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Reprogramming of Mouse Fibroblasts to iPS Cells in Stirred Suspension Bioreactors using Physical and Genetic Methods

by

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Abstract

The clinical application of stem cells depends on the availability of pluripotent cells that are not restricted by ethical, immunological and technical considerations. The recent development of the derivation of induced pluripotent stem cells (iPSCs) from somatic cells is opening a new era in developmental biology. Having potential advantages in regenerative medicine, iPSCs are similar to their embryonic stem cell (ESCs) counterparts and possess all of the essential criteria such as pluripotency, self-renewal and potency. Despite major improvements in the methods of iPSC generation and expansion, the process still remains inefficient and poorly characterized.

This thesis describes the development of a novel approach for the derivation and expansion of iPSCs. Initially, a study conducted to evaluate the potential of using the stirred suspension bioreactor (SSB) system for large scale differentiation of murine ESCs into cardiomyocytes. Despite the fact that we could differentiate ESCs into cardiomyocytes, surprisingly, we found that the SSB suppressed differentiation, in favor of maintaining pluripotency. The effect was presumed to be due to effect of fluid shear stress (FSS) on the cells. Based on the findings that SSBs favor pluripotency over differentiation, we examined this environment for the long term expansion and maintenance of iPSCs in an undifferentiated state. Our results demonstrated that SSBs yield a 58-fold expansion of undifferentiated pluripotent iPSCs over 4 days. *In vitro* and *in vivo* characterization further confirmed the existence of fully functional and undifferentiated pluripotent iPSC aggregates following long term passaging in SSBs.

Subsequently, we developed a novel method for the efficient and expedited derivation of iPSCs in SSBs. We found that suspension bioreactors increase both the

kinetics and efficiency of iPSC derivation and can provide a selective advantage to enhance cellular reprogramming, presumably through application of shear stress. The resulting suspension-derived iPSCs (SiPSCs) resembled ESCs in their *in vitro* and *in vivo* characteristics, such as teratoma and chimera formation and displayed germ line transmission competency. The findings presented in this thesis show that SSBs not only suppress differentiation but also provide a novel environment for the expansion and maintenance of iPSCs as well as their efficient derivation.

Preface

The following manuscript was reprinted:

Chapter II (Partly) (with permission from John Wiley and Sons, Inc.)

Shafa M, Krawetz R, Rancourt DE. Returning to the stem state: epigenetics of recapitulating pre-differentiation chromatin structure. Bioessays. 2010 Sep; 32(9):791-9. Review

Chapter III (Biomed Central open access)

Shafa M, Krawetz R, Zhang Y, Rattner JB, Godollei A, Duff HJ and Rancourt DE. Impact of stirred suspension bioreactor culture on the differentiation of murine embryonic stem cells into cardiomyocytes. BMC Cell Biology. 2011 Dec 14; 12(1):53.

Chapter IV (with permission from John Wiley and Sons, Inc.)

Shafa M, Sjonnesen K, Yamashita A, Liu S, Kallos MS and Rancourt DE. Expansion and long term maintenance of induced pluripotent stem cells in stirred suspension bioreactors. J Tissue Eng Regen Med. 2011 Jul 14. doi: 10.1002/term.450. [Epub ahead of print]

Chapter V (Nature publishing group, no permission needed)

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Other Achievements not Reflected in this Thesis

Book Chapter

Stem Cell Epigenetics and Human Disease. Mehdi Shafa and Derrick E. Rancourt. Elsevier. 2012. In press.

The Chromatin State of Pluripotent Cells. Mehdi Shafa and Derrick E. Rancourt. Nova Science Publishers, Inc. 2012. In press.

Conference Presentations

1. **Stem Cell Network annual meeting conference. Montreal, Canada, Nov 2-4, 2009.** Mehdi Shafa, Henry J. Duff, Derrick E. Rancourt. Enhancement of ES cell cardiomyocytes differentiation in suspension culture. **(Poster presentation)**

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Efficient and expedited derivation of mouse Induced Pluripotent Stem Cells in Stirred Suspension Bioreactors. (**Podium presentation**)

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To my beautiful and wonderful wife Neda and my sons Rahman and Ryan-I feel so fortunate to have you all in my life.

Dedication

To **Neda**, who gave me her true love and continued support all the way throughout our journey. Neda, you are such a special part of me; you are everything that matters in my life. The more I learn about you, the more I realize why I love you so much.

Table of Contents

List of Tables

List of Figures and Illustrations

List of Symbols, Abbreviations and Nomenclature

Symbol	*Definition*
ALP	*-alkaline phosphatase*
ASCs	*- adult stem cells*
BSA	*- bovine serum albumin*
DMEM	*- Dulbecco's modified Eagle's medium*
DNA	*- deoxyribonucleic acid*
EB	*- embryoid body*
ECM	*- extracellular matrix*
EGCs	*- embryonic germ cells*
ESCs	*- embryonic stem cells*
Et-Br	*- ethidium bromide*
FACS	*- fluorescent-activated cell sorting*
FBS	*- fetal bovine serum*
FSS	*-fluid shear stress*
H&E	*- hematoxylin and eosin*
HLA	*- human leukocyte antigen*
HSCs	*- hematopoietic stem cells*
ICM	*- inner cell mass*
iPSCs	*- induced pluripotent stem cells*
LIF	*- leukemia inhibitory factory*
MEF	*- murine embryonic fibroblast*

MET	*- mesenchymal-epithelial transition*
MSCs	*- mesenchymal stem cells*
NSCs	*- neural stem cells*
PBS	*- phosphate buffered saline*
PCR	*- polymerizing chain reaction*
qRT-PCR	*-quantitative real time – polymerase chain reaction*
RNA	*- ribonucleic acid*
SCID	*- severe combined immunodeficiency*
SiPSCs	*- suspension-derived iPSCs*
SSB	*- stirred suspension bioreactor*

Units of Measurement

bp	*-base pair*
d	*- day*
g	*- gram*
hr	*- hour*
M	*- moles/L*
mg	*- milligram*
min	*- minute*
mL	*- millilitre*
mM	*- millimolar*
ng	*- nanogram*

nm	- *nanometer*
rpm	- *revolutions per minute*
µg	- *microgram*
µL	- *microlitre*
µm	- *micrometer*
µM	- *micromolar*

Epigraph

Our greatest glory is not in never falling,

but in rising every time we fall.

~Confucius

Chapter One:

Introduction

Embryonic stem cells (ESCs) are derived from the inner cell mass (ICM) of pre-implantation blastocysts (Evans and Kaufman 1981; Thomson, Itskovitz-Eldor et al. 1998). They are endowed with two unique properties: unlimited self-renewal capacity and the stable developmental potential to give rise to various terminally differentiated cell types. Induced pluripotent stem cells (iPSCs) are another source of pluripotent stem cells (PSCs) that have been recently derived by forcing expression of transcription factors (Takahashi and Yamanaka 2006; Takahashi, Tanabe et al. 2007). They share similar characteristics with ESCs, which make them a suitable cell source for therapeutic applications, and basic research. One major research area is regenerative medicine, which focuses on the application of cell therapies for the treatment of human degenerative diseases. In addition, PSCs can also be used as model systems for understanding human development in addition to elucidating the pathophysiology of specific disorders.

Several technical obstacles must be overcome before human PSCs clinical application can be a reality. These include but are not limited to: 1) immunological rejection upon transplantation, 2) ethical considerations pertaining to their derivation, 3) potential tumorigenic properties following cell therapy, and 4) scalable production.

One of the challenging areas, which has hindered the therapeutic application of PSCs, is the potential for immune rejection of cells following allogenic transplantation. Although application of immunosuppressive drugs can reduce the immune response, it will not completely resolve the attack and the patients can be highly vulnerable to

infection. An alternative strategy to hamper the immunological graft rejection of transplanted cells would be the establishment of a clinical-grade HLA-haplotype bio-bank of pluripotent cell lines with enough diversity. A banking system will also decrease the cost of clinical applications, since the complete cell therapy for all of the patients costs too much.

The possibility of constructing such a human ESC bank was first suggested for the United Kingdom population (Taylor, Bolton et al. 2005). They estimated the number of donor ESC lines needed to cover the whole UK population; only ten donors who are homozygous for common HLA types can provide a complete HLA-A, HLA-B and HLA-DR match for 37.7% of recipients and an acceptable match for about 67% of the recipients. In the Japanese population, a bank size of only 50 iPSC lines is adequate to afford match for three loci in about 90% of people (Nakatsuji, Nakajima et al. 2008). The number of donors and the percentage of population coverage can be adjusted for each population. Another advantage of these kinds of centralized bio-banks is the availability of reliable, robust, and well-characterized ESCs/iPSCs for researchers. Development of such banking systems necessitates the generation of adequate number of cells through a scalable and reproducible process.

Another obstacle that prevents the clinical application of ESCs is the ethical objections attributed to their derivation procedures. Currently, the process of deriving human ESC requires the destruction of human embryos. The recent derivation of iPSCs through direct cellular reprogramming seems to avoid the complicated ethical and moral disputes surrounding the derivation of ESCs, albeit with caveats. Although iPSCs bypass some ethical concerns associated with destroying an embryo, there are still ethical

roadblocks regarding their potential usage. For example, the relative low cost and ease of their derivation, make it potentially possible to create gametes for reproduction purposes.

The generation of mouse and human iPSCs from terminally differentiated cells is providing an alternative source of pluripotent stem cells. This method is empowering the field of regenerative medicine and is opening a new era in developmental cell biology. Originally, iPSCs were generated by introducing defined transcription factor genes using viral vectors (Takahashi and Yamanaka 2006; Yu, Vodyanik et al. 2007). Since 2006, the field has investigated the derivation of iPSCs from various mouse and human somatic cells with combinations of different approaches such as plasmids, proteins, mRNA and microRNAs. Similar to their ESCs equivalent, iPSCs have all of the functional pluripotency criteria such as self-renewal, clonality and potency. They can differentiate to form all three germ layers *in vitro* and *in vivo*. iPSCs have the prominent advantage over ESCs in that their derivation does not need human embryos or oocytes. In addition to its application in basic biology, iPSC technology has also provoked enthusiasm for establishing novel therapies for degenerative disorders. This will be enabled by deriving iPSCs from patients bearing genetic mutations. These specific iPSCs will be useful for elucidating diseases on a cell/tissue level and developing potential therapeutics. Hence, generation of high quality iPSCs can enhance the application of these cells for developmental biology and, ultimately, allow their use in clinical therapies.

Tumorigenicity or indiscriminate mass formation by pluripotent stem cell differentiation products is still considered a challenging safety concern preventing clinical application (Amariglio, Hirshberg et al. 2009). It has been shown that even a trace number of residual undifferentiated cells can produce teratomas upon *in vivo*

transplantation (Wernig, Zhao et al. 2008). Pre-differentiation of these cells toward a single cell lineage is one means to remove the tumor formation risk of transplanted cells, but the absence of a reliable and robust directed differentiation method has restricted PSC application in humans. Even in the presence of such protocols, impure populations of differentiated cells may exist (Clarke and van der Kooy 2009). Thus far, several approaches have been developed to bypass this impediment including pre-selection of differentiated cells through flow cytometry and lineage selection using antibiotic selectable markers.

Despite advances in the field, the process of iPSC generation remains inefficient and poorly characterized. One of the greatest challenges for the translation of ESC/iPSC technology to the bedside has been the scalable and robust production of these cells (Kirouac and Zandstra 2008). Currently, there is a "bioprocess gap" between the potential applications of iPSCs and their inadequacy, which necessitates a suitable plan for large-scale production protocols.

The large scale production of ESCs has been achieved by the utilization of stirred suspension bioreactors (SSBs). SSBs offer several benefits over the conventional use of adherent tissue culture flasks. First, these systems expedite the large-scale expansion of ESCs with less cost. Second, they supply a homogenous and consistent culture environment, which eliminates time consuming feeding, costly passaging procedures, risk of contamination and decreases culture variability, including the possibility of controlling culture variables such as pH and oxygen concentration. Recently, our lab has shown that SSBs are a favourable and scalable environment for the controlled expansion of murine

and human ESCs as aggregates (Cormier, zur Nieden et al. 2006; zur Nieden, Cormier et al. 2007; Krawetz, Taiani et al. 2010).

The robustness of the controlled stirred suspension bioreactors allows the potential generation of large quantities of iPSCs on a clinical scale. Suspension bioreactor technology offers the potential to simplify the process of deriving and expanding iPSCs for therapeutic application. When used to its fullest potential, SSBs could offer high capacity derivation/expansion of iPSCs using a fraction of the time, while greatly reducing labour and culture-ware costs. If iPSC therapies are going to become a reality in the future, then methods to uniformly derive and expand cells are necessary.

1.1 Thesis objectives

The original goal of this thesis was to develop a bioprocess for generating cardiomyocytes from mouse ESCs/iPSCs in the suspension bioreactor. However, during the course of these experiments, we discovered that the SSB environment preserved pluripotency in cells that otherwise were fated to become cardiomyocytes. This observation changed the direction of this thesis towards developing a new method for generating and expanding induced pluripotent stem cells in the suspension bioreactor.

Therefore, this thesis focuses on the application of SSBs for the differentiation of ESCs as well as derivation and expansion of iPSCs. SSBs were selected as a new environment to explore the impact of fluid shear stress (FSS) on the differentiation of ESCs and also the derivation/expansion of iPSCs. Mouse fibroblasts were chosen for reprogramming experiments due to the ability to check the full developmental potential

of derived cells via chimera formation and germline transmission. This thesis is divided into three research aims:

(i) To develop an effective bioprocess environment for scalable differentiation of mouse ESCs into cardiomyocytes in SSBs,

(ii) To evaluate the effectiveness of SSBs for the expansion and long term maintenance of mouse iPSCs, and

(iii) To develop a novel method for combinational cellular reprogramming within SSBs using physical and genetic methods and to generate relevant cell numbers through an economically efficient bioprocess.

1.2 Thesis overview

The thesis will start with an overview of stem cells and their classifications with specific emphasis on the adult stem cells (ASCs), ESCs and iPSCs. Different examples of pluripotent and adult stem cells and the advantage and disadvantage of each category will be discussed. In addition, the regulation of pluripotency in ESCs will be presented along with an emphasis on iPSC generation and a summary of the epigenetic regulation during iPSC derivation. At the end of the literature review chapter, different systems for the differentiation of ESCs into cardiomyocytes and the promise of ESCs and iPSCs in regenerative medicine will be discussed.

The body of this thesis will be presented as a manuscript-style format and will incorporate three manuscripts. The first manuscript presented in this thesis (Chapter III) explains a project that was carried out to develop an effective bioprocess for the differentiation of mouse ESCs toward cardiomyocytes in SSBs. The scalable production

of ESC-derived cardiomyocytes in a SSB system has previously been demonstrated (Zandstra, Bauwens et al. 2003; Niebruegge, Nehring et al. 2008). However, in this study, we discovered that suspension bioreactor culture systems have the ability to suppress cardiomyocyte differentiation, and induce 'transient' pluripotency within a defined differentiation protocol. In contrast to adherent culture, ESCs induced to differentiate toward cardiomyocytes in suspension bioreactors retain their ability to form ESC-like colonies, express pluripotency markers and form teratomas after exposure to differentiation condition for 25 days. Although the exact impact of FSS on the stirred cells in the SSB has yet to be identified, we believe that shear stress plays an important mechanistic role in bioreactor-induced pluripotency. This hypothesis will be further elaborated in the Discussion chapter.

Based on the finding in Chapter III that SSBs favour pluripotency rather than differentiation, we decided to evaluate the SSB environment for the long term expansion and maintenance of murine iPSCs in an undifferentiated state. *In vitro* differentiation into different lineages in addition to *in vivo* teratomas formation revealed the existence of fully functional and undifferentiated pluripotent iPSCs following long term passaging in SSBs. We found that SSB culture represents an efficient process for the large-scale expansion and maintenance of murine iPSCs, which is an important first step in the clinical application of their human counterparts. The findings from the expansion and maintenance of iPSCs are presented in the second manuscript (Chapter IV) of this thesis.

Since our results in previous chapters showed that the effects of shear stress were favourable toward pluripotency, we hypothesized that SSBs provided a selective advantage for enhancing iPSC reprogramming through the application of shear stress. A

subsequent study was undertaken to examine the role of SSBs in increasing the efficiency and kinetics of murine embryonic fibroblasts (MEFs) reprogramming. Interestingly, we found that murine iPSCs can be generated more quickly and efficiently within SSBs. By applying this method to MEFs, millions of fully reprogrammed iPSCs could be derived in 10 days, making it 100- to 1000-fold more efficient than conventional adherent culture methods. The resulting suspension-derived iPSCs (SiPSCs) resemble ESCs in their *in vitro* and *in vivo* characteristics. Furthermore, SiPSCs display a normal karyotype, form teratomas, produce viable chimeras, and display germ line transmission competency. Our results suggest that the suspension culture environment provides a selective advantage for enhancing iPSC generation. The findings are presented in the third manuscript in this thesis (Chapter V).

Although the projects outlined herein have focused on the use of mouse cells, this work provides a critical cornerstone for future investigations to establish similar expansion and derivation methods for human cells. Furthermore, our findings will provide insight into the effects of FSS in maintaining pluripotency and will open a new avenue for application of SSB systems in iPSC derivations and expansion. We believe that SiPSC technology has the potential to enhance and standardize iPSC research, bringing it to clinical application more quickly.

Chapter Two:

Literature Review

2.1 Stem Cells

Stem cells are described functionally as cells that have the ability to self-renew, as well as the capacity to give rise to differentiated cells (Smith 2001; Weissman, Anderson et al. 2001). There are some definite characteristics attributed to stem cells. First of all, stem cells are able to self-renew through numerous cell cycle divisions, while preserving their undifferentiated state without oncogenic transformation. A second property of stem cells is potency; stem cells can produce multiple types of differentiated cells with distinctive gene expression profiles and specific cellular morphologies (Lanza 2009). The third and the most significant characteristic is clonogenicity, which means that each single stem cell has the ability to generate more stem cells.

The concept of the stem cell originated from the ground-breaking work of Till and McCulloch. In 1961, they clearly provided evidence of the existence of stem cells within bone marrow and showed that not all proliferating cells are identical. Prior to this time, all proliferating cells were regarded as stem cells. Their approach was to investigate the cells with the capability to repopulate hematopoietic tissues following cellular depletion of the tissue by radiation (Till and McCulloch 1961). In 1963, they defined two key properties of stem cells: self-renewal and potency (Becker, McCulloch et al. 1963). These studies provided the important foundation for subsequent human bone marrow transplantation therapy.

Stem cells are often classified with regard to their source of derivation or potency state. As mentioned, potency defines the cells potential to differentiate into various cell types. Stem cells have different levels of potency (totipotency, pluripotency, multipotency and uniptency) depending on where they come from. Totipotency is the ability of stem cells to differentiate into any cell type including embryonic and extraembryonic lineages (Lanza 2009). Cells generated from the first few divisions of the fertilized oocyte are totipotent and can give rise to the entire organism.

Pluripotency refers to stem cells that have the capability to differentiate into any cell type in the body (Lanza 2009). Since these cells cannot form extraembryonic tissues such as placenta, they lack the potential to construct the whole organism. Pluripotent cells are normally derived from the inner cell mass (ICM) of the blastocyst. Recently, a new source of pluripotent cells named induced pluripotent stem cells (iPSCs) has been achieved by artificially reprogramming somatic cells. The features of iPSCs will be discussed in detail later in this chapter.

Unlike pluripotent stem cells, multipotent progenitor cells can only differentiate to a limited number of lineages depending on their developmental stage, while uniptency refers to the capacity of stem cells to develop into only one type of cell (Lanza 2009).

