from the author

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[1] D. Orlic, J. Kajstura, S. Chimenti, D. M. Bodine, A. Leri, P. Anversa, *Ann. N.Y. Acad. Sci.* **2001**, *938*, 221.
[2] D. A. Taylor, B. Z. Atkins, P. Hungspreugs, T. R. Jones, M. C. Reedy, K. A. Hutcheson, D. D. Glower, W. E. Kraus, *Nat. Med.* **1998**, *4*, 929.
[3] M. J. Evans, M. H. Kaufman, *Nature* **1981**, *292*, 154.
[4] M. Koike, S. Sakaki, Y. Amano, H. Kurosawa, *J. Biosci. Bioeng.* **2007**, *104*, 294.
[5] V. A. Maltsev, J. Rohwedel, J. Hescheler, A. M. Wobus, *Mech. Dev.* **1993**, *44*, 41.
[6] M. D. Ungrin, C. Joshi, A. Nica, C. Bauwens, P. W. Zandstra, *PLoS One* **2008**, 3.
[7] T. C. Doetschman, H. Eistetter, M. Katz, *J. Embryol. Exp. Morph.* **1985**, *87*, 27.
[8] H. Kurosawa, *J. Biosci. Bioeng.* **2007**, *103*, 389.
[9] G. M. Keller, *Curr. Opin. Cell Biol.* **1995**, *7*, 862.
[10] A. G. Smith, *Annu. Rev. Cell Dev. Biol.* **2001**, *17*, 435.
[11] S. Niebruegge, A. Nehring, H. Bar, M. Schroeder, R. Zweigerdt, J. Lehmann, *Tissue Eng. Part A* **2008**, *14*, 1591.
[12] P. W. Burridge, D. Anderson, H. Priddle, M. D. B. Munoz, S. Chamberlain, C. Allegrucci, L. E. Young, C. Denning, *Stem Cells* **2007**, *25*, 929.

[13] F. Sarvi, T. Arbatan, P. P. Y. Chan, W. Shen, *RSC Adv.* **2013**, *3*, 14501.
[14] P. Aussillous, D. Quere, *Nature* **2001**, *411*, 924.
[15] E. Bormashenko, R. Balter, D. Aurbach, *Appl. Phys. Lett.* **2010**, 97.
[16] L. Mahadevan, Y. Pomeau, *Phys. Fluids* **1999**, *11*, 2449.
[17] E. Bormashenko, Y. Bormashenko, A. Musin, Z. Barkay, *ChemPhys Chem* **2009**, *10*, 654.
[18] J. Tian, T. Arbatan, X. Li, W. Shen, *Chem. Commun.* **2010**, *46*, 4734.
[19] Y. Xue, H. Wang, Y. Zhao, L. Dai, L. Feng, X. Wang, T. Lin, *Adv. Mater.* **2010**, *22*, 4814.
[20] T. Arbatan, A. Al-Abboodi, F. Sarvi, P. P. Y. Chan, W. Shen, *Adv. Healthcare Mater.* **2012**, *1*, 467.
[21] T. Arbatan, L. Li, J. Tian, W. Shen, *Adv. Healthcare Mater.* **2012**, *1*, 79.
[22] E. Bormashenko, R. Balter, D. Aurbach, *Int. J. Chem. Reactor Eng.* **2011**, 9, Note S10.
[23] G. McHale, M. I. Newton, *Soft Matter* **2011**, *7*, 5473.
[24] K. S. O'Shea, *Biol. Reprod.* **2004**, *71*, 1755.
[25] F. Sarvi, Z. Yue, K. Hourigan, M. C. Thompson, P. P. Y. Chan, *J. Mater. Chem. B* **2013**, *1*, 987.
[26] A. J. Engler, M. A. Griffin, S. Sen, C. G. Bonnetnann, H. L. Sweeney, D. E. Discher, *J. Cell Biol.* **2004**, *166*, 877.
[27] J. Hescheler, B. K. Fleischmann, S. Lentini, V. A. Maltsev, J. Rohwedel, A. M. Wobus, K. Addicks, *Cardiovasc. Res.* **1997**, *36*, 149.
[28] M. Ema, S. Takahashi, J. Rossant, *Blood* **2006**, *107*, 111.
[29] T. Motoike, D. W. Markham, J. Rossant, T. N. Sato, *Genesis* **2003**, *35*, 153.
[30] E. C. Svensson, R. L. Tufts, C. E. Polk, J. M. Leiden, *Proc. Natl. Acad. Sci. U.S.A.* **1999**, *96*, 956.
[31] K. R. Boheler, J. Czyz, D. Tweedie, H. T. Yang, S. V. Anisimov, A. M. Wobus, *Circ. Res.* **2002**, *91*, 189.
[32] T. J. Lints, L. M. Parsons, L. Hartley, I. Lyons, R. P. Harvey, *Development* **1993**, *119*, 419.
[33] S. Baba, T. Heike, K. Umeda, T. Iwasa, S. Kaichi, Y. Hiraumi, H. Doi, M. Yoshimoto, M. Kanatsu-Shinohara, T. Shinohara, T. Nakahata, *Stem Cells* **2007**, *25*, 1375.
[34] K. Rajala, M. Pekkanen-Mattila, K. Aalto-Setala, *Stem Cells Int.* **2011**, Article ID 383709.
[35] A. M. Samarel, *Am. J. Physiol.-Heart C* **2005**, *289*, H2291.
[36] K. S. O'Shea, *Anat. Rec.* **1999**, *257*, 32.
[37] C. Mauritz, K. Schwanke, M. Reppel, S. Neef, K. Katsirntaki, L. S. Maier, F. Nguemo, S. Menke, M. Haustein, J. Hescheler, G. Hasenfuss, U. Martin, *Circulation* **2008**, *118*, 507.
[38] N. Azpiazu, M. Frasch, *Genes Dev.* **1993**, *7*, 1325.
[39] C. Biben, R. P. Harvey, *Genes Dev.* **1997**, *11*, 1357.
[40] I. Lyons, L. M. Parsons, L. Hartley, R. L. Li, J. E. Andrews, L. Robb, R. P. Harvey, *Genes Dev.* **1995**, *9*, 1654.
[41] M.-X. Zhu, J.-Y. Zhao, G.-A. Chen, *Exp. Cell Res.* **2013**, *319*, 21.
[42] S. Gajovic, P. Gruss, *Int. J. Dev. Biol.* **1998**, *42*, 225.
[43] G. R. Martin, M. J. Evans, *Proc. Natl. Acad. Sci. U.S.A.* **1975**, *72*, 1441.
[44] T. Arbatan, W. Shen, *Langmuir* **2011**, *27*, 12923.
[45] K. J. Livak, T. D. Schmittgen, *Methods* **2001**, *25*, 402.
[46] A. Arshi, Y. Nakashima, H. Nakano, S. Eaimkhong, D. Evseenko, J. Reed, A. Z. Stieg, J. K. Gimzewski, A. Nakano, *Sci. Technol. Adv. Mater.* **2013**, 14.