Stem cells can originate from adult, fetal and embryo tissues. Based on this classification, there are three types of stem cells: adult stem cells (ASCs; also known as somatic stem cells), embryonic stem cells (ESCs) and embryonic germ cells (EGCs).

2.1.1 Adult stem cells

Adult stem cells (ASCs) are derived from various adult somatic tissues of the developed organism such as liver, lung, brain, skin, blood and bone marrow. These cells are considered to be multipotent with more restricted cellular repertoire than pluripotent cells. In a living organism, adult stem cells are responsible for the replenishment of tissues upon growth and injury (Van Zant 2003).

Hematopoietic stem cells (HSCs) are among the best characterized adult stem cells. HSCs are multipotent stem cells that have the unique capacity to self-renew and to differentiate to generate all the blood cell types from both the myeloid and lymphoid lineages (Kondo, Wagers et al. 2003). They have the potential to replenish the lympho-hematopoietic system when transplanted into irradiated animals (Jacobson, Simmons et al. 1951). These rare cells reside in the bone marrow of adult organisms. HSCs can also be obtained from the peripheral blood following pre-treatment with certain cytokines, such as granulocyte-colony stimulating factor (G-CSF), which stimulates HSC proliferation (Papayannopoulou 1999). Other sources of HSCs include umbilical cord blood, the fetal hematopoietic system and *in vitro* hematopoietic differentiation of ESCs or EGCs. Despite the fact that bone marrow transplantation has been widely employed during the last few decades to treat patients with malignant, congenital and degenerative disorders, there are some major roadblocks for their clinical applications. First, HSCs lack the ability to proliferate and differentiate properly to their progenies *in vitro.* Second, there is a lack of accurate knowledge to distinguish HSCs from other cells existing in bone marrow or the bloodstream.

A second example of ASCs is neural stem cells (NSCs). The discovery of NSCs in the mammalian central nervous system (CNS) has provided new insights into the neurogenic process during development. These cells are at the centre of intensive studies for their potential applications in the cell therapy of brain injuries and the treatment of neurodegenerative disorders (Gage, Ray et al. 1995; McKay 1997). Until recently, it was a common belief that the developed brain loses its ability to regenerate new neurons. However, new studies have demonstrated the existence of neural progenitor cells that reside in the germinal area of the forebrain called sub-ventricular zone (Reynolds and Weiss 1992; Weiss, Dunne et al. 1996). It is possible to isolate and expand NSCs from mammalian CNS by a variety growth conditions. When cultured in the presence of epidermal growth factor (EGF) and fibroblast growth factor-2 (FGF-2), they form floating aggregates called neurospheres (Bez, Corsini et al. 2003). NSCs are characterized by their life-long potential to self-renew and to differentiate into the array of more specialized neural cells such as neurons, astrocytes and oligodendrocytes.

Another type of well-studied category of adult multipotent stem cells is mesenchymal stem cells (MSCs). They are the natural precursors of multiple mesodermal lineages such as osteoblasts, chondrocytes, adipocytes, myoblasts, and types of connective tissues (Kraus and Kirker-Head 2006). MSCs have been derived from the bone marrow of different species such as human, rabbit, dog and pig (Chamberlain, Fox et al. 2007). In addition, these cells have been isolated from a number of other tissues including cartilage, adipose, synovium, skeletal muscle, and placenta (Chamberlain, Fox et al. 2007). MSCs are rare in bone marrow, representing about 1 in 10,000 nucleated cells and their number changes dramatically with age (Caplan 2007). MSCs

have been shown to retain their ability to self-renew through long-term passaging and to differentiate using specific growth factors (Kraus and Kirker-Head 2006).

MSCs are of extreme therapeutic interest because they have the potential to treat a wide range of degenerative disorders. They have several advantages over other types of stem cells, which make them a promising source for tissue engineering and cell-based therapies. First, they can be used in autologous transplantation, decreasing the risks of immune-rejection and complications of transplantation. Second, they have high proliferation rates and differentiation capabilities. Third and most importantly, MSCs avoid the ethical considerations that surround the use of human ESC and EGCs (see below). Despite the above mentioned advantages, there are still several barriers for their full potential application in clinical trials. The characterization of MSCs is a complex process due to the lack of exclusive surface marker expression (Vogel, Grunebach et al. 2003). This fact makes it difficult to isolate appropriate cell population based on a universal surface marker. The other limiting factor is the severe aging of MSCs in culture due to the rapid shortening of the telomeres (Mora and Rojas 2008). When compared to young donors, adult MSCs have already gone through some enormous shortening of their telomere length (Baxter, Wynn et al. 2004).

In general, ASCs are particularly useful in clinical transplantations. Adult stem cells have several advantages as well as disadvantages. Unlike ESC and EGCs, these cells are not tumorigenic and have fewer ethical issues, which facilitate their clinical application. Furthermore, the autologous transplantation of ASCs can prevent immune rejection of cells in the recipient. With appropriate quality control and testing, allogenic ASCs can also be used. Despite these advantages, ASCs have limited differentiation

capacity and a very restricted life span when cultured *in vitro*, which restricts their clinical applications. In addition, they have not been found in all adult tissues, making ESCs in these cases the only choice to achieve the required cell type. A major challenge regarding clinical use of multipotent cells is isolation of enough quantities of cells for cell therapy. This can be complicated by idiopathic factors, which make autologous transplantation problematic.

ESCs are typically derived from the inner cell mass of 4-5 days embryo. Considering some differences, all of the embryonic cells are considered pluripotent stem cells, which indicate that they show full developmental capacity, being able to differentiate into cells from three germ layers *in vivo* and *in vitro* and contribute to chimera formation including germline transmission. More details about pluripotent stem cells will be discussed below.

2.1.2 Embryonic Stem Cells (ESCs)

Pluripotent stem cell lines were first isolated from a form of malignant germ cell tumor called teratocarcinoma (Kahan and Ephrussi 1970). These embryonal carcinoma (EC) cell lines have the capacity to differentiate into a variety of cells from three germ layers. EC cells exhibit similar expression patterns to the cells present in the ICM, which led to the idea that these cells are the counterpart of pluripotent cells in the ICM (Rossant and Papaioannou 1984). Studies on EC cells during subsequent years after their isolation provided the intellectual and methodological principles for the derivation and maintenance of mouse ESCs.

Mouse embryonic stem cells (ESCs) were first derived directly from the inner cell mass (ICM) of a preimplantation blastocyst using culture systems previously established for mouse EC cells (Evans and Kaufman 1981; Martin 1981). The resulting cells revealed unrestricted proliferative capacity, and were pluripotent. The establishment of mouse ESCs normally involves cultivating dissected ICMs on fibroblast feeder cells, derivation of new ESC lines takes place by expansion of the outgrowth into colonies. Mouse ESCs are pluripotent and have the capability to remain undifferentiated and proliferate indefinitely *in vitro,* which distinguishes them from adult stem cells. Mouse ESCs can be maintained *in vitro* in their undifferentiated state and recapitulate characteristics of ICM cells (Jaenisch and Young 2008). They also have the ability to differentiate into all three embryonic germ layers *in vitro* and when injected into immunocompromised mice (Martin 1981). Unlike EC cells, ESCs are karyotypically normal cells and have the ability to integrate at a high frequency to various tissues in chimeras and transmit through the germline. Furthermore, several mouse ESC lines have been reported to form entire viable newborns through tetraploid complementation assay (Bradley, Evans et al. 1984). These characteristics thus provided a useful method to introduce genetic modifications to the mouse germ line, which will be discussed later.

Since the derivation of mouse ESCs, the mechanism of their self-renewal and differentiation has been a matter of intense investigation. Initially, feeder cells were used to promote ESC self-renewal while inhibiting differentiation. The unknown factor secreted from the feeder cells was later defined as a member of the interleukin-6 family named leukemia inhibitory factor (LIF) (Smith, Heath et al. 1988; Williams, Hilton et al. 1988). This discovery allowed the expansion of ESCs on gelatinized tissue culture plates

free from feeder cells using serum and recombinant LIF. Binding of LIF to its receptor triggers two series of intercellular signaling events, the JAK-Stat and the Ras/mitogen-activated protein kinase (MAPK) pathway (Ying, Nichols et al. 2003).

Mouse ESCs have a distinct recognizable morphology to the experienced eye including prominent nucleoli and a high nuclear-to-cytoplasmic ratio. They also express various markers of pluripotent stem cells including alkaline phosphatase, stage specific embryonic antigen-1 (SSEA-1), Sox2, POU class transcription factor Oct4 gene (Oct3/4), homeobox protein Nanog, and zinc finger protein Rex1 (Chambers and Tomlinson 2009).

In the absence of LIF, mouse ESCs can differentiate *in vitro* into an embryo-like 3D cystic structure known as an embryoid body (EB) containing cells belonging to three embryonic germ layers. ESCs are normally induced to form cell aggregates by culturing on non-adherent plastic surface using hanging drop culture or spinner flasks. Upon aggregation, the cells start to spontaneously differentiate into multiple cell lineages. In mouse, the classical assay for demonstrating the pluripotency is the ability of ESCs to contribute to three germ layers via teratoma formation as well as chimera formation; however, the most stringent test for pluripotency is the germline transmission assay (Hoffman and Carpenter 2005).

Teratomas are benign tumors of multiple cell lineages that contain differentiated derivatives of all three germ layers (mesoderm, ectoderm and endoderm). Teratoma formation normally involves subcutaneous injection of undifferentiated cells into the thigh of immune deficient mice (Lanza 2009). After three to four weeks, the tumor-like tissues are examined by histology. In some cases, complex structures, such as teeth, hair

and neural tube can be formed in teratomas, which provide an experimental model to investigate the development of these structures.

During the chimera formation, about 10-15 mouse ESCs are normally microinjected into the single mouse blastocyst. Injected blastocysts are then transferred into the uteri of pseudopregnant recipient females. Pluripotent mouse ESCs retain their ability to participate in normal embryonic development, even after long term expansion and manipulation *in vitro*. The progeny of ESCs contribute to both somatic tissue and germ cells with variable percentages.

Beside their potential application in cell therapies, ESCs can be a vehicle for producing transgenic animals. Transgenic animals have been widely used as experimental models to investigate phenotypic changes upon introducing a particular genetic modification in the germline. Since being developed, this technique has become a vital tool to study developmental process in the mouse. Knock-out, knock-in and conditionally mutant mice can be generated with this method. After microinjection of genetically modified ESCs into blastocysts of different strain, chimeric mice with two different coat colors will be generated. Subsequently, chimera breeding will give rise to offspring harboring the recombinant gene.

Since the first derivation of human ESCs in 1998 (Thomson, Itskovitz-Eldor et al. 1998), there has been controversy about whether these cells are exactly the counterpart of mouse ESCs. Despite the fact that both mouse and human ESCs have apparent common origin from the ICM and have the capacity to proliferate *in vitro*, their functional and phenotypic characteristics are substantially different.

Murine ESCs produce tight, rounded colonies, while human ESCs form looser and flatter cell clumps. Human ESCs have a longer doubling time and are sensitive to single cell passaging. Although both ESCs express common pluripotency gene markers such as alkaline phosphatase, Oct4, Nanog, Sox2, they differ in the expression of other markers including Rex1, and the stage-specific embryonic antigens (SSEA)-family of surface glycoproteins (Ginis, Luo et al. 2004). For example, murine ESCs express SSEA-1 in their undifferentiated state, while human cells express SSEA-3 and SSEA-4. Human ESCs start to express SSEA-1 only after differentiation (Thomson, Itskovitz-Eldor et al. 1998).

The most prominent difference between the two species is the regulation of self-renewal. Mouse ESCs need leukemia-inhibiting factor (LIF)/Stat3 and BMP4 signaling to support their self-renewal, while human ESCs require feeder cells, various growth factors and depend on bFGF and Activin A/TGFβ signaling (Ying, Nichols et al. 2003; Xu, Peck et al. 2005). However, a number of publications have established feeder-free, xeno-free and serum-free protocols for the derivation and maintenance of human ESCs (Lu, Hou et al. 2006; Ludwig, Levenstein et al. 2006; Yao, Chen et al. 2006).

It has been shown that human and mouse ESCs differ systematically by certain criteria including core transcriptional regulation, pattern of gene expression, genome-wide promoter occupancy of pluripotency factors and X chromosome inactivation (Boyer, Lee et al. 2005; Silva, Rowntree et al. 2008). All of the above dissimilarities between mouse and human ESCs have provoked important questions concerning the biological equivalency of these two cell types.

The cell population within the ICM normally transforms into the epiblast, which eventually produces all adult tissues through a process known as gastrulation (Lindstrom 1999). It is believed that ESCs are the *in vitro* equivalent of the epiblast, as they have the potential to form all somatic lineages and in mice to generate germ line competent chimeras. Recently, a different type of pluripotent stem cell termed epiblast stem cell (EpiSC) has been derived from postimplantation epithelialized murine embryos, and found to share major features of human ESCs, such as tissue culture requirements, intolerance to single cell passaging and flattened colony morphology (Brons, Smithers et al. 2007; Tesar, Chenoweth et al. 2007; Bao, Tang et al. 2009). Similar to human ESCs, EpiSCs are dependent on the Activin/Nodal signaling pathway and the X chromosome has been inactivated in female cell lines. In contrast to mouse ESCs, EpiSCs are inefficient in producing chimeras and fail germline transmission, even though they express the core pluripotency markers such as Nanog, Oct4 and Sox2. However, they can generate teratomas composed of three germ layers (Brons, Smithers et al. 2007; Tesar, Chenoweth et al. 2007). Interestingly, they can be reprogrammed into bona fide ESCs by re-expression of Krueppel-like factor 4 (Klf4) or using 2i chemical inhibitors (Guo, Yang et al. 2009; Hanna, Markoulaki et al. 2009). It has been already reported that the dual inhibition (2i) of glycogen synthase kinase-3 (GSK3) and mitogen-activated protein kinase signalling by CHIR99021 and PD0325901 promotes reprogramming of mouse somatic cells to ground state pluripotency (Silva, Barrandon et al. 2008).

Ethical and legal considerations make it impossible to confirm pluripotency of human ESC through chimera formation and germline transmission assays. Therefore, pluripotency of these cells can only be assessed by *in vitro* differentiation and by

teratoma formation. Furthermore, the similarities between mouse EpiSCs and human ESCs raise fundamental questions regarding the true origin, and *in vivo* equivalent of human ESCs during development (Lovell-Badge 2007).

Several technical obstacles must be overcome before human ESCs can be employed clinically. These include but are not limited to ethical considerations pertaining to their embryonic derivation and the tumorigenic properties after cell therapy. Human ESCs are usually derived from the ICM of blastocyst-stage embryos (Thomson, Itskovitz-Eldor et al. 1998). These pluripotent cells have also been derived from other sources including single cell biopsies from morula stage embryos (Chung, Klimanskaya et al. 2008) and somatic cell fusion with existing hES cell lines (Cowan, Atienza et al. 2005). Human ESC lines are established from surplus embryos that have been collected by *in vitro* fertilization (IVF) and donated for research purposes. However, the derivation of human ESCs by all of the mentioned methods requires the destruction of human embryos, which has raised significant ethical and legal considerations.

Recently a new source of pluripotent stem cells, termed induced pluripotent stem cells (iPSCs) has been generated that could be an alternative to ESCs. These cells circumvent some of the ethical concerns associated with the derivation of human ESCs and offer tremendous potential for generating disease-specific pluripotent cells. More details about iPSCs will be discussed in the following section.

A second obstacle to ES cell therapy is the potential for tumor development. Although the teratoma formation tendency of human ESCs is a valuable test of pluripotency, this characteristic causes a challenging technical and safety issue. This problem arises from the fact that the presence of one or a few undifferentiated ESCs *in*

vivo may result in the formation of a teratoma at the transplant site (Hentze, Soong et al. 2009).

EGCs are another type of pluripotent cells. They have the same characteristics as ESCs with respect to their differentiation potential and contribution to the germline of chimeric mice (Labosky, Barlow et al. 1994). Similar to ESCs, they have indistinguishable morphological characteristics upon cultivation on feeder cells; however they are derived from primordial germ cells (PGCs). PGCs are a population of cells located near the epiblast not undergoing cellular specification (Saitou, Barton et al. 2002). Subsequently, they migrate to and colonize the genital ridges. In this stage, PGCs can be isolated and propagated *in vitro* under appropriate culture condition to produce EGCs (Matsui, Zsebo et al. 1992). The most prominent difference between ESCs and EGCs is that the latter may demonstrate substantial imprinting of particular genes, which inhibits the generation of chimeric mice by some EGC lines (Surani 1998; Surani 2001).

In summary, being pluripotent, ESCs are a unique tool to inform us about the earliest molecular and cellular processes that regulate normal development. These cells may also provide us a model to study the pathways involved in both pluripotency and self-renewal and highlights their promising role in cellular therapies for the treatment of human regenerative disorders.

2.1.2.1 Regulation of pluripotency in ESCs

The significant characteristics of ESCs, including self-renewal, maintenance of pluripotency and differentiation to different adult lineages, necessitate the existence of specific molecular mechanisms during early development. The mechanism by which

ESCs choose between pluripotency and differentiation seems to rely on specific molecular factors. Their self-renewal and differentiation depends upon several different signals including growth factors and their particular microenvironmental niche or extra cellular matrix (ECM). The properties of ESCs endow them the potentiality for cell therapy and tissue engineering. However, many obstacles hinder their potential clinical applications. One of these impediments is their tendency to experience spontaneous differentiation *in vitro* (Amit and Itskovitz-Eldor 2002). Therefore, the molecular mechanism that regulates ESCs "stemness" has to be elucidated before their utilization in widespread clinical applications.

Progression from the pluripotent state to a differentiated phenotype is usually accompanied by distinguished alterations in cellular function, which are predetermined by global gene expression patterns during early development. Importantly, the genes responsible for self-renewal are down-regulated, while most of the lineage-specific genes are up-regulated.

The regulatory mechanisms that control self-renewal are not yet fully understood; however, the critical role of transcription factors like Oct4, Sox2 and Nanog has been elucidated (Pan and Thomson 2007). Until recently, most of the studies have focused on the transcriptional network and its regulation. However, several recent lines of evidence emphasize that ESC differentiation and early development largely rely on the flexibility of epigenetic modifications (Reik 2007). Therefore, the mechanism of gene regulation during development is not only dependent upon a network of transcription factors, but also on epigenetic changes, such as the covalent alterations of histones (Iizuka and Smith 2003), ATP-dependent chromatin remodeling (Lusser and Kadonaga

2003), exchange of histones and histone variants (Ahmad and Henikoff 2002) and DNA methylation at CpG islands (Bird and Wolffe 1999), all of which have been found to play fundamental role in preserving pluripotency and blocking differentiation (Figure 2.1).

2.1.3 Induced pluripotent stem cells (iPSCs)

The concept of nuclear reprogramming has received much interest over the last few decades as it can offer an unlimited and patient-matched source of cells for cell replacement therapy. There are two major techniques for somatic cell reprogramming including somatic cell nuclear transfer (SCNT) and induced pluripotent stem cell (iPSCs). Nuclear reprogramming was firstly established by Gurdon *et al* in 1958, where Xenopus were cloned from somatic cells by replacing nuclei of oocytes with intestine (Gurdon, Elsdale et al. 1958). Since then, SCNT has been successfully demonstrated in mammalian cells, and resulted in the birth of Dolly the sheep (Campbell, McWhir et al. 1996).

In 2006, a milestone was achieved by artificially creating pluripotent stem cells from mouse and then human adult somatic cells (Takahashi and Yamanaka 2006; Takahashi, Tanabe et al. 2007; Yu, Vodyanik et al. 2007). In two independent studies led by Thomson and Yamanaka, ES-like cells called “induced pluripotent stem (iPS) cells” were generated from adult human fibroblasts by viral transduction of four defined pluripotency transcription factor genes. Yamanaka’s group used Oct4, Klf4, c-Myc and Sox2, while Thomson’s group applied Nanog and Lin28 instead of c-Myc and Klf4. These iPSCs morphologically resemble ESCs; they express cell surface markers characteristic of ESCs, have a normal karyotype, express telomerase and demonstrate

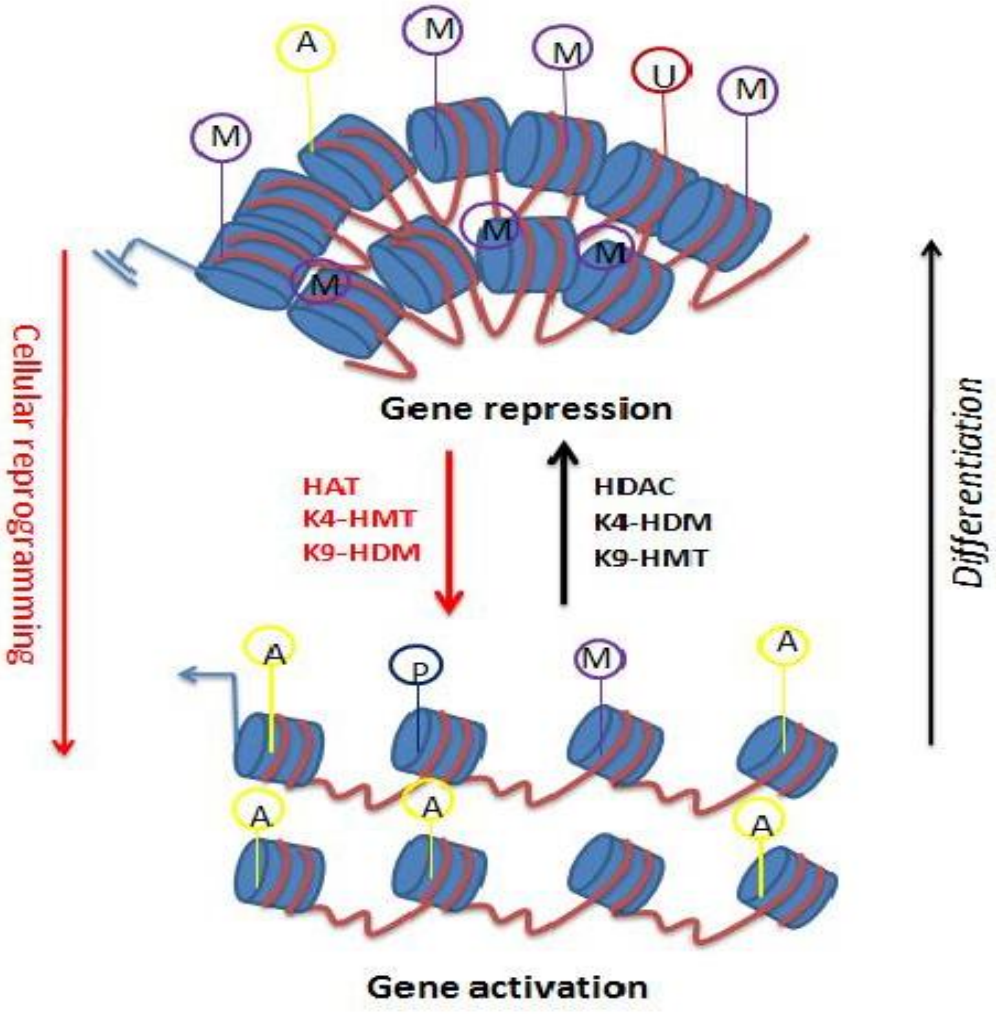

Figure 2.1. Histone modifications during development and cellular reprogramming. Regulation of gene expression largely relies on transcription factor circuitry and chromatin modifications. Different histone modifications occur as cells differentiate resulting in a transcriptionally less-permissive chromatin. Histone modifications (methylation (M), acetylation (A), ubiquitination (U), and phosphorylation (P)) have to be re-set as differentiated cells are reprogrammed to induced pluripotency. HAT: histone acetyl transferase, K4-HMT: histone lysine H3 (K) 4 methyl transferase, K9-HMT: histone lysine H3 (K) 9 methyl transferase, HDAC: histone deacetylase, K4-HDM: histone lysine H3 (K) 4 demethylase and K9-HDM: histone H3 lysine (K) 9 demethylase.

multi-lineage differentiation in both embryoid bodies and teratomas. Since 2006, the field has expanded to generate iPSCs from various mouse and human somatic cells using different approaches, as well as using various forms of gene transformation, protein transduction and microRNAs (Okita, Nakagawa et al. 2008; Stadtfeld, Nagaya et al. 2008; Kaji, Norrby et al. 2009; Soldner, Hockemeyer et al. 2009; Woltjen, Michael et al. 2009; Zhou, Wu et al. 2009; Yang, Li et al. 2011).

Several lines of evidence emphasize the importance of epigenetic marks during the generation of iPSCs and the concomitant reversal of cell fate (Rodriguez, Velkey et al. 2007; Zaehres and Scholer 2007). It has been postulated that cell destiny can be reset through alteration of lineage–limited epigenetic patterns such as histone acetylation/methylation and DNA methylation. Experimental data suggest that mouse derived-iPSCs have the ability to acquire the chromatin signature of pluripotent ESCs and can be transmitted through the germline (Okita, Ichisaka et al. 2007).

The role of each reprogramming factor has been investigated in more detail since the initial iPSC studies. Although, the application of Oct4 and Sox2 is indispensible for iPSC generation, c-Myc and Klf4 significantly increase the efficiency. Due to large differences in epigenetic status between ESCs and their differentiated progeny, Oct4 and Sox2 cannot find their targets in somatic cells. It has been proposed that c-Myc and Klf4 alter the structure of chromatin enabling these two core factors access to their targets, thereby increasing the expression of downstream genes (Takahashi and Yamanaka 2006). For example, among the four Yamanaka factors, c-Myc is a well-characterized proto-oncogene transcriptional activator and a modulator of DNA replication. It has been shown that c-Myc induces the up-regulation of Gcn5 (histone acetyl transferase gene),

which is a key player in histone structure, and therefore might improve the accessibility of target genes to Oct4. Klf4 is also acetylated by p300 (acetyl transferase protein) and has the ability to control gene transcription through regulation of histone acetylation (Evans, Zhang et al. 2007). These results are in agreement with the open chromatin theory of ESC self-renewal and pluripotency.

Using cDNA microarray and ChIP approaches, it has been proposed that Oct4 regulates the expression of over 350 genes in ESCs including several epigenetic modifiers (Matoba, Niwa et al. 2006). Two histone demethylases, Jmjd1a and Jmjd2c have been identified to be part of the groups of genes regulated by Oct4 (Loh, Zhang et al. 2007). This study also confirmed that Jmdj2c is recruited to the Nanog promoter (a key component of the ESC transcriptional network) and demonstrated that upon depletion of Jmdj2c subsequent differentiation could only be rescued by ectopic expression of Nanog. These results clearly show that Oct4 both directly and indirectly controls genes required for maintaining the open, accessible chromatin state required for self-renewal and pluripotency. They also suggest a positive feedback loop between transcriptional circuitry and epigenetic modification. In this way, ESC transcription factors regulate the expression of chromatin remodeling genes and, in turn, help to unveil chromatin conformation in promoter regions of target genes allowing for self-regulation of the epigenetic network.

2.1.3.1 Epigenetic regulation during iPSC generation

In 1942, Waddington first mentioned “epigenetics” to describe how genetic interactions may contribute to phenotype. It is important to emphasize that the definition

of a gene as a stretch of DNA had not been identified yet in the 1940s. He later proposed his famous "epigenetic landscape" model in 1957 by comparing the early developmental differentiation with a ball travelling down a canal, which starts from a fertilized totipotent embryo and ends up as different lineage-committed cells. According to this developmental concept, cells move through different one-way branched valleys inside the canal and select their ultimate irreversible cellular fates during this trip (Waddington 1957). As they reach the end of each valley (lineage), they are obliged to stay in that valley and cannot jump over boundaries into other branches or return to their starting point. With regard to iPSC generation, it has been suggested that the four reprogramming transcription factors push cells backward in this canal by removing specific epigenetic hurdles, which under normal conditions stabilize cells in their differentiated status/valley (Yamanaka 2009).

Based on Waddington's epigenetic model, Yamanaka proposed a stochastic model for iPSC generation (Yamanaka 2009). This model is based on a concept, which is the exact reversal of that proposed by Waddington. Specifically, cells experience four different events during reprogramming. As they are rolling back up the slope toward pluripotency, some cells are prevented from moving back up the slope by epigenetic bumps and hence they will have the ability to self-renew. The second group of cells will be partially reprogrammed and without continuous expression of exogenous factors, they lose their pluripotency and roll back towards a specific lineage. The third group may trans-differentiate due to insufficient and improper expression of ectopic factors. The fourth group does not begin to travel but instead undergo apoptosis or cellular senescence (Figure 2.2). According to this scenario and based on a stochastic view of ESCs, any

gene or molecule that has the capability to facilitate this movement up the slope and prevent the cell from rolling back would enhance the derivation of iPSCs.

The endogenous loci of all reprogramming factors are heavily methylated in somatic cells, whereas their loci are hypomethylated in ESCs and iPSCs (Imamura, Miura et al. 2006). For iPSC derivation, all of these promoters need to be reactivated by demethylation enzymes. Since the factors used in direct reprogramming do not have known demethylating activity, this event requires other downstream activated epigenetic modifiers. Based on CpG methylation differences between lineage-specific cells and ESCs, one group demonstrated an essential role for CpG methylation as an epigenetic modifier in iPSC generation. Doi et al found that CpG methylation patterns can discriminate iPSCs, ESCs and fibroblasts. They suggested that epigenetic reprogramming involves the same differentially methylated regions (DMRs) of CpG island "shores" that mark normal differentiation (Doi, Park et al. 2009). It has been also revealed that specific loci in iPSCs remain semi-reprogrammed, which means that the methylation pattern of iPSCs differs from ESCs.

Another obstacle toward iPSC generation is the requirement for histone modification and remodeling during reprogramming. Although, it has been shown that histone H4 within the promoters of reprogramming genes is deacetylated in somatic cells, it is hyperacetylated in iPSCs and ESCs. Several studies indicate that the H3 and H4 histones within the Oct4 and Nanog promoters are hyperacetylated (a hallmark of active genes) (Hattori, Nishino et al. 2004; Kimura, Tada et al. 2004). Among the four factors, only c-Myc has the ability to modify chromatin. It mediates chromatin modification by

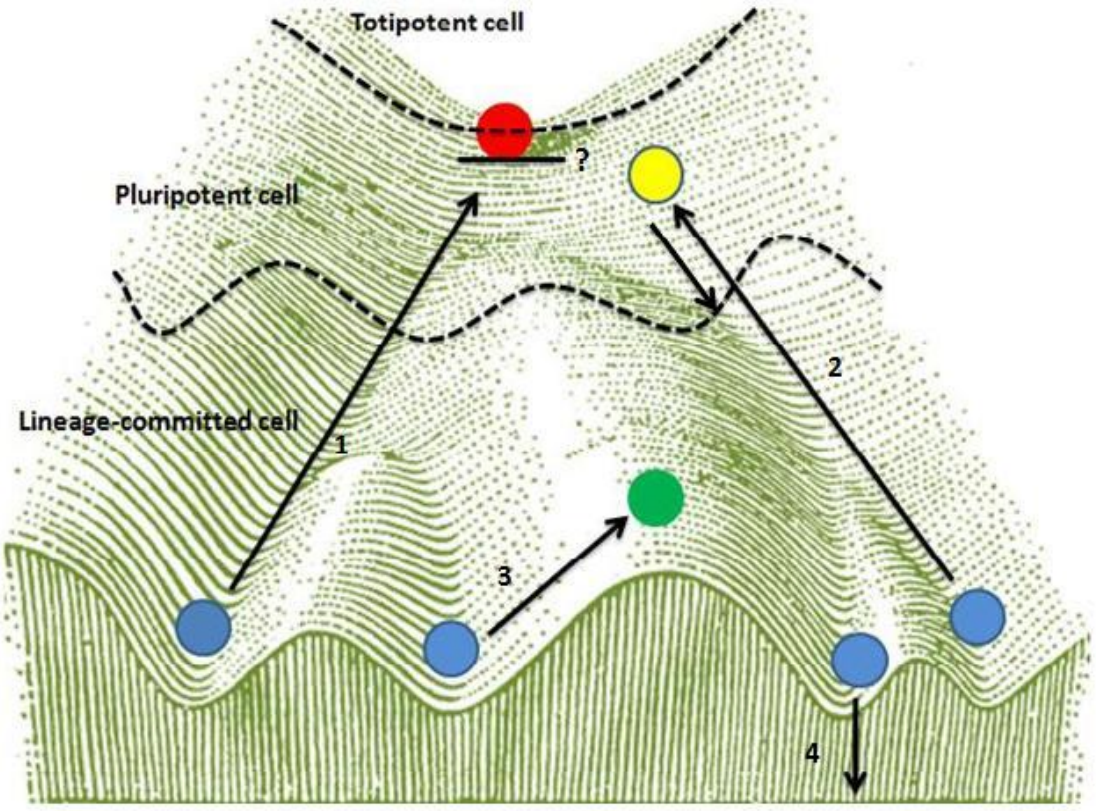

Figure 2.2. The stochastic model for iPSC generation. The model proposed by Yamanaka is based on the Waddington model. (1) Reprogrammed cells may encounter four possible scenarios. One group of cells is blocked by epigenetic barriers and begins self-renewal. (2) Other cells are trapped inside a semi-reprogrammed state due to inefficient epigenetic modification and travel back down their valley in the absence of ectopic expression of pluripotency factors. (3)Some cells may move to other neighboring valleys and transdifferentiate into other cell types, due to inefficient expression of reprogramming factors. (4) The final group experience apoptosis or cellular senescence.

increasing the expression of Gcn5 recruiting it for the modification of target genes (Knoepfler, Zhang et al. 2006).

Another factor in the derivation of iPSCs is the state of histone methylation. ESCs and iPSCs are tagged by activator histone modifications, which are marked by H3K4me3 and demethylation of lysine 9 within pluripotency genes. These two histone modifications show the bivalent chromatin characteristics of pluripotent genes, which are accomplished by simultaneous methylation at H3K27me3 and H3K4me3. Recent studies suggest that ESCs employ a novel and unusual mechanism for controlling gene expression for lineage-specific genes, which is silent in pluripotent ESCs, but may be expressed upon differentiation. Recently, a ‘‘histone bivalent’’ model (active and inactive histone modifications) has been proposed for ESCs (Azuara, Perry et al. 2006). According to this model, some lineage-specific genes are simultaneously marked with both repressive and active modifications. In comparison to differentiated cells, lineage-specific genes are kept silent by chromatin modification, but may be poised for subsequent rapid induction as ESCs choose between embryonic lineages. This bivalent model supports the notion that ESC pluripotency and self-renewal is maintained by the activity of differentiation genes, which are in a silent but poised state. For efficient generation of iPSCs, it is required that this histone modification be achieved either through genetic approaches or with the aid of some small molecules.

Reflecting on the role of chromatin remodeling during reprogramming, researchers have recently applied small molecules to circumvent these epigenetic blocks and enhance the generation of iPSCs. Several different chemical inhibitors for histone deacetylases, as well as DNA and histone methyltransferases have been used in

combination with genetic factors (Huangfu, Maehr et al. 2008; Shi, Do et al. 2008). Kubicek et al. found a small-molecule inhibitor of G9a histone methyltransferase, BIX-01294, could enhance the induction of reprogramming in neural stem cells while replacing Oct4 (Kubicek, O'Sullivan et al. 2007). Since G9a is a down-regulator of Oct4 during early development, they suggested that BIX-01294 enhances iPSC formation by inhibiting G9a and subsequently releasing Oct4 from negative regulation. This group also generated iPSCs from mouse embryonic fibroblasts (MEFs) with only two factors; Oct4 and Klf4 in the presence of BIX-01294. Interestingly, Valproic acid (VPA) increased the efficiency and kinetics of reprogramming by 100-fold in a four factor system. VPA could also replace either Klf4 or c-Myc in reprogramming and enabled iPSC generation with only Oct4 and Sox2 from human fibroblasts (Huangfu, Osafune et al. 2008). VPA is a potent histone deacetylating (HDAC) agent and facilitates cellular reprogramming by inducing an open chromatin conformation (Huangfu, Osafune et al. 2008). These results imply that DNA methylation, histone methylation and histone deacetylation contribute to epigenetic hurdles, which have to be overcome for successful iPSC generation (Table 2.1).

Consistent with a chromatin remodeling strategy to enhance iPS derivation, several studies have also shown that other small molecules including L-type Ca^{2+} channel agonist (BayK8644) and lysine-specific demethylase 1 inhibitor (Parnate) can improve mouse and human somatic cell reprogramming (Shi, Desponts et al. 2008). Recently, the role of Wnt/β-catenin signaling pathway has been revealed in promoting ESCs pluripotency and self renewal (Miyabayashi, Teo et al. 2007). It has been demonstrated in several studies that the GSK3 inhibitor (CHIR99021) can significantly improve the

Name	Function	Effects	Reference
RG-108	DNA methyl transferase inhibitor	Promote MEFs reprogramming	(Shi, Desponts et al. 2008)
BIX-01294	G9 histone methyl transferase inhibitor	Reprogramming of neural progenitor cells and MEFs	(Huangfu, Maehr et al. 2008; Shi, Desponts et al. 2008)
Valproic Acid	Histone deacetylase inhibitor	Enhanced reprogramming of mouse and human fibroblasts	(Huangfu, Maehr et al. 2008; Shi, Desponts et al. 2008)
SAHA	Histone deacetylase inhibitor	Enhances reprogramming of mouse fibroblasts	(Huangfu, Maehr et al. 2008)
Trichostatin-A	Histone deacetylase inhibitor	Enhances reprogramming of mouse fibroblasts	(Huangfu, Maehr et al. 2008)
5-azacytidine	Methyl transferase inhibitor	Enhanced reprogramming of mouse fibroblasts	(Huangfu, Maehr et al. 2008; Mikkelsen, Hanna et al. 2008)

Table 2.1. Chemicals with epigenetic modification properties used to enhance iPSC generation**.**

reprogramming efficiency of MEFs and also facilitate the reprogramming of MEFs transduced by only Oct4 and Klf4 (Li, Zhou et al. 2009; Desponts and Ding 2010). Inhibition of GSK3 prevents β-catenin degradation and enhances its subsequent translocation to the nucleus to activate expression of specific genes (discussed in Chapter VI). MEK inhibitor molecule (PD0325901) has also been used to stabilize and select completely reprogrammed iPSCs (Shi, Do et al. 2008).