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Appendix 3

Golden Gate Assembly Protocol-Detailed

Golden Gate TALEN assembly

This is an expanded and slightly modified TAL assembly protocol published in the original form in Cermak, et al., 2011 (http://dx.doi.org/10.1093/nar/gkr218)
Modifications to the published protocol by Michelle Christian, Colby Starker and other members of Dan Voytas' lab.

Reagents (those highlighted are often not found in most labs and need to be ordered specifically for this protocol):

A) Set of 60 library vectors (see the file TALEN_golden_gate_library_stock_plate_sheet)
B) 10X T4 DNA ligase buffer (NEB)
C) Quick ligase or T-4 DNA ligase (NEB)
D) restriction endonuclease BsaI (NEB)
E) restriction endonuclease Esp3I (Fermentas or Fisher [FERER0452])
F) Plasmid-Safe nuclease (Epicentre Biotechnologies E3110K)
G) 10mM ATP
H) chemically competent cells
I) SOC
J) LB plates and liquid media with Tetracycline (10mg/l), Spectinomycin (50mg/l), Ampicillin (50mg/l)
K) X-gal/IPTG
L) Miniprep kit (Qiagen)

For screening/sequencing of transformants:

Primers

pCR8_F1: ttgatgcctggcagttccct
pCR8_R1: cgaaccgaacaggcttatgt
TAL_F1: ttggcgtcggcaaacagtgg
TAL_R2: ggcgacgaggtggtcgttgg
SeqTALEN_5-1 catcgcgcaatgcactgac (use this for sequencing in place of TAL_F1)

AND/OR restriction endonucleases – for restriction screening (NEB)
AflII
XbaI
BstAPI or StuI
AatII

BspEI

DAY1

1. Choose your TALEN RVD sequence: N = number of RVDs (12-31)

   If the TALEN length is 12-21:

   2. Pick plasmids for the RVDs 1-10 (e.g. pNI1, pNN2, pHD3, pHD4….) + destination vector pFUS_A

   3. Pick plasmids 11 up to N-1 + destination vector pFUS_B#N-1
      (pFUS_B plasmids are labeled B1-B10 but they are used for RVDs 11-30 – if the RVD #N-1 is 19 or 29, use the same destination vector pFUS_B9)

   If the TALEN length is 22-31:

   2. Pick plasmids for the RVDs 1-10 + destination vector pFUS_A30A, pick plasmids for the RVDs 11-20 + destination vector pFUS_A30B

   3. Pick plasmids 21 up to N-1 + destination vector pFUS_B#N-1

Note: pFUS_B plasmids are labeled B1-B10 but they are used for RVDs 11-30 – if the RVD #N-1 is 19 or 29, use the same destination vector pFUS_B9)

4. Mix golden gate reaction **#1** – for each set of vectors separately:

   1-10 + pFUS_A;
   11-(N-1) + pFUS_B(N-1)
   or
   1-10 + pFUSA30A
   11-20 + pFUSA30B
   21-(N-1) + pFUS_B(N-1)

   a) 150ng of each module vector + 150ng of pFUS vector.
   b) 1µl BsaI
   c) 1µl Quick ligase or T-4 DNA ligase (QL is higher efficiency, but T-4 is much cheaper and works fine)
   d) 2µl 10X T4 DNA ligase buffer (to final concentration of **1X**)
   e) H_2O up to **20µl total reaction volume**

Note: Published protocol indicates using 20µL reactions, but we find 10µL reactions are reliably effective (same concentrations as in published protocol). have done ½ reactions (same concentrations, only 10µL total volume). If a particular cloning reaction is somewhat difficult (failed more than once), it may be useful to use a 20µL reaction.

5. Run cycle: **10x** (37˚C/5min + 16˚C/10min) + 50˚C/5min + 80˚C/5min
   With this cycle you will get hundreds of white colonies with 90-100% efficiency.

6. Plasmid-Safe nuclease treatment: this destroys all unligated linear dsDNA fragments including incomplete ligation products with lower number of repeats fused; and cut and linearized vectors. The incomplete, shorter fragments would be cloned into the destination vector *in vivo* by recombination in the bacterial cell, if not removed (the start of the first repeat and the end of the last repeat are in the destination vector backbone, so the backbone has homology to each repeat module as they differ only in RVDs)
   To each of your golden gate #1 reactions add:
   a) 1ul 10mM ATP
   b) 1ul Plasmid-Safe nuclease
   Incubate at 37˚C/1h

Note: The Plasmid-Safe nuclease manual says you should inactivate the enzyme by heating the reaction to 70˚C for 30 minutes, but our experience, for bacterial transformatin, inactivation is not necessary

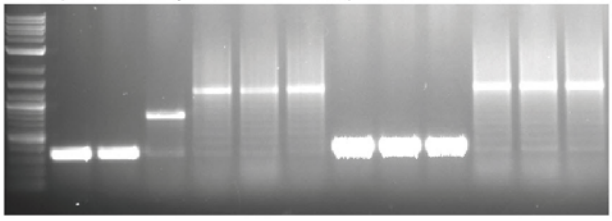
7. Transform your chemically competent cells (we use 5ul of the GG reaction)

8. Plate on $Spec^{50}$ plates + 40µL of 20mg/mL X-gal +40µL of 0.1M IPTG. When plating transformations of the pFUSB vectors that have fewer repeats (especially less than 6 repeats), be careful to not plate all the cells as the efficiency is so high you can't pick single colonies on day 2.

DAY2

9. Pick 1-3 white colonies from each plate and check by **colony PCR** using primers pCR8_F1 and pCR8_R1 (primers are the same for each pFUSA, pFUSA30A, pFUSA30B, pFUSB1-10 vector). PCR program: Anneal at 55º, extend 1.75min, cycle 30-35X. You should get a band around your expected size (~1.2KB for vectors with 10 repeats), but you will also get smearing and a 'ladder' of bands starting at ~200bp and every 100bp up to ~500bp. This is the sign of a correct clone and is the result of the repeats in the clones.