As mentioned earlier, the carcinogenic capacity of undifferentiated pluripotent cells such as ESCs and iPSCs remains a challenging hurdle toward their clinical cell-replacement therapy. Recently, an alternative roadmap to direct cell lineage conversion has been developed that can potentially circumvent the possibility of tumor formation upon transplantation. The idea of reprogramming across various lineages may provide a variety of immunologically matched cells directly from patients. In this approach, one type of mature differentiated somatic cell, such as a fibroblast, is directly transformed into another cell type without passing through the fully pluripotent iPSC state. Successful transdifferentiation of mouse and human somatic cells has been reported for different types of cells including cardiomyocytes (Ieda, Fu et al. 2010; Efe, Hilcove et al. 2011), neurons (Ambasudhan, Talantova et al. 2011; Pfisterer, Wood et al. 2011), dopaminergic neurons (Caiazzo, Dell'Anno et al. 2011; Pfisterer, Kirkeby et al. 2011), spinal motor neurons (Berry, Gursel et al. 2011; Son, Ichida et al. 2011), blood progenitors (Szabo, Rampalli et al. 2010), chondrogenic (Hiramatsu, Sasagawa et al. 2011; Outani, Okada et al. 2011), EpiSC (Han, Greber et al. 2011) and hepatocytes (Sekiya and Suzuki 2011; Swenson 2012). The technique of direct dedifferentiation appears to be easier than the iPSC derivation process and can therefore shorten the reprogramming period. Direct cell

fate conversion also reduces the number of required factors and hence may contribute to the production of safer reprogrammed cells.

2.1.4 Differentiation of ESCs and iPSCs into cardiomyocytes

A prominent aspect of ESC research concentrates on revealing the mechanisms of differentiation from the pluripotent ESCs and iPSCs to various terminally differentiated cell lineages. This differentiation capacity makes ESCs and iPSCs an appealing cell source for cell/tissue repair in regenerative medicine. The *in vitro* differentiation of pluripotent cells into cardiomyocytes makes it also possible to study the developmental aspects of cardiomyocytes.

ESC differentiation into cardiomyocytes was first reported by Kehat et al (Kehat, Kenyagin-Karsenti et al. 2001). Various methods have been developed for induction and purification of cardiomyocytes derived from human and mouse ESCs and iPSCs. Different growth factors, chemicals, extracellular matrix, suspension bioreactor and genetic approaches have been investigated so far (Edwards, Harris et al. 1983; Wobus, Kaomei et al. 1997; Takahashi, Lord et al. 2003; Zandstra, Bauwens et al. 2003; Kawai, Takahashi et al. 2004; Baharvand, Azarnia et al. 2005; Yoon, Yoo et al. 2006; Taha, Valojerdi et al. 2007; Taha and Valojerdi 2008). Cardiomyocytes have been reported to comprise from 0.5 to 5% of the cells in randomly differentiating ESC cultures (Lanza 2009). Accordingly, a great effort has been devoted to enhance cardiomyocyte yield during in vitro differentiation. Toward this goal, studies have shown that several chemicals and growth factors can be used to induce differentiation of mESC and hESC to cardiomyocytes in different culture conditions. Dimethyl sulfoxide (DMSO) and all-trans

retinoic acid (RA) have been shown to induce cardiomyocytes differentiation in murine P19 embryonic carcinoma cells (Edwards, Harris et al. 1983) and mouse ESCs (Wobus, Kaomei et al. 1997). However, neither DMSO nor RA enhanced hES cell cardiomyocyte differentiation when procedures were similarly applied (Xu, Police et al. 2002). 5-azacytidine (5-aza-dC) induces the differentiation of mesenchymal stem cells into cardiomyocytes, presumably via demethylation of DNA (Yoon, Yoo et al. 2006). It has been also shown by several studies that ascorbic acid (Takahashi, Lord et al. 2003), BMP-4 (Taha, Valojerdi et al. 2007; Taha and Valojerdi 2008), Fibroblast Growth Factor 2 and Bone Morphogenetic Protein 2 (Kawai, Takahashi et al. 2004) can enhance mESC differentiation to cardiomyocytes in a time and concentration-dependent manner .

The ultimate goal in regenerative medicine is production of large-scale and highly purified differentiated cells such as cardiomyocytes that are suitable for cell transplantation. From a commercial point of view, the capability to produce clinically relevant cell numbers through an economically viable bioprocess would be a critical and basic prerequisite. Cell transplantation therapies aimed at repairing tissue damage because of myocardial infarction would necessitate the successful seeding of almost 5-10×10^8 donor adult cardiomyocytes per patient (Al-Radi, Rao et al. 2003). The robust generation of such huge number of cardiomyocytes would only be feasible in controlled SSBs capable of high-density differentiation of ESC in clinical scale.

Currently, most protocols use adherent culture to differentiate ESCs into cardiomyocytes (Wobus, Kaomei et al. 1997; Ventura and Maioli 2000; Sachinidis, Fleischmann et al. 2003; Kawai, Takahashi et al. 2004; Baharvand, Azarnia et al. 2005; E, Zhao et al. 2006; Sato, Takahashi et al. 2006; Taha, Valojerdi et al. 2007). However,

there are major downsides attributed to these static culture environments such as being time consuming and having variability from culture to culture. On the other hand, SSBs propose several advantages over the routine use of static culture flasks. It has been shown that these systems promote the large-scale expansion and differentiation of the cardiomyocytes in a homogeneous culture environment (Zandstra, Bauwens et al. 2003) and decrease the risk of culture variability. SSBs have already been adapted in our lab to expand and differentiate ESCs into osteogenic and chondrogenic cell lineages (Cormier, zur Nieden et al. 2006; zur Nieden, Cormier et al. 2007; Taiani, Krawetz et al. 2010). However, the results revealed that the suspension bioreactor environment enhances ESC pluripotency, while suppressing differentiation efficiency. In another study, scalable production of EBs and the possibility of engineering cardiac tissue was evaluated using one type of rotary cell culture system (RCCS), termed slow turning later vessel (STLV) (Wang, Wei et al. 2006). EB formation efficiencies were almost 2.0 times higher than that of liquid suspension in a Petri dish. Recently, SSBs were employed to induce differentiation of ESCs toward heart cells. The ESCs were encapsulated in poly-L-lysine-layered liquid core alginate beads. Encapsulated ESCs yielded higher expression of cardiac markers such as Nkx2.5- and GATA4 compared to static culture system (Jing, Parikh et al. 2010).

Recent studies have also shown that cardiomyocytes, smooth muscle cells, and multiple types of endothelium can be differentiated from mouse iPSCs (Mauritz, Schwanke et al. 2008; Narazaki, Uosaki et al. 2008). Both groups found that iPSC–derived cardiomyocytes are structurally and functionally similar to ESC–derived

cardiomyocytes. They used the EB formation method to differentiate iPSCs into cardiomyocytes.

2.2 Promise of ESCs and iPSCs in Regenerative Medicine

Regenerative medicine is the process of producing viable, normal and functional cells and tissues to replace or regenerate human damaged cells, tissues, or organs in order to restore or establish normal function (Daar and Greenwood 2007). It is one of the few multidisciplinary areas that combines expertise from a variety of different kinds of sciences. The ultimate goal of regenerative medicine is to establish techniques to cure previously untreatable disorders. The spectrum of regenerative medicine is huge and the technology encompasses different research areas like biomaterial engineering, developmental and stem cell biology, tissue engineering and transplantation science.

A central platform of regenerative medicine is human cells including ESCs/iPSCs and adult stem cells. Establishment of a proper cellular model of a specific or in some cases rare human genetic disorders using human iPSCs is a valuable model to investigate the etiology, pathophysiology and mechanism of diseases.

For decades*, in vitro* culture of human cells has played a vital role in biomedical investigations. This technique allows scientists to have a better understanding of different normal and pathogenic conditions *in vitro*. Although some animal models are available in the field of human genetic disorders, there are few reliable *in vitro* models, which researchers can work on. This issue definitely impedes researchers to find proper answers to critical questions in normal human development in addition to pathological conditions.

Most of the patients affected with genetic disorders have different phenotypes even in single gene diseases, implying that other related gene defects can change the symptoms of a specific disease (Summers 1996; Sidransky 2006). So, it can be an advantage to develop a cellular model of genetic disease to investigate the molecular pathways corresponding to specific genetic disease. Modeling of human inherited disorders can be achieved via two major techniques; each of them has their own limitations.

a) Cell culture. This technique suffers from several drawbacks. Firstly, many of the cell cultures derived from patients have a limited life-span and so cannot be propagated indefinitely *in vitro,* which in turn restricts their study. Secondly, the number of tissues that can be derived is restricted in human. This means that it is impossible to maintain cell culture models for all human tissues. Thirdly, extracted primary cell lines allow scientists to investigate only a short period of human cellular and molecular development depending on the cell type and the tissue derived. Finally, most of the cell lines currently available to researchers around the globe are derived from cancerous tissues or have been transformed in a way to be immortal (Grimm 2004), so they may harbor chromosomal aberrations or mutations.

b) Animal models: Although this approach has helped scientists simulate some of the human genetic disorders in various animals, it has its own disadvantages. Importantly, most of the animals are not a complete representative of the actual human disease (Dvash and Benvenisty 2004). Although some similarities exist between human and animal models, there are major dissimilarities as shown by molecular pathways, biochemical interactions and even development. Furthermore, some of the abnormalities

and mutations in human do not exist in laboratory animals. For example, cystic fibrosis, a genetically inherited disease of the exocrine glands in human, has no known counterpart in animals.

One of the other most promising benefits of ESCs and iPSCs is that large numbers of them can be relatively easily produced in culture; while adult stem cells are rare in mature tissues and methods to grow them have not been well established. This is an important distinction, as large numbers of cells are needed for stem cell replacement therapies. One of the major concerns related to ESCs is the ethical issue that seems to be almost overcome by reprogramming of adult cells (fibroblast, lymphocyte, etc) to generate iPSCs.

An amenable approach for establishing immortal cultures of pluripotent iPSCs from patients would not only promote disease research but also build a basis for the generation of autologous cell therapies that would prevent immune rejections after transplantation. We should consider that cell therapy using patients' own cells has been one of the ultimate aims in stem cell research for years (Findikli, Candan et al. 2006). These patient specific iPSCs can be potentially differentiated into proper cell types for studying the effects of new drugs, toxicity testing and disease development *in vitro*.

The potential of patient-derived iPSCs in regenerative medicine is enormous. It will remove the existing ethical hurdles related to human ESCs and also can potentially eliminate the problem of immune rejection. These cells could be genetically modified so as to cure different genetic diseases such as Type 1 diabetes, Alzheimer's, Parkinson's, cardiomyopathy, hemophilia and muscular dystrophy that involves specific cellular pathology. Considering the first step toward iPS cell therapy, the field is now trying to

characterize them to distinguish any dissimilarity between iPSCs and ESCs. If they are similar from the genetic and epigenetic point of view, the source of a worldwide bank could be histocompatible-reprogrammed iPS cells from different donors. Large amounts of patient histocompatible cells could also be produced *ex vivo* due to the unlimited self-renewal capacity of iPSCs. These cells would generate a large amount of specific differentiated cells required for tissue engineering. Adapting ESCs defined *in vitro* differentiation approaches for iPSCs, they are going to revolutionize the stem cell biology field and provide cell therapy and cure for degenerated tissues and organs. Although iPSC technology is still at its early stage, the field has been experiencing substantial growth in recent years. Efforts are currently focused on better understanding of reprogramming process and to establish a standard method for characterization of iPSCs. This will enable them to be used in clinical applications. Furthermore, continued studies to identify and characterize other novel molecules that control the reprogramming efficiency may ultimately lead to the establishment of efficient and gene-free reprogramming to pluripotent state. The application of chemical approaches in biology and specifically in the area of stem cell and regenerative medicine has become increasingly available. Small molecules are offering higher level of specific regulation over complex stem cell network. There is no doubt that small molecules with the capability to change cell fate will be a promising tool in stem cell biology, iPSC generation and regenerative medicine.

Chapter Three:

Stirred Suspension Bioreactor Suppresses ESC Differentiation

In the following manuscript, Mehdi Shafa contributed to the experimental design, data collection and analysis (Figures 3.1-3.7, 3.9), and preparation of the manuscript.

Impact of stirred suspension bioreactor culture on the differentiation of murine embryonic stem cells into cardiomyocytes

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Running Title: Effect of bioreactor culture on murine ESC differentiation

Keywords: Embryonic Stem Cell, Cardiomyocytes, Differentiation, Stirred Suspension Bioreactor

3.1 Abstract

Background: Embryonic stem cells (ESCs) can proliferate endlessly and are able to differentiate into all cell lineages that make up the adult organism. Under particular *in vitro* culture conditions, ESCs can be expanded and induced to differentiate into cardiomyocytes in stirred suspension bioreactors (SSBs). However, in using these systems we must be cognizant of the mechanical forces acting upon the cells. The effect of mechanical forces and shear stress on ESC pluripotency and differentiation has yet to be clarified. The purpose of this study was to investigate the impact of the suspension culture environment on ESC pluripotency during cardiomyocyte differentiation.

Results: Murine D3-MHC-neo^r ESCs formed embyroid bodies (EBs) and differentiated into cardiomyocytes over 25 days in static culture and suspension bioreactors. G418 (Geneticin) was used in both systems from day 10 to enrich for cardiomyocytes by eliminating non-resistant, undifferentiated cells. Treatment of EBs with 1mM ascorbic acid and 0.5% dimethyl sulfoxide from day 3 markedly increased the number of beating EBs, which displayed spontaneous and cadenced contractile beating on day 11 in the bioreactor. Our results showed that the bioreactor differentiated cells displayed the characteristics of fully functional cardiomyocytes. Remarkably, however, our results demonstrated that the bioreactor differentiated ESCs retained their ability to express pluripotency markers, to form ESC-like colonies, and to generate teratomas upon transplantation, whereas the cells differentiated in adherent culture lost these characteristics.

Conclusions: This study demonstrates that although cardiomyocyte differentiation can be achieved in stirred suspension bioreactors, the addition of medium

enhancers is not adequate to force complete differentiation as fluid shear forces appear to maintain a subpopulation of cells in a transient pluripotent state. The development of successful ESC differentiation protocols within suspension bioreactors demands a more complete understanding of the impacts of shear forces on the regulation of pluripotency and differentiation in pluripotent stem cells.

3.2 Background

Embryonic stem cells (ESCs) are derived from the inner cell mass (ICM) of pre-implantation embryos (Thomson, Itskovitz-Eldor et al. 1998). These ESCs have the ability to remain undifferentiated and proliferate indefinitely *in vitro*, while maintaining the potential to differentiate into all three embryonic germ layers (Thomson, Itskovitz-Eldor et al. 1998; Reubinoff, Pera et al. 2000). An important aspect of ESC research focuses on elucidating the mechanisms of differentiation from the pluripotent ESC to various terminally differentiated cell types. This differentiation capacity makes ESCs an attractive cell source for cell/tissue replacement therapies for the treatment of degenerative human diseases. Moreover, ESCs can also be used as a model system for understanding human genetic disease by elucidating the pathophysiology of specific genetic disorders, including but not limited to cardiac disorders.

The *in vitro* differentiation of ESCs into cardiomyocytes provides an opportunity to study the developmental aspects of cardiomyocytes. Cardiomyocytes are terminally differentiated muscle cells in the adult mammalian heart, which do not divide. Although a small percentage of the cells may be capable of proliferation (Anversa and Kajstura 1998), this is not sufficient for regeneration after myocardial injury. The ultimate goal in

cardiac regenerative medicine is to produce in large-scale, highly purified cardiomyocytes that are suitable for cell transplantation. Such cell transplantation therapies would require the successful seeding of as many as 1.0×10^9 donor cardiomyocytes per patient (Al-Radi, Rao et al. 2003).

From a commercial perspective, the ability to generate such clinically relevant cell numbers through an economically viable bioprocess is a priority. The robust generation of such large cardiomyocyte numbers could only be feasible in controlled bioreactors capable of high-density ESC differentiation. Currently, most protocols use static culture to differentiate ESCs into cardiomyocytes (Wobus, Kaomei et al. 1997; Ventura and Maioli 2000; Sachinidis, Fleischmann et al. 2003; Kawai, Takahashi et al. 2004; Baharvand, Azarnia et al. 2005; Sato, Takahashi et al. 2006; Taha, Valojerdi et al. 2007). Although routinely used for ESC culture and differentiation, static culture flasks can only support a pre-clinical research project. Alternatively, stirred suspension culture bioreactors offer several advantages over the conventional culture methods. We and others have previously demonstrated that suspension bioreactors can support large-scale expansion of the ESCs over extended passages, while retaining their pluripotency (Cormier, zur Nieden et al. 2006; zur Nieden, Cormier et al. 2007).

The scalable production of ESC-derived cardiomyocytes in a suspension bioreactor system has previously been demonstrated using a retinoic acid based protocol (Zandstra, Bauwens et al. 2003; Niebruegge, Nehring et al. 2008). However, since we have previously observed that the suspension bioreactor environment enhances ESC pluripotency, while suppressing differentiation efficiency (Taiani, Krawetz et al. 2010), we sought to investigate whether this phenomenon would also occur during

cardiomyocyte differentiation. This study demonstrates that suspension bioreactor culture systems do indeed have the ability to inhibit differentiation, and even induce 'transient' pluripotency within a defined differentiation protocol presumably due to influence of shear stress on the cells. Our data show that in contrast to static culture, ESCs induced to differentiate toward cardiomyocytes in suspension bioreactors retain their ability to express pluripotency markers and form teratomas.

3.3 Materials and Methods

ESC culture

Murine D3-MHC-neo^r ESCs (a gift from Dr. Peter Zandstra, University of Toronto) were maintained in the pluripotent state on gelatin-coated tissue culture dishes with inactivated mouse embryonic fibroblasts (MEFs) in high glucose Dulbecco's modified Eagles medium (DMEM; Invitrogen) supplemented with 15% FBS (Invitrogen), 0.1mM non-essential amino acids, 50 U/mL Streptomycin and 50 U/mL Penicillin, 0.1 mM β-mercaptoethanol (Gibco), and 1000 U/mL Leukemia Inhibitory Factor (LIF) (ESGRO, Chemicon). The cells were sub-cultured every second day on MEF feeders. Expansion of pluripotent ESCs in stirred suspension bioreactors was carried out as previously described (Cormier, zur Nieden et al. 2006; zur Nieden, Cormier et al. 2007).

Cardiomyocyte differentiation

Differentiation of D3-MHC-neo^r ESCs was performed in a 125 ml suspension bioreactor (Corning Style Spinner Flask, NDS Technologies Inc. Fig 1A) by seeding

about 5×10^4 cells/ml into ESC medium without LIF. As an accepted procedure, removal of LIF triggers a series of events that cause the spontaneous differentiation of mESCs. Formed embryoid bodies (EBs) were treated with 1mM ascorbic acid and 0.5% DMSO from day 3 to the end of the experiment to induce cardiomyocyte differentiation. The culture media was changed every 4 days. An agitation rate of 100 rpm was used to achieve the shear stress of 6.1 dyne/cm^2 in suspension as described previously (Cormier, zur Nieden et al. 2006). Differentiation on static culture was performed with the hanging drop method. Briefly, cell suspensions were diluted to 400-500 cells/20µl in differentiation medium. Each cell suspension was pipetted onto the inner surface of the tissue culture plate cover and cultured for 3 days. The suspended droplets were transferred into a 100 mm bacteriological plate with differentiation media and incubated for 5 days to form EBs and then plated on gelatin-coated 24-well tissue culture plates for further differentiation. Both ascorbic acid (1mM) and DMSO (0.5%) were added to induce cardiomyocyte differentiation from day 3 to the end of the differentiation (Day 25). Each differentiation procedure (static or bioreactor) was run in triplicate. Similarly, all of the characterization experiments were performed three times.

As the cells carry the MHC-neo^r transgene, the expression of the neomycin resistance gene in ESC-derived cardiomyocytes enables their selection using G418 during *in vitro* differentiation. G418 (400µg/ml) was added to the cultures from day 10 in order to enrich for cardiomyocytes by eliminating non-resistant, undifferentiated cells. In order to examine the pluripotency of differentiated cells, EBs derived from suspension bioreactor (without drug selection) were dissociated by trypsin for 5 min followed by plating on gelatin-coated culture plates using mESC media with LIF. Differentiated cells

in static culture were also trypsinized for 3 min and replated on gelatin-coated plates using mESC media with LIF (above).

Teratoma formation assay.

CB-17 severe combined immunodeficient (SCID) mice were ordered from Taconic Company and housed in the animal facility of the Faculty of Medicine, University of Calgary. Animal protocols were performed as approved by the University of Calgary Health Science Animal Care Committee. Cells were harvested from both bioreactor and static cultures after three weeks of differentiation using Trypsin/EDTA. Four mice were injected with differentiated cells of each group: 1) bioreactor without G418 selection, 2) bioreactor with G418 selection, 3) static differentiation without selection, and 4) static differentiation with selection. Cells (1.0×10^6) in a total volume of 100 µl PBS were injected into the skin fold of the inner thigh. After 21 days, the animals were sacrificed and tissues were dissected and examined by histological procedures. Briefly, 4% paraformaldehyde (PFA) was used to fix the tissue overnight at 4^oC. After dehydration by increasing concentrations of ethanol, the tissue was embedded in paraffin. Sections were stained with hematoxylin and eosin (H&E) and examined for different types of tissues by light microscopy. All of the cell injections were performed by Dr. Rancourt's lab technician, Mrs Shiying Liu.

Immunofluorescence

Aliquots of ESC aggregates, taken from the bioreactor at various intervals from day 0 to day 25 of differentiation, were washed with PBS, and fixed overnight in 4% PFA

in PBS at 4°C. Aggregates were then washed three times with PBS, permeabilized in 0.5% saponin in PBS at 4°C overnight, rinsed three times with PBS, then blocked in 3% BSA at 4°C overnight. The primary antibodies for murine Nanog, Oct4 and α-MHC (MF-20) (Santa Cruz Biotechnology, Inc. CA, USA) were diluted 1:50 in 3% BSA and added to the aggregates (overnight at 4°C). Approximately 20-25 aggregates were incubated in 50 µl of antibody solution. The aggregates were then washed 3 times with PBS and blocked again overnight at 4°C. Following the blocking step, the aggregates were incubated overnight at 4°C with Alexa-fluor 488 secondary antibody and TOTO-3 (Molecular Probes). TOTO-3, a carbocyanine dimer stain with far-red fluorescence, was used as a nuclear counterstain. After incubation, the aggregates were washed 3 times with PBS and mounted on slides with mountant (9:1 glycerol:PBS). Spacers (0.25mm) were attached to slides before mounting to avoid aggregate deformation. Slides were analyzed by confocal microscopy (Zeiss 510) using 488, 568 and 633nm filters. To process the images, LSM image browsing software was used (Carl Zeiss, Germany).

For immunofluorescence staining of single cardiomyocytes, EBs were dissociated with collagenase or accutase (Life technologies) for 15 min at 37°C. Mechanical dissociation of beating areas was performed under a stereo dissecting microscope using a 27G1/2 needle attached to a 1 ml syringe. Dissociated cells were transferred to fresh media and washed 2-3 times with Dulbecco's Phosphate Buffered Saline (DPBS) and incubated with trypsin for 5-7 min. The cells were plated in 24-well gelatin-coated plates or 35 mm dish for 2 days and then stained with cardiac specific antibody α-MHC.

Semi-quantitative and real-time PCR

RT-PCR was utilized to evaluate the expression of the pluripotency and cardiac-specific transcripts. RNA was isolated from derived aggregates at various stages of differentiation in static (Day 0, 5, 10, 15, 20) and bioreactor (Day 0, 5, 10, 15, 20 and 25) cultures. The RNeasy Mini Kit (Qiagen) was used to isolate RNA according to the manufacturer's instructions. The RNA concentration was measured by spectrophotometer (BioPhotometer, Eppendorf). Total RNA (1 μg) was transcribed into cDNA using an oligo dT primer and SuperScript III cDNA synthesis Kit (Invitrogen). PCR amplification was carried out in a final volume of 20 μl using Taq DNA polymerase (Invitrogen) using the following steps: 94°C for 3 min, 94°C for 30 sec, 55°C for 45 sec and 72°C for 1 min. Gene primer sets used in the amplification reactions were designed and blasted (NCBI) for mouse ESC specificity. Primer sequences are listed in Table 3.1. GAPDH was used as an internal standard. PCR products were separated on a 1.5% agarose gel, stained with ethidium bromide, visualized and photographed on a UV trans-illuminator.

For quantitative, real-time RT-PCR, standard curves were derived from dilution of the amplicons. Real Time RT-PCR was performed in an iCycler iQ system (BioRad) using a SYBR green PCR master mix (BioRad). The following cycles were used: 3 min at 95°C as initial denaturation, followed by 30 sec at 95°C, 30 sec annealing at 57°C and 30 sec extension at 72°C for 45 cycles. Melting curves were generated at the end of each run to ensure the presence of a single amplicon. Expressions was normalized to GAPDH, a housekeeping gene and compared to static culture. Semi-quantitative and real-time PCR experiments were performed in triplicate.

Transmission electron microscopy (TEM)

Cells were pelleted, washed in PBS and fixed in 3% glutaraldehyde in Millonig's phosphate buffer for 1 hour at RT. Post-fixation was in 2% OsO_4 for 20 minutes. The cells were dehydrated in ethanol, and then embedded in Polybed 812 resin (Polysciences, Warrington, PA). Polymerization was performed at 37°C for 24 hours. Silver-gray sections were cut with an ultramicrotome (Leica) equipped with a diamond knife, stained with uranyl acetate and lead citrate, and then examined in a H-7000 Hitachi electron microscope. Fifteen separate samples from suspension bioreactor were used for TEM.

Chronotropical responses of derived cardiomyocytes

Beating cardiomyocytes outgrowths were investigated at day 14 of differentiation (4 days after G418 selection). The frequency of beating was calculated by the number of cardiomyocyte pulsations per minute. The beating rate was determined before and after application of each drug. After the addition of drugs, the cardiac bodies were incubated for 5 min before measurement. The variation in beating was measured by the difference of the pulsation rates between pre and post addition of each drug. Twenty separate samples were tested for each drug. The following drugs (all from Sigma) were used: isopernaline (10^{-3} M, 117H1382), phenylephrine (10^{-3}M, P-6126), diltiazem (10^{-3}M, D2521), and Bay K8644 (10^{-3} M, B-133).

Flow cytometry

At different time points of differentiation, EBs were harvested from the bioreactor and investigated for expression of pluripotency markers (Oct4, Nanog and Sox2) and

cardiac marker α-MHC by flow cytometry. Accutase (eBioscience) treatment for 15 min followed by pipetting was used to dissociate EBs to a single cell. Dispersed cells were washed one time with PBS. The pellet was resuspended in 1 ml of 4% PFA in PBS then washed 3×5min with 4 ml PBS. The cells were permeabilized in 2ml of 0.5% saponin for 15 min at room temperature. They were washed once with 2 ml PBS for 5 min and resuspended in 3%BSA/PBS for 30 min at 37°C. The primary antibody (approximately 1 μg per 1 million cells) was conjugated with 5 μl of Comp.A (Invitrogen) and incubated 5 min at room temperature. Then 5 μl of Comp B was added and incubated 5 min. The conjugated primary antibody was then added to cell suspension and incubated for 60 min at 37°C. The cells were washed 3 times with PBS and resuspended in 200 μl FACS tubes. FACS experiments were performed using a BD FACSVantage SE™ System at the University of Calgary's Flow Cytometry Facility.

Statistical analysis.

Results are expressed as mean ± SD. Significance of chronotropical responses of derived cardiomyocytes and quantitative RT-PCR results was evaluated by the Student's t-test. Difference were considered statistically significant at values of $p<0.05$. Statistical analysis (ANOVA) was performed for the FACS results using GraphPad Prism4 (GraphPad Software) and significance was set at $p<0.05$.

Gene	Sequences	Product Size	Indication
Oct4	Forward: 5'-GGCGTTCTCTTTGGAAAGGTGTTC-3' Reverse: 5'-CTGGAACCACATCCTTCTCT-3'	312bp	Pluripotency
Sox2	Forward: 5'-CACAACTCGGAGATCAGCAA-3' Reverse: 5'-CTCCGGGAAGCGTGTACTTA-3'	190bp	Pluripotency
Nanog	Forward: 5'-AAGCAGAAGATGCGGACTGT-3' Reverse: 5'-GTGCTGAGCCCTTCTGAATC-3'	232bp	Pluripotency
α-MHC	Forward: 5'- CTGCTGGAGAGGTTATTCCTCG-3' Reverse: 5'-GGAAGAGTGAGCGGCGCATCAAGG-3'	301bp	Cardiomyocyte
ALCAM	Forward:5'-CTTGCACAGCAGAAAACCAA-3' Reverse: 5'-TAGACGACACCAGCAACGAG-3'	190bp	Cardiogenesis
PCAM-1	Forward: 5'-TGCAGGAGTCCTTCTCCACT-3' Reverse: 5'-ACGGTTTGATTCCACTTTGC-3'	245bp	Early mesoderm
ANF	Forward: 5'-TGATAGATGAAGGCAGGAAGCCGC-3' Reverse:5'-GGATTGGAGCCCAGAGTGGACTAGG-3'	203bp	Cardiomyocyte
GAPDH	Forward: 5'-AACTTTGGCATTGTGGAAGG-3' Reverse: 5'-ACACATTGGGGGTAGGAACA-3'	223bp	Housekeeping gene

Table 3.1. Primer sequences used in RT-PCR and Quantitative-PCR

3.4 Results

Tumorgenicity of bioreactor-derived cardiomyocytes

The elegant study by Zandstra *et al*., demonstrated that ESC derived cardiomyocytes could indeed be generated in a scalable stirred suspension bioreactor provided cells were lineage selected (Zandstra, Bauwens et al. 2003). However, based on our previous observation demonstrating increased tumorigenesis of ESC-derived chondrocytes and osteoblasts in stirred suspension bioreactors, we were interested to determine if this effect was eliminated within the G418 (Geneticin) selected population. Using D3-MHC-neo^r ESCs from the Zandstra study, we induced cardiomyocyte differentiation in static and suspension culture for teratoma analysis. The resultant cardiomyocytes from both bioreactor and static culture (with and without G418 selection) were injected into SCID mice and after 21 days, were harvested and processed for histopathology studies. Interestingly, lineage-selected cardiomyocytes from both bioreactor and static differentiation cultures produced no tumor growth *in vivo* suggesting that drug selection for terminal markers may indeed be a reliable tool for the scalable production of ESC derivatives. As expected, static and bioreactor differentiated cells without drug selection during differentiation produced teratomas; however, tumors developed from bioreactor cultured ESCs were significantly larger (~10 mm^2) than static derived tumors (2-3 mm^2). Furthermore, teratomas formed from bioreactor ESCs contained cells representative of all three germ layers; whereas, cells from static culture did not (Fig. 3.1). Based on this result, we undertook the characterization of cardiac bodies generated in the bioreactor, in addition to an analysis of gene expression during differentiation.

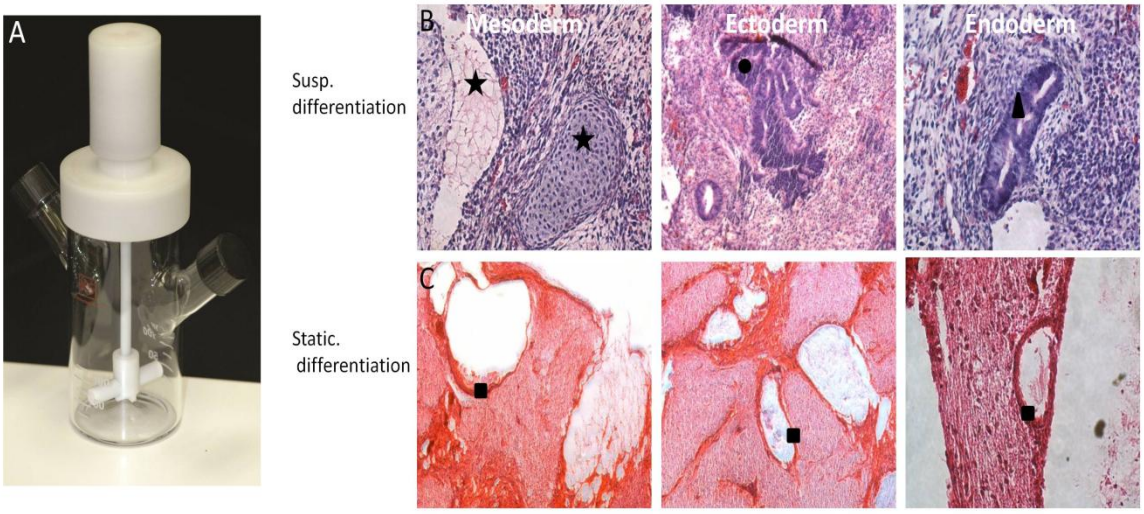


Figure 3.1. Pluripotency of bioreactor differentiated cells as shown by tumor formation in SCID mice. A) The 125-mL NDS stirred-suspension bioreactor. B) Sections of teratoma generated from non-drug selected pluripotent mESCs in bioreactor showing cells from all three germ layers: (★) Mesoderm; (●) Ectoderm; (▲) Endoderm (H&E staining). C) Teratomas derived from mESCs after differentiation under static culture system. The majority of teratomas contain unorganized tissue. Only some gut-like epithelial cells were recognizable (■).

Expression of pluripotency and cardiac markers before/after drug selection in suspension and static culture conditions

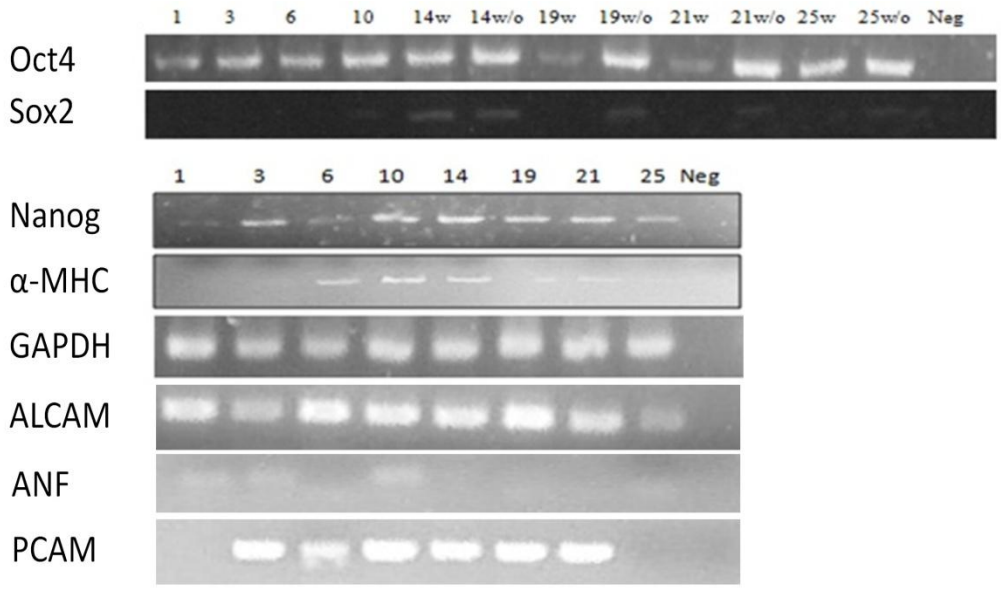
RT-PCR results confirmed the expression of cardiac genes in addition to the expression of pluripotency markers during differentiation in the suspension culture bioreactor. Cardiac markers were examined in non-drug selected bioreactors. α-MHC (myosin heavy chain) gene expression was up-regulated on day 6 through day 14 in EBs and decreased gradually to the end of the differentiation experiment. The early mesodermal marker PCAM-1(Platelet endothelial cell adhesion molecule-CD31) began to be expressed on day 3 and persisted until day 21. Expression of other cardiogenesis lineage genes such as ALCAM (activated leukocyte cell adhesion molecule, CD166) and ANF (atrial natriuretic factor) showed that the ESCs successfully differentiated to cardiomyocytes in the suspension bioreactor. Among the examined pluripotency markers, Oct4 was consistently expressed on all days during differentiation, surprisingly, even with drug selection present from day 10 of the differentiation protocol. Sox2 expression was absent after G418 selection, but was still expressed in the bioreactor without lineage selection. Nanog expression in the non-drug selected bioreactor fluctuated during the first 10 days of differentiation, with expression being up-regulated at day 14 and decreasing gradually from that time point onward (Fig. 3.2A). In static culture, Nanog expression was downregulated on day 5 and the pluripotency markers Oct4 and Sox2 were completely absent on day10 after LIF removal (Figure 3.2B). The cardiac marker α-MHC started to be expressed on day 5 after LIF removal and persisted until the end of experiment. Quantitative RT-PCR in the non-drug selected bioreactors showed that Oct4, Sox2, and Nanog expression persisted during differentiation in the bioreactor culture

compared to static culture. The maximum fold increase in expression was on day 15 for Sox2 and Nanog and day 20 for Oct4 (Fig. 3.3).

Since RT-PCR is a global gene expression assay, Fluorescence-Activated Cell Sorting (FACS) analysis was used to confirm these marker results at the cellular and protein level in non-drug selected cells. FACS showed that the pluripotency marker Oct4 was expressed during all time points of differentiation examined (Fig. 3.4 A, C). In the absence of LIF and G418 selection, the expression of Oct4 positive cells increased from ~20% on day 10 to ~55% on day 25 in the bioreactor. Correspondingly, expression of the cardiac marker, α-MHC (MF-20), increased from ~6.5% on day 10 to ~20% by day 20 with a decrease to ~14% by day 25. Surprisingly, a small population of cells (<10%) expressed both Oct4 and α-MHC (validated with isotype controls) within bioreactors without drug selection. These double positive cells reached a plateau on day 15 during differentiation (Fig. 3.4A). Confocal microscopy also was used to further confirm and validate the existence of Oct4 and Nanog positive cells (Fig. 3.4B).

Interestingly, bioreactor-differentiated cells (without drug selection) retained their ability to form ES-like colonies on gelatin coated plates after 25 days differentiation. However differentiated cells in static culture lost this ability (Figure 3.5). Confocal microscopy also confirmed the simultaneous expression of α-MHC and Oct4 in cells within the bioreactor-differentiated EBs (Figure 3.6).

A



B

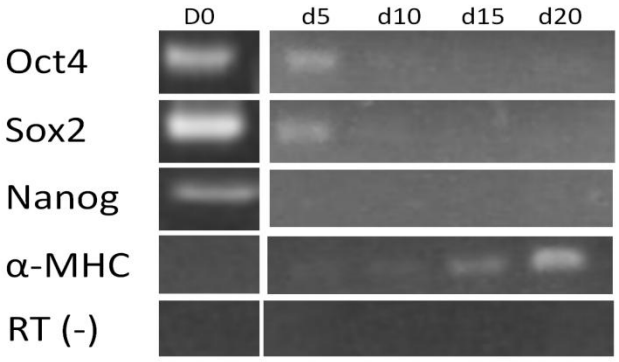


Figure 3.2. Expression of pluripotency and cardiac markers during differentiation in suspension and adherent cultures. A) RT-PCR showed the expression of pluripotency as well as cardiac lineage genes during differentiation in the suspension bioreactor. The G418 used from day 10 of differentiation to select cardiomyocytes: w: with drug; w/o: without drug, Neg: Negative control. The numbers show days past differentiation. Cardiac markers were examined in non-drug selected bioreactors. B) RT-PCR showed the absence of pluripotency markers gene expression during static differentiation. The expression of the cardiac lineage gene α-MHC increased during differentiation in static culture in non-drug selected cultures.