   Example of Colony PCR results for pFUS vectors:



Note: Lanes 2 and 3 are negative pFUS clones (empty). Lane 4 contains the 'correct' clone for this pFUS. pFUS clones that only contain 1 or 2 repeats are very similar in size to empty pFUS clones – check the size carefully. Lanes 5,6,7,11,12,13 show the 'laddering' effect well. DNA ladder is NEB's 2Log

10. Start the over-night cultures with the correct clones

DAY3

11. Miniprep the plasmids:
    pFUS_A with first 10 repeats cloned (**A**)
    pFUS_B with 11-(N-1) repeats cloned (**B**)
    or
    pFUS_A30A with first 10 repeats cloned (**A1**)
    pFUS_A30B with second 10 repeats cloned (**A2**)
    pFUS_B with 21-(N-1) repeats cloned (**B**)

12. Optional restriction digestion testing/sequencing:
    Use enzymes AflII and XbaI (same for all destination vectors) to cut out the array of fused repeats: 1048bp for pFUS_A vectors, different sizes depending on number of repeats cloned for pFUS_B vectors
    and/or sequence with primers pCR8_F1, pCR8_R1

13. Mix golden gate reaction **#2**

a) 150ng of each vector **A** and **B** (or **A1**, **A2** and **B**)
b) 150ng of respective **pLR vector** – this is the vector containing the last "half-repeat" including the last RVD, choose it according to your TALEN sequence – it is the last RVD in the sequence (there are 5 pLR vectors – pLR-HD, pLR-NG, pLR-NI, pLR-NN, pLR-NK)
c) 75ng of destination vector pTAL1, 2, 3, or 4
d) 1µl Esp3I
e) 1µl Quick ligase or T-4 Ligase (this reaction is so efficient that T-4 ligase is always sufficient)
f) 2µl 10X T4 DNA ligase buffer or Tango buffer (the buffer for the Esp3I enzyme)
g) H_2O up to 20µl

14. Run cycle: 10x(37˚C/5min + 16˚C/10min) + 37˚C/15min + 80˚C/5min for hundreds of white colonies, OR **37˚C/10min + 16˚C/15min + 37˚C/15min + 80˚C/5min** for tens of white colonies* **This 1 cycle reactions is sufficient for the second GG reaction, and this is what the Voytas lab usually does.**

15. Transform your competent cells (use 5ml of the reaction)

Note: Plasmid-Safe nuclease treatment is not necessary in this case, because the final destination vector has no homology with the inserted repeats

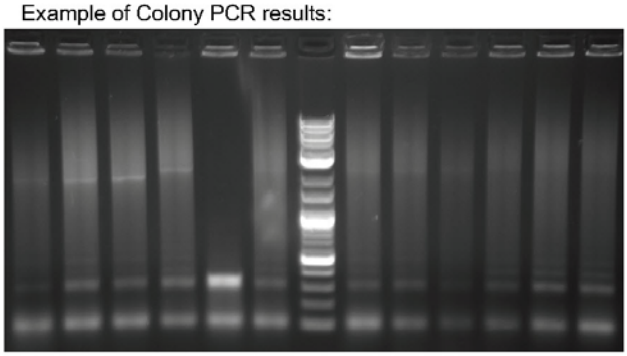
16. Plate on Carb(Amp)^{50} plates + X-gal and IPTG (see above). After you gain confidence/experience with the GG cloning, it's reasonable to skip the IPTG/X-gal for the pTAL cloning.

DAY4

17. Pick 1-3 white colonies and check by colony PCR using primers TAL_F1 and TAL_R2 using these conditions:
    a. Anneal at 55º, extend 3 minutes, cycle 30-35X

b. Very often, you can't see the band of the size you expect, but instead see a smear and the 'ladder' effect – again, this is the sign of a correct clone.

18. Run on a gel, choose a correct clone and start an over-night culture
    Example of Colony PCR results:



Note that in lane 5, you don't see very much 'smear' around 3KB (Ladder is NEB's 2Log), which indicates that this clone is NOT correct. In most other cases in the above gel you can see faint bands around 2-3 KB, which are the correct length for the completed TALs in the picture above. For TALs with >22 repeats, it is common to fail to amplify enough full-length TAL to see on a gel, however if you can see the 'smear' those clones are almost always correct. The 'ladder effect' is evident in some of the lanes (4, 11,12,13).

DAY5

19. Miniprep the pTAL vectors containing your final full-length TALEN

20. Optional restriction digestion testing/sequencing:
    Use enzymes BstAPI (or StuI) and AatII to cut out the final array of repeats – check on a gel
    You can use BspEI enzyme, which cuts only in HD repeats (except HD1 repeats) – on the gel you'll get a pattern resembling your TALEN sequence and HD repeats position

Note: there is no BspEI site in the first (i.e. #1) and the last (i.e. #N) HD repeat

and sequence with primers SeqTALEN 5-1 and TAL_R2

21. Your TALEN is ready to use in a yeast-based DNA cleavage assay. Or further cloning, you can cut the TALE domain out using BamHI or combination of XbaI and EcoRV (blunt)