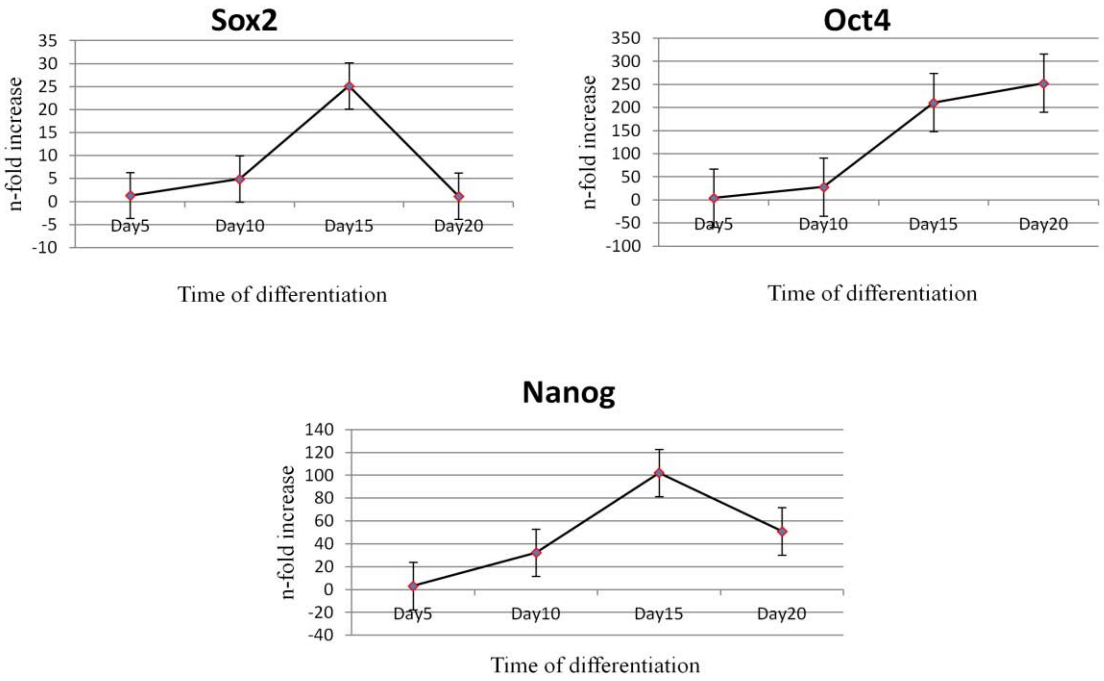


Figure 3.3. Real-time PCR showed the expression of pluripotency markers in suspension bioreactor. Q-PCR results in non-drug selected cultures revealed the fold increase of gene expression in suspension bioreactor compared to static culture during the time course of cardic differentiation. Values represent mean ± SD of 3 independent experiments. $p<0.05$.

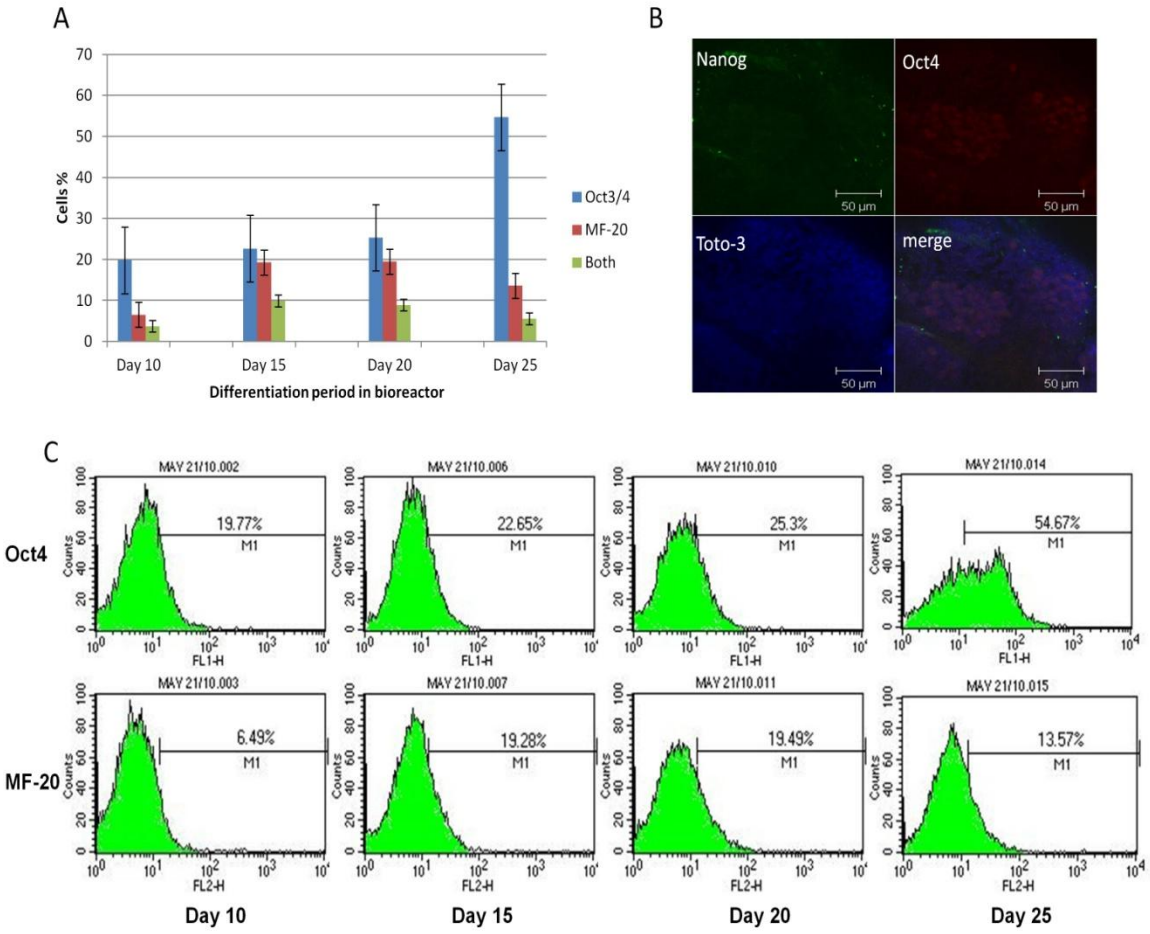


Figure 3.4. Expression of pluripotency and cardiac markers after 25 days in suspension culture. A) Fluorescence-activated cell sorting (FACS) analysis of Oct4 and MF-20. The percentage of cells expressing Oct4 increased from 20% to 54%. The maximum percentage of cells expressing MF-20 was on day 20 and declined afterward. A population of cells was expressing both markers at the same time during differentiation. Values represent mean±SD of 3 independent experiments. $p<0.05$. B) Confocal microscopy confirmed the expression of Oct4 and Nanog in a sub-population of cells 20 days after differentiation in bioreactor. C) Distribution of cell populations in suspension culture system as revealed by FACS analysis of Oct4 and MF-20 genes.

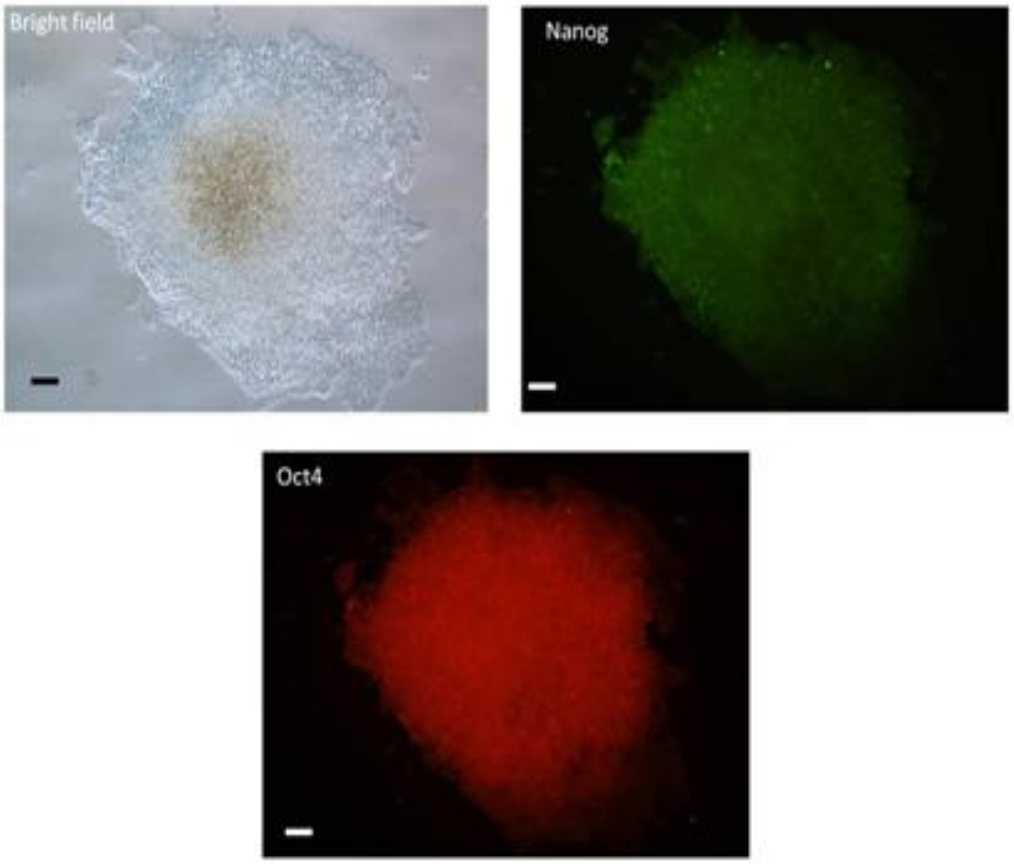


Figure 3.5. Pluripotency of bioreactor-differentiated embryoid bodies. Differentiated ESCs retained their ability to form ESC-like colonies upon plating on gelatin-coated culture plates. The morphology of the colonies as well as expression of pluripotency markers showed the pluripotency of differentiated cells after 25 days in suspension bioreactor without drug selection. Immunostaining was performed with antibodies against Nanog and Oct4.

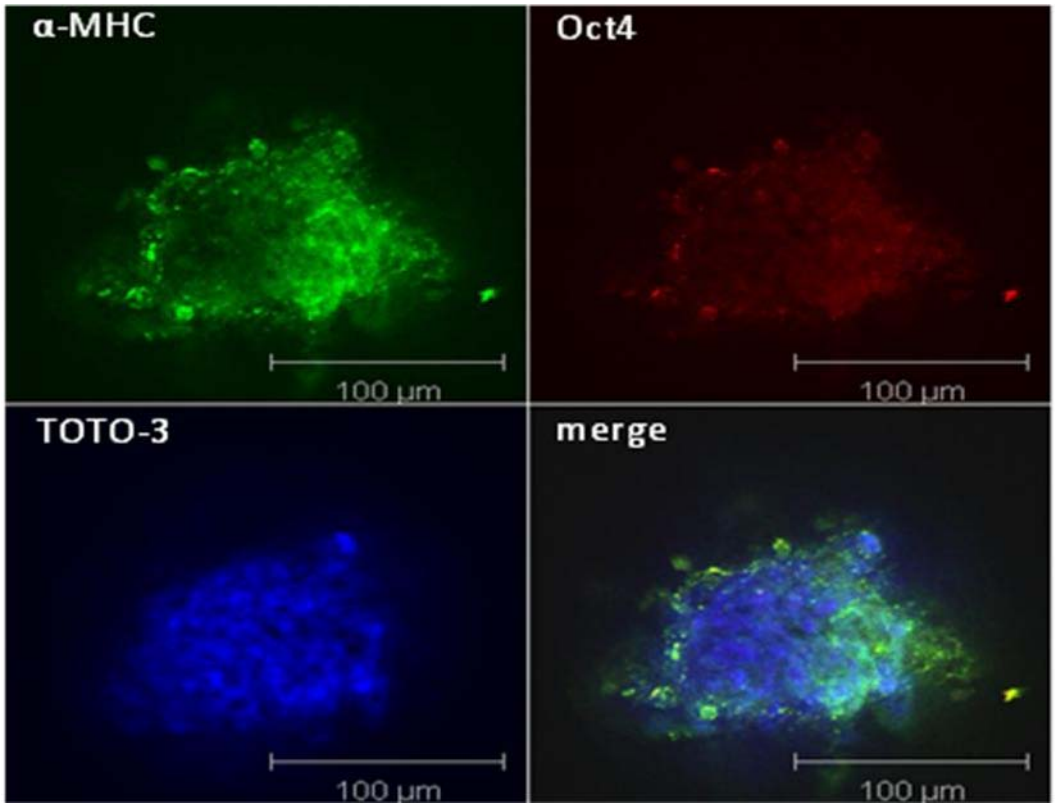


Figure 3.6. Expression of pluripotency marker Oct4 and cardiac marker α–MHC in suspension biore actor. Confocal microscopy of da y 25 E Bs showed the simultaneous expression of α-MHC a nd Oct4 withi n the bioreactor-derived aggregates. Scale bar: 100µm.

Characterization of bioreactor-derived cardiomyocytes

With the observation that pluripotent cells persisted through induced differentiation and the possibility of 'bipotent' $Oct4^{+}/\alpha\text{-}MHC^{+}$ cells, it was next imperative to characterize and test the functionality of the bioreactor derived cardiomyocytes. Within the bioreactor, ESCs aggregated one day after the initial inoculation of single cells without LIF (Fig. 3.7A), with aggregates increasing in size during differentiation. The first beating EBs were seen on day 12, after removal of the LIF and remained for the rest of differentiation period (Fig. 3.7B). Moreover, the cardiac outgrowths of dissociated aggregates kept beating for almost 45 days with beating rates ranging from 29-60 beats/min. The maximum percentage of beating EBs (50%) was on day 14 after differentiation (Fig. 3.7C). Conversely, the appearance of the first beating EBs in the hanging drop method was on day 6, even before moving to suspension culture.

After selection with G418, the resistant derived- cardiomyocytes in the bioreactor had the ultrastructure phenotype of differentiated cardiomyocytes as revealed by light and electron microscope. Dissociated cells were used for light microscope visualization, as well as immunofluorescence staining. After dissociation; the cells produced clusters of spontaneously contractile areas, which were positive for α-MHC as revealed by immunostaining with the MF-20 antibody (Fig. 3.7D). Light microscope showed rounded mononuclear cells with definite subcellular compartments. For TEM, whole cardiac bodies were prepared. Electron microscope revealed the presence of cardiomyocyte-specific ultrastructures including myofibrils with distinct Z-, I-, H- and A-bands, as well as cell-cell adhesion structures, such as gap junctions and desmosomes (Fig. 3.8).

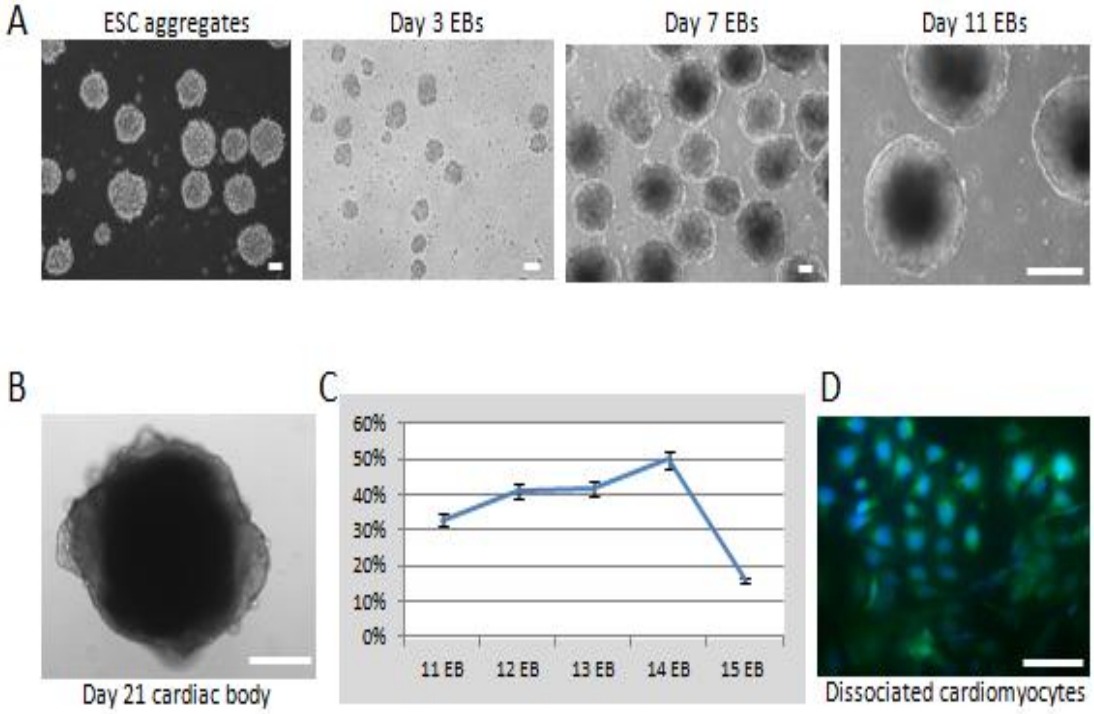


Figure 3.7. Mouse MHC-neo^r ESCs line differentiation in suspension culture. A) Murine ESC aggregates. The size of EBs was growing over time period of differentiation. B) Beating cardiac body (CB) 21 days after differentiation. The CBs were beating rhythmically with various rates. C) Percentage of beating EBs (AA+DMSO). The maximum percentage of beating was 50% on day 14 of EBs. D) Dissociated cardiomyocytes stained with specific cardiac antibody Myosin sarcomere (MF-20) green and DAPI-blue. Scale bars: 100μM.

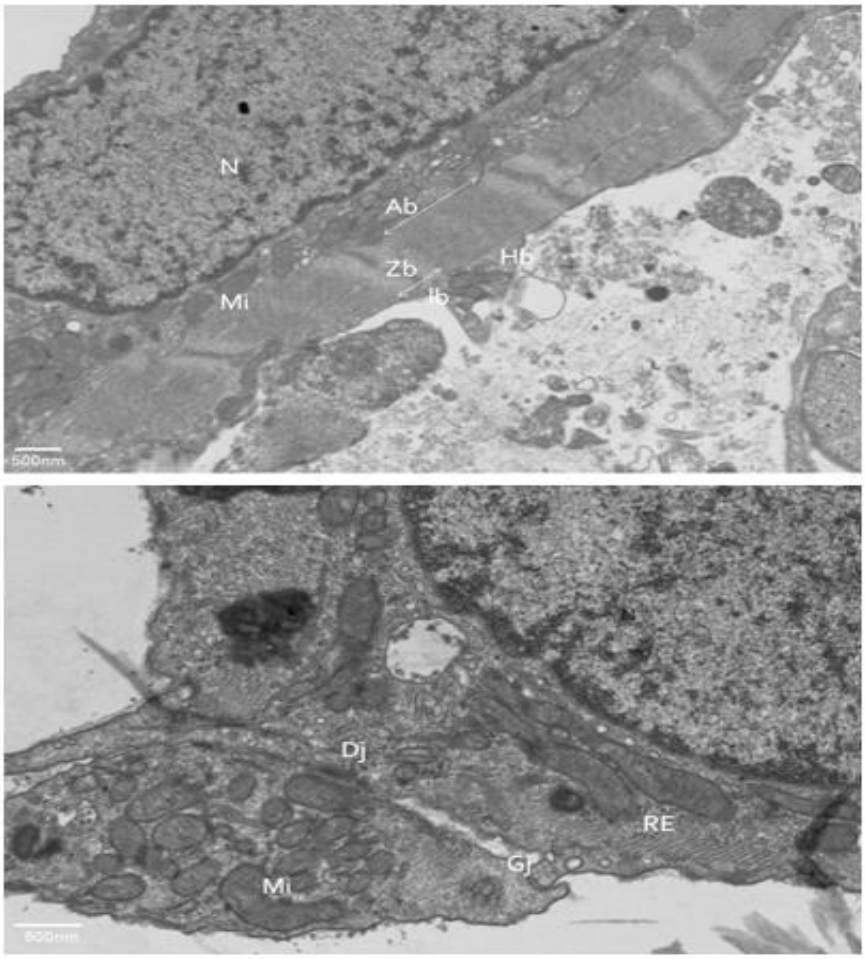


Figure 3.8. Electron microscopy of ESC-derived cardiomyocytes. Mouse MHC-neo^r ESCs line differentiation in suspension culture. Beating cardiac bodies showed specific subcellular ultrstructure of cardiomyocytes using electron microscopy. The presence the cardiomyocytes specific structure including nucleus and myofibrils, mitochondria, endoplasmic reticulum, gap junctions and desmosomes was shown. Hb: H-band; Ib: I-Band; Ab: A-band; Zb: Z-band; Dj: Desmosome Junction; GJ: Gap junction; Mi: Mitochondria; RE: Reticulum Endoplasmic; N: Nucleus. Thanks to Dr. Jerome Rattner for performing TEM.

Pharmacological responses of ESC-derived cardiomyocytes

Spontaneously contracting cardiomyocytes were examined to check if they retain the ability to respond to pharmacological agents. Upon administration of isoprenaline (β1-adrenergic receptor agonist), phenylephrine (α-adrenergic receptor agonist) and BayK8664 (Ca^{2+} channel activator), the beating frequency of cardiac bodies were enhanced. However, the chronotropical effect of diltiazem (Ca^{2+} channel blocker) treatment was negative on cardiomyocyte beating rate (Fig. 3.9A). The highest increased frequency was observed for BayK8664, which was reversed to the previous beating rate about 4 hours after removal of the drug through changing the media (Fig. 3.9B). This highlights the existence of developed functional Ca^{2+} ion channels in cardiomyocytes. These characterization experiments demonstrate that the cells produced were not only positive for cardiac specific markers, but also displayed the properties of functional cardiomyocytes.

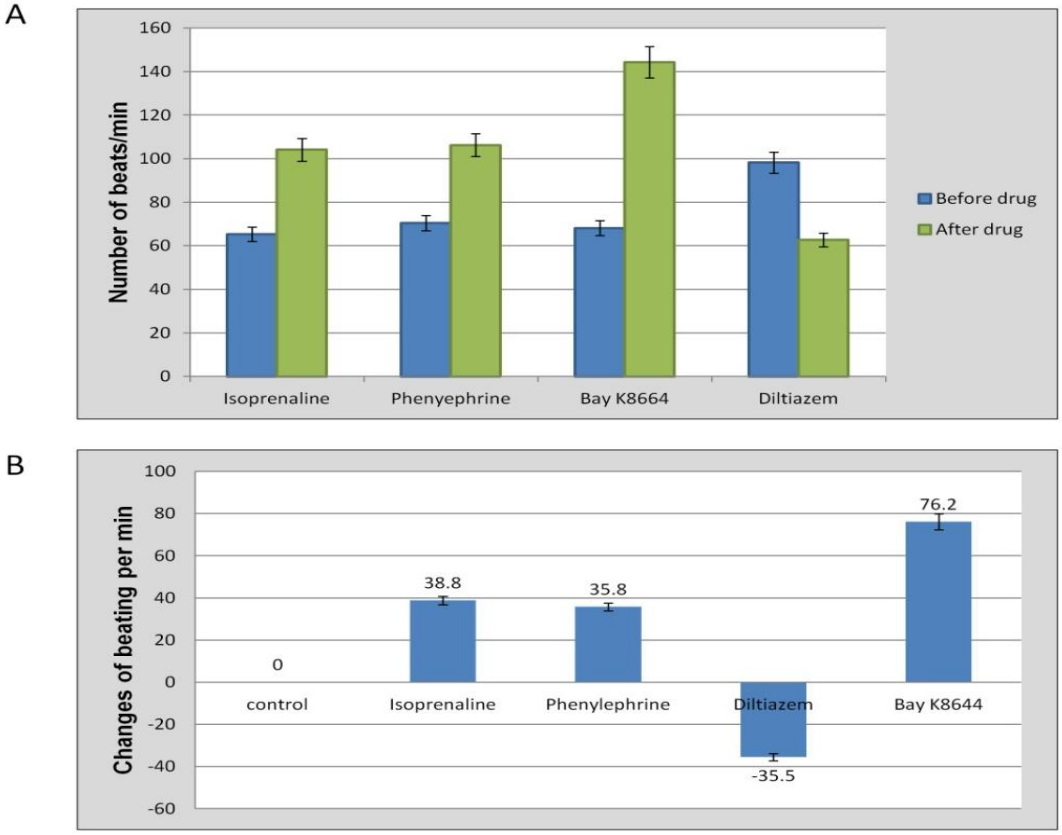


Figure 3.9. Chronotropical responses of bioreactor-derived cardiomyocytes. A) Variation in the number of beatings after drug application. The highest change was due to application of Bay K8664. B) Changes in beating rate. The frequency of changes in beating clearly showed the development of mature ion channels in the membrane of cardiomyocytes, which enabled them to respond to drugs ($P<0.05$).

3.5 Discussion

The large-scale production of differentiated cells suitable for clinical transplantation is a fundamental objective of regenerative medicine research. The replacement of damaged cells using *in vitro* differentiated functional cardiomyocytes has received more attention in the last decade with clinical trials on the horizon. Despite many developments in the stem cell field, we still lack the ability to produce an adequate number of suitable differentiated cells that can meet clinical requirements. We conducted this study to evaluate the tumorigenicity of bioreactor-derived cardiomyocytes, since we have observed that ESCs differentiated in stirred suspension culture maintain their pluripotency compared to those differentiated in static culture. In this study we observed that by day 12 following the removal of LIF in suspension, ESCs underwent efficient differentiation into cardiomyocytes in the presence of ascorbic acid and DMSO. Expression of the early mesodermal marker (PCAM) and cardiomyocyte specific markers (ALCAM (CD166) (Hirata, Murakami et al. 2006), ANF (Temsah and Nemer 2005) and α-MHC (Bin, Sheng et al. 2006)) clearly confirmed the differentiation of ESCs into cardiomyocyte in stirred suspension culture. Similarly, in static culture, cells efficiently made EBs three days after the removal of LIF and the derived-cardiac bodies showed characteristics of cardiomyocytes including expression of the molecular marker α-MHC. It has previously been shown that both ascorbic acid (AA) and DMSO enhance differentiation of mouse ESCs into cardiac myocytes. It has been suggested that AA induces permissive changes enabling cardiomyocyte differentiation, while DMSO has been also shown to activate essential cardiogenic transcription factors, such as GATA-4

and Nkx-2.5. However, the exact related mechanism for triggering these genes is still not very well known (Paquin, Danalache et al. 2002; Takahashi, Lord et al. 2003).

Ultra-structural studies by electron microscopy revealed the specific subcellular sarcomeric organization of cardiomyocytes, such as Z-banding. H- and A-bands, and intercalated discs including desmosome and gap junction were also obvious in derived cells. The chronotropical responses of cardiomyocytes confirmed the existence of specialized Ca^{+2} channels as well as α1 and β1-adrenergic receptors.

Importantly, no tumors were generated after cardiomyocyte lineage selection was used to eliminate cells not expressing the α-MHC-neo^r gene. Based on these experiments, we assume that functional cardiomyocytes had been produced that did not pose a risk of tumor formation *in vivo*. However, it should be emphasized that even in the presence of drug selection, ESCs differentiated in stirred suspension culture still maintained the expression of pluripotency markers. This trend was even more apparent in cultures not undergoing drug selection pressure, where a sub-population expressed both Oct4 and α-MHC simultaneously in the same cell. It is important to remember, however, that drug selection did negate tumor formation signifying that the expression of pluripotent genes is either: (i) not maintained once the cells are taken out of expansion, or (ii) not sufficient to reprogram a cell that has undergone terminal differentiation.

Furthermore, the results presented here show that even after differentiation toward cardiomyocytes, a sub-population of cells (54%) still express Oct4 demonstrating some link between Oct4 and the environment. This fact is further confirmed by the difference observed in teratoma formation between static and suspension cultures. The suspension bioreactor retained the pluripotency of cells to a degree that ESCs formed teratomas after

transplantation to SCID mice containing cells representative of all three germ layers after differentiation. However, cells from static culture only formed an unstructured cell population. Although not a teratoma, a tumor mass was still generated. Although lineage selection using transgenic constructs is a useful research tool, this approach cannot be applied clinically. Hence, our observation that bioreactor-derived cardiomyocytes maintain hallmarks of pluripotency has significant implications. Before the large production of cardiomyocytes in suspension bioreactors will be possible, it will be necessary to eliminate bioreactor-induced pluripotency.

We have recently evaluated the application of suspension bioreactor culture for the generation of cartilage and bone tissue (Taiani, Krawetz et al. 2010). Unlike the static culture environment, bioreactor-differentiated aggregates caused teratoma formation when implanted subcutaneously into SCID mice, which implicated the existence of pluripotent cells in the bioreactor even after 30 days of suspension culture. Upon closer analysis of cells within aggregates, we discovered that cells on the ridges of the aggregate expressed the greatest amount of Oct4 in locations that would be exposed to the greatest amount of laminar fluid flow. This result suggested the possibility that the maintenance of pluripotency within bioreactor differentiation cultures was the result of fluid shear stress. In the current study, much like all our other bioreactor studies, we apply an agitation rate of 100 rpm (shear stress of 6.1 dyne/cm^2) in order to optimize mass transfer and avoid necrosis, which otherwise occurs at lower velocities (Cormier, zur Nieden et al. 2006).

We believe that liquid shear stress in the stirred suspension bioreactor plays an important mechanistic role in bioreactor induced pluripotency. Shear stress can modulate

gene expression through mechanotransduction, where physical signals are sensed at the cell periphery, transduced into biochemical signals within the cell, ultimately resulting in cell responses, including changes in gene expression (Vogel and Sheetz 2006; Vogel and Sheetz 2009). Previous studies in other cells have demonstrated that shear stress can induce the nuclear translocation of ß-catenin into the nucleus (Norvell, Alvarez et al. 2004; Avvisato, Yang et al. 2007). As ß-catenin is an important regulator of pluripotency (Dreesen and Brivanlou 2007), we are interested in the role of the canonical Wnt signaling pathway in this induced pluripotency process. Recently, using a LEF/TCF-GFP reporter system, we have confirmed that ß-catenin nuclear occupancy is considerably increased over controls when cells are exposed to 6.1 dynes/cm^2 shear stress compared to lower levels of shear generated by lower velocities of stirring (Brad Day, unpublished data). Following nuclear translocation, β-catenin forms a complex with LEF/TCF transcription factors. This complex interacts with the specific sequences in the promoter regions of certain genes to activate transcription.

Recently, Saha et al. has shown that biaxial cyclic strain above a certain threshold inhibits human ESCs differentiation and enhances their self-renewal without selecting against growth or survival of differentiated or undifferentiated cells (Saha, Lin et al. 2006). This group later suggested that strain may induce autocrine or paracrine signaling through TGFβ (Transforming growth factor beta) superfamily ligands in human ESCs since the TGFβ superfamily activation of Smad2/3 was necessary for suppression of spontaneous differentiation under strain (Palecek, Saha et al. 2008).

Despite the fact that stirred suspension cultures are very useful for the generation of a large number of undifferentiated cells, we have found that the addition of medium

enhancers is not adequate to force complete differentiation of the population in suspension bioreactors. By elucidating the exact mechanism(s) by which liquid shear stress may contribute to promoting pluripotency and preventing differentiation, we will be able to create an efficient environment for both the production of large quantities of pluripotent stem cells, and their differentiated progeny. This is an important objective for human regenerative medicine, as lineage selection using transgenes will not be possible.

3.6 Conclusions

Although stirred suspension bioreactors are very advantageous for the scalable generation of undifferentiated cells, addition of medium enhancers is not sufficient to induce complete differentiation. Our observation that bioreactor-derived cardiomyocyte cultures maintain characteristics of pluripotency has significant implications for human regenerative medicine. We believe that liquid shear stress in the stirred suspension bioreactor plays an important mechanistic role in bioreactor induced pluripotency, which needs further investigation.

3.7 Acknowledgement and Funding

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Chapter Four:

Expansion of iPSCs in Stirred Suspension Bioreactors

In the following manuscript, Mehdi Shafa contributed to the experimental design, data collection and analysis (Figures 4.1-4.9, 4.10 A,C-E and 4.11), and preparation of the manuscript.

Expansion and long term maintenance of induced pluripotent stem cells in stirred suspension bioreactors

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Keywords: induced pluripotent stem cells; stirred suspension bioreactor; cell expansion; pluripotency

4.1 Abstract

Induced pluripotent stem cells (iPSCs) can provide an important source of cells for the next generation of cell therapies in regenerative medicine, in part due to their similarity to embryonic stem cells (ESCs). Patient-specific iPSCs represent an opportunity for autologous cell therapies, which are not restricted by immunological, ethical and technical obstacles. One of the technical hurdles that must be overcome before iPSCs can be clinically implemented is the scalable, reproducible production of iPSCs and their differentiated progeny. All of the iPSC lines established thus far have been generated and expanded with static tissue culture protocols, which are time-consuming and suffer from batch-to-batch variability. Alternatively, stirred suspension bioreactors (SSBs) propose several benefits and their homogeneous culture environment facilitates the large-scale expansion required for clinical studies at less cost. We have previously developed protocols for expanding murine and human ESCs as undifferentiated aggregates in SSBs. Resulting cells were karyotypically normal, expressed pluripotency markers and could be differentiated into all three germ lineages both *in vitro* and *in vivo*. In this study, we demonstrate that SSBs yield a 58-fold expansion of undifferentiated pluripotent iPSCs over 4 days. *In vitro* differentiation into cartilage, bone and cardiomyocytes lineages in addition to *in vivo* teratomas formation further confirmed the existence of fully functional and undifferentiated pluripotent iPSC aggregates following long term passaging. SSB culture represents an efficient process for the large-scale expansion and maintenance of iPSCs, which is an important first step in their clinical application.

4.2 Introduction

The potential therapeutic application of stem cells in regenerative medicine relies upon the accessibility of pluripotent cells that are not restricted by ethical, technical, and immunological concerns. In 2006, a milestone was achieved in cell biology by artificially deriving pluripotent stem cells from mouse and later human somatic cells. In two independent studies led by Thomson and Yamanaka, ES-like cells called "induced pluripotent stem cells (iPSCs)" were generated from mouse embryonic fibroblasts and adult human somatic cells by viral transduction of four defined pluripotency transcription factor genes (Takahashi and Yamanaka 2006; Takahashi, Tanabe et al. 2007; Yu, Vodyanik et al. 2007). Yamanaka's group used Oct4, Klf4, c-Myc and Sox2 and Thomson's group applied Nanog and Lin28 instead of c-Myc and Klf4. These iPSCs resembled mouse and human ESCs morphologically, expressed cell surface markers characteristic of ESCs, demonstrated multilineage differentiation in both embryoid bodies and teratomas, had a normal karyotype and expressed telomerase.

There are several notable benefits of iPSCs. Most importantly, these cells don't have the ethical considerations, which currently impose a hurdle to human embryonic stem cell (hESC) investigations. The ability to induce an adult cell to act like an ESC might also enable bypassing other controversial methods for creating pluripotent cells, notably somatic cell nuclear transfer, a technique that has additional ethical issues and is extremely difficult to do routinely, as unfertilized human eggs are required.

Another critical advantage of iPS cell technology is that, in theory, it allows for the creation of cell lines that are genetically customized to a patient (Hanna, Wernig et al. 2007). The issue of rejection, in which the body's immune system identifies implanted

cells or tissues as foreign and attacks them, is another obstacle to the therapeutic application of cell-based therapies. If cells for therapy can be generated using a patient's own cells, the issue can be potentially rendered.

However, several technical hurdles must be overcome before iPSCs can be used in clinical implementation. One of these obstacles is the scalable reproducible production of iPSCs in adequate quantities for their successful future clinical applications. Although commonly used static culture flasks are renowned for ESC culture, they become impractical beyond pre-clinical research. There are also major disadvantages associated with static culture systems such as time-consuming feeding and passaging, and culture-to-culture variability due to non-homogenous culture conditions. Alternatively, stirred suspension bioreactors (SSBs) offer several benefits over the conventional use of static culture flasks. First, these systems facilitate the large-scale expansion required for clinical studies with less cost and second, providing a homogenous culture environment, which decreases the risk of culture variability. Also, there is a “bioprocess gap” between the scarcity of stem cells and the potential high demand, necessitating a suitable plan for large-scale expansion procedures for both ESCs and iPSCs.

Previously, results have been presented showing different culture systems for the proliferation and expansion of mouse and human ESCs and iPSCs. In a study, manual fed-batch shake flask bioreactors were employed to proliferate mouse ESCs as single cells by abrogating E-Cadherin mediated cell-cell contacts (Mohamet, Lea et al. 2010). Although this method could result in proliferation and maintenance of ESCs in an undifferentiated state, it needs E-cadherin blocking antibodies, which increases the cost of culture. Olmer et al., developed a defined medium to standardize long term expansion

of human ESCs and iPSCs as aggregates in a suspension culture system (Olmer, Haase et al. 2010). They demonstrated that the suspension culture system could yield a high proliferation rate, but their system was not suitable for controlled large scale expansion of clinically relevant numbers of iPSCs. Larijani et al. also reported long-term maintenance of undifferentiated human ESCs and iPSCs in a suspension culture system. Although they did not employ suspension bioreactors and achieved low expansion rates, the authors emphasized the usefulness of SSBs for large scale expansion of iPSCs (Larijani, Seifinejad et al. 2011).

Recently, we and others showed that SSBs are a favorable, scalable environment for the controlled expansion of murine ESCs as aggregates (Fok and Zandstra 2005; Cormier, zur Nieden et al. 2006; zur Nieden, Cormier et al. 2007; Kehoe, Lock et al. 2008). Furthermore, we found that SSB culture may induce pluripotency and reduce the differentiation efficiency of mESCs in serum-containing media through fluid shear stress (Taiani, Krawetz et al. 2010). We have also successfully developed protocols for the expansion of hESCs in SSBs, which yielded a 25-fold expansion of pluripotent hESCs over 6 days (Krawetz, Taiani et al. 2010).

Currently, iPSCs are derived and cultured in static conditions, which have the same disadvantages mentioned above for ESCs (Takahashi and Yamanaka 2006; Takahashi, Tanabe et al. 2007; Yu, Vodyanik et al. 2007; Woltjen, Michael et al. 2009). Expansion and maintenance of large quantities of iPSCs, through an economical and reproducible protocol, will facilitate further research as well as future clinical applications. The current study sought to show that murine iPSCs can be maintained and expanded in suspension culture bioreactors without loss of pluripotency over a long-term

period. The iPSC aggregates that were generated retained the ability to express pluripotency markers, as well as the potential for multi-lineage differentiation *in vitro* and *in vivo.*

4.3 Materials and Methods

Preparation of reprogramming retroviral vectors

Plat-E retroviral packaging cells (Cell Biolabs, San Diego, CA) were prepared for plasmid transfections by seeding 8×10^6 cells per 100 mm dish (one dish for each reprogramming gene). Cells were maintained in FP medium, which consists of DMEM containing 10% FBS and 50 U of penicillin/streptomycin. The next day, each pMXs retroviral plasmid DNA (Oct4, Sox2, Klf4, and c-Myc; Addgene plasmids 13366, 13367, 13370 and 13375 respectively, Cambridge, MA) (Kitamura, Koshino et al. 2003) was introduced into Plat-E cells using Fugene 6 transfection reagent (Roche, 815075) according to the manufacturer's recommendations. Briefly, 27 µl of Fugene 6 transfection reagent was added to 300 µl of Opti-MEM in a 1.5 mL tube. 9 µg of each retroviral vector was added into the prepared Fugene/OpiMEM tube drop by drop and incubated for 15 min. Each Vector/Fugene 6 complex was added dropwise into the Plat-E dishes and incubated overnight at 37°C, 5% CO_2. The following day, medium was removed and replaced with 10 mL fresh FP medium. Forty eight hours post-transduction virus-containing medium from each transfection was collected, filtered through 0.45µm Acrodisc filter (Pall Life Sciences) and supplemented with 4 µg/mL Polybrene.

Generation of miPSCs in static culture system

In the current study, we used two iPSC lines, RS-2 and iPS-3. RS-2 line was generated, expanded and characterized in our lab and iPS-3 was derived in Dr. Marek Michalak's lab at the University of Alberta. The iPS-3 line was then expanded and characterized in our lab. In summary, for derivation of the RS-2 line, CD1 mouse embryo fibroblasts (MEFs) were seeded at 8×10^5 cells/100 mm dish. Equal parts of Oct4, Sox2, Klf4, and c-Myc retroviral conditioned medium were mixed together and added to the MEFs in dishes. Cells were incubated overnight at 37°C in 5% CO_2. The following day, supernatant was removed and replaced with fresh FP medium. Three days after infection, FP medium was replaced by mESC medium supplemented with LIF (see below). The ESC-like colonies were selected and expanded over a two to three week period according to standard procedures (see below).

miPSC culture

miPSCs were grown as a monolayer in the pluripotent state on gelatin-coated tissue culture dishes with inactivated MEF feeder cells in miPSC/ES medium (high glucose DMEM (Invitrogen) supplemented with 0.1 mM non-essential amino acids, 50 U/mL penicillin, 50 U/mL streptomycin, 0.1 mM β-mercaptoethanol (Gibco), 1000 U/mL LIF (ESGRO, Chemicon) and 15% FBS (Invitrogen). Cells were sub-cultured every second day on inactivated-MEFs feeder cells to prevent their differentiation.

Expansion of pluripotent miPSCs in SSB was carried out as previously described for mouse ESCs (Cormier, zur Nieden et al. 2006; zur Nieden, Cormier et al. 2007) with some modifications. Briefly, 5×10^6 miPSCs (passage 14) were inoculated in

a 125 mL suspension bioreactor (NTS Technologies, Vinland, NJ) with 100 mL miPSC medium at a cell density of 5×10^4 cells/mL miPSC medium. Every four days, the miPSC aggregates were dissociated and passaged using 0.25% Trypsin/EDTA and mechanical dissociation. Dissociated cells were reintroduced to the bioreactor at the same concentration (5×10^6 cells/100 mL) as a single-cell suspension and agitated at 100 rpm. SSBs were run for a total of thirty-two days (eight passages).

Growth rate

Samples (2.0 mL) were taken daily from the bioreactors at specific times following inoculation. While miPSC aggregates were dissociated with 0.25% trypsin/ EDTA, total cell numbers and viabilities were calculated using a hemocytometer combined with Trypan blue staining. The cell-fold expansion, average growth rate and average doubling time were calculated using the inoculation and passaging day viable cell densities.

Chromosome counts

After dissociating miPSC aggregates on day 16 and 32 in the bioreactor, cells were cultured overnight in static culture conditions without feeder cells. The following day, cells were incubated with 0.2 μg/mL colcemid at 37°C for 1 h, trypsinized, resuspended, and incubated in 2-3 mL of aqueous 0.068 M KCl for 15 min at 37°C. They were then fixed with fresh 3:1 methanol:glacial acetic acid, followed by three rinses with fixative. Single cells were dropped on the precleaned slides to spread chromosomes.

The dried slides were stained with Giemsa solution. Forty separate metaphase spreads were examined to evaluate cell ploidy.

Immunofluorescence

miPSCs aggregates were dissociated by accutase for 15 min at 37°C. Cells were plated on 35 mm gelatin-coated dishes for 2 days and then stained with antibodies for pluripotency markers including Oct4, Rex1 and Nanog. Briefly, cells were washed twice with PBS and then fixed with 4% paraformaldehyde/PBS for 15 min at room temperature. Cells were then washed twice with PBS (10 min each) and permeabilized with 0.1% Triton X-100 (Sigma, T8532) in PBS for 10 min at room temperature. After washing twice with PBS, the cells were treated with blocking solution (3% BSA in PBS) for 1 hr. The primary antibodies (Santa Cruz Biotechnology, CA) were diluted to working concentrations in blocking solution (1:200) and incubated overnight at 4°C. The next day, cells were washed three times with PBS and incubated with secondary antibody (1:500) overnight at 4°C. The cells were washed with PBS three times and examined under a fluorescence microscope.

RT-PCR and Q-PCR

RNA was isolated from bioreactor-derived aggregates at various stages of maintenance in the bioreactor (day 0, 16 and 32) using RNeasy Mini Kit (Qiagen, 74106) according to the manufacturer's instructions. RNA concentrations were determined by spectrophotometer (BioPhotometer, Eppendorf). Reverse transcription polymerase chain reaction (RT-PCR) was carried out to evaluate expression of the pluripotency transcripts

before and during bioreactor expansion period. Total RNA (1 μg) was transcribed into cDNA using oligo dT primer and SuperScript III cDNA synthesis Kit (18080-51, Invitrogen). PCR amplification was performed in a final volume of 20 μl using Taq DNA Polymerase (Invitrogen) consisted of the following steps: 94°C for 3 min, 94°C for 30 sec, 55°C for 45 sec and 72°C for 1 min. Primer sets used in the amplification reactions were designed and blasted (NCBI) for mouse specificity. GAPDH was used as internal standard. PCR products were separated on a 1.5% agarose gel and stained with ethidium bromide, visualized and photographed on a UV transluminator. Quantitative PCR (Q-PCR) was performed in an ABI7900HT Fast Real-Time PCR System. RT reaction mix was prepared using High Capacity cDNA Archive Kit (Applied Biosystems, 4322171). TaqMan gene expression assays (Applied Biosystems; CA, USA) for mouse Oct4, Nanog, Sox2 and 18S genes were employed according to manufacturer's protocols to quantify the expression of genes during different time course of maintenance. Expressions was normalized to 18S and compared to day 0.

In vitro differentiation

For chondrogenic and osteogenic differentiation, we used the micro-mass approach as described previously (Yamashita, Krawetz et al. 2009). Briefly, miPSCs were dissociated using 0.25% trypsin/EDTA and seeded in high-density micromass spots ($1.0x\ 10^5$ cells per 10 μl spot) for 2 hrs. Medium was added to each dish without dissociating the spots. For chondrogenic differentiation, we used differentiation media containing DMEM (Gibco), 1% non-essential amino acids (Invitrogen), 50 U/mL penicillin and 50 μg/mL streptomycin (Invitrogen), 0.1 mM 2-mercaptoethanol

(Invitrogen), 1% insulin, transferrin and selenium (ITS) (Invitrogen), 1% FBS (Gibco), 10 ng/mL TGF-β1 (PeproTech), 10 ng/mL BMP-2 (PeproTech), and 50 μg/mL ascorbic acid (Sigma). Medium was changed every 2 days. At day 5, differentiated miPSCs formed aggregates within the micro-mass. These aggregates were transferred to static suspension culture in 6 cm petri dishes containing chondrogenic differentiation media 1% ITS, 1% FBS, 10 ng/mL BMP-2 and 50 μg/mL AA (Yamashita, Nishikawa et al. 2010). Medium was changed every 3-5 days. Following 3 weeks differentiation, these aggregates were stained using alcian blue and collagen 2 (COL2) immunofluorescence.

For osteogenic differentiation, we used differentiation media containing DMEM (Gibco), 1% non-essential amino acids (Invitrogen), 50 U/mL penicillin and 50 μg/mL streptomycin (Invitrogen), 0.1 mM 2-mercaptoethanol (Invitrogen), 15% knock-out serum replacement (KSR), 50 μg/mL ascorbic acid, 10 mM β-glycerophosphate (β-GP) (Sigma) and 100 nM dexamethasone (Dex) (Sigma). After 5 days, the aggregates were transferred to static suspension culture. Medium was changed every 3-5 days. Following 3 weeks differentiation, these aggregates were stained by Alizarin Red S and osteocalcin (OC) immunofluorescence.

Cardiomyocyte differentiation was performed using the hanging drop method. Briefly, the cells were diluted to 400-500 cells/20 μl by differentiation medium. The cell suspension was pipetted onto the inner surface of the tissue culture plate cover and cultured for 3 days. The suspended droplets were transferred into a 100 mm bacteriological plate with differentiation media and incubated for 5 days and then plated to gelatin-coated 24-well tissue culture plate for further differentiation. Ascorbic acid (10^{-3} M) was applied to induce cardiomyocytes differentiation from the day 0 of EBs until

the end of the differentiation (day 20). Differentiation of miPSCs was also performed in 125 mL suspension bioreactor to evaluate the EB formation capacity of cells in suspension culture. About 5×10^4 cells/mL were seeded into iPS medium without LIF. Both ascorbic acid (10^{-3} M) and DMSO (0.5%) were applied to induce cardiomyocyte differentiation in suspension bioreactors from the day 3 of EBs until the end of the differentiation (day 25).

Teratoma formation assay

CB-17 SCID mice were purchased from Charles River Company and housed in the animal facility of the Faculty of Medicine, University of Calgary. Animal protocols were performed as approved by the Animal Care Committee of the University of Calgary. Cells were harvested from the bioreactor at the end of the experiments using Trypsin/EDTA. Mice were injected with the miPSCs at the start and at the end of the maintenance period. About 10^6 cells in a total volume of 100 µl PBS were injected subcutaneously in the inner thigh. After 21 days, animals were dissected and tissues were examined by histological methods. Briefly, 4% paraformaldehyde was used to fix the tissue overnight at 4°C. After dehydration, the tissue was embedded in paraffin. Sections were stained with H&E and examined for different types of tissues by light microscope. All of the cell injections were performed by Dr. Rancourt's lab technician, Mrs. Shiying Liu.

Colony-forming cell assay

For the CFC (colony forming cell) assay, three different concentrations of miPSCs (1.0×10^5, 1.5×10^5 and 2.0×10^5) were seeded onto gridded gelatin-coated dishes and grown in miPSC maintenance medium containing 1000 U/mL of LIF. After 5 days incubation at 37°C/5% CO_2 culture condition, emerging colonies were stained for alkaline phosphatase and counted. The number of differentiated colony outgrowth (negative) versus undifferentiated (positive) were distinguished and counted.

Alkaline phosphatase assay

miPSC aggregates were stained for alkaline phosphatase. Briefly, just before each passage, aggregates were taken from the bioreactor and transferred to a 15 mL tube. After centrifugation and aspirating excess medium, aggregates were fixed with 4% paraformaldehyde in PBS for 2-3 min. The fixative was then aspirated and the cells rinsed with PBS. The ALP staining reagent was prepared according to manufacturer's protocol (Millipore Corporation). The aggregates were stained for 15 min at room temperature. The percentage of positive red aggregates were recorded by counting fifty separate aggregates.

4.4 Results

iPSCs generated using adherent culture showed characteristics of pluripotency

One of the mouse ESC lines (RS-2) was characterized *in vivo* and *in vitro* to confirm its fully reprogrammed status (Fig. 4.1, 4.2A and 4.2Ba). iPS-3 line was generated using lentiviral vectors in Dr. Marek Michalak's lab at the University of

Alberta. RT-PCR and teratoma formation assay in SCID mice were performed to confirm the pluripotent state of the cells. (Fig.4.2A and 4.3). iPS-3 cells showed normal karyotype after derivation (Fig 4.2Bb).

Long term maintenance of miPSCs as aggregates in SSBs.

Previously, we developed methods for expanding murine embryonic stem cells (mESCs) as pluripotent aggregates in SSBs (Cormier, zur Nieden et al. 2006; zur Nieden, Cormier et al. 2007). In the present study, we sought to investigate whether our method could be extended to murine induced pluripotent stem cells (miPSCs). Two iPSC lines, RS-2 and iPS-3, were used in this study. Over the first 24 h after inoculation, iPSCs formed small tight aggregates similar to those observed with mESCs, when stirred at 100 rpm. To prevent the cells from differentiating, aggregates were trypsinized into single cells every 4 days after expanding in suspension. The cells were then reintroduced into the suspension bioreactor, inoculated at a cell density of 5×10^4 cells/mL. Using this approach, miPSC lines subsequently underwent 8 passages (P1-P8) for a total of 32 days of suspension culture. The morphology of iPS aggregates for both RS-2 and iPS-3 were similar to ESC aggregates (Fig 4.4A,B) and remained unchanged over the course of each passage (passages 5-8) (Fig. 4.4C). Viable cell density and cell viability were determined through daily cell counts of the suspension culture.

The apparent growth rate, cell-fold expansion per passage and average doubling time were calculated using the inoculation and final viable cell densities for each passage as shown in Table 4.1. Growth rates were lower, but consistent with those characteristic

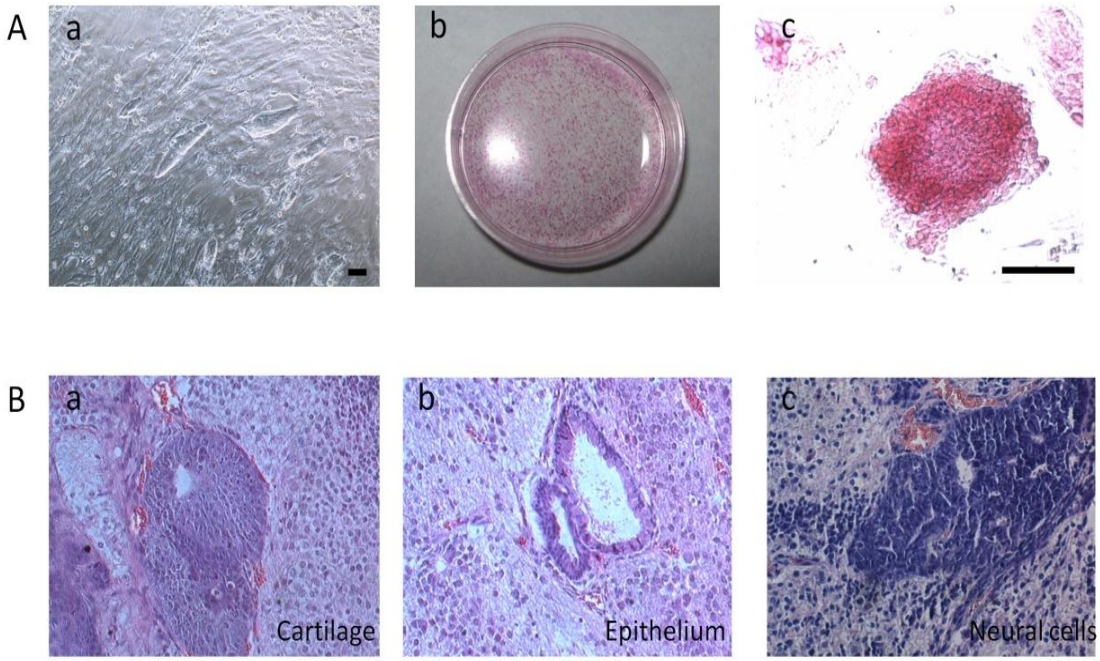


Figure 4.1. Characterization of RS-2 iPS Cells Derived from Mouse Embryonic Fibroblasts (MEFs). (A) Morphology of RS-2 iPSC line. a: Undifferentiated RS-2 colonies on inactivated mouse embryonic fibroblast feeder layer. b: RS-2 colonies on MEF feeder. Cells were stained for Alkaline Phosphatase (ALP) expression. c: Single RS-2 iPS colony positive for ALP. (B) Hematoxylin and eosin staining shows a teratoma from iPS cells (RS-2) containing multiple tissues, including cartilage, epithelium and neural cells. Scale bars=100 μm.

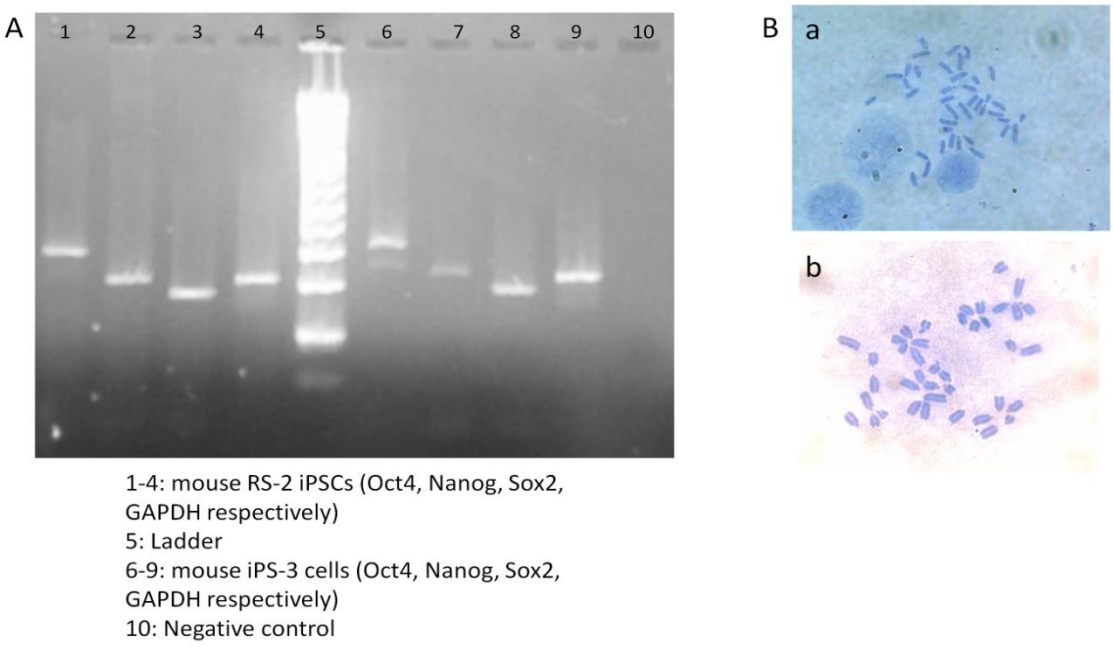


Figure 4.2. RT-PCR confirmed the expression of core pluripotency markers in RS-2 and iPS-3 cell lines. (A) RT-PCR analysis of ES cell marker genes in RS-2 and iPS-3 cells. (B) Both RS-2 and iPS-3 cell lines showed normal karyotype (a: RS-2 cell line, b: iPS-3 cell line).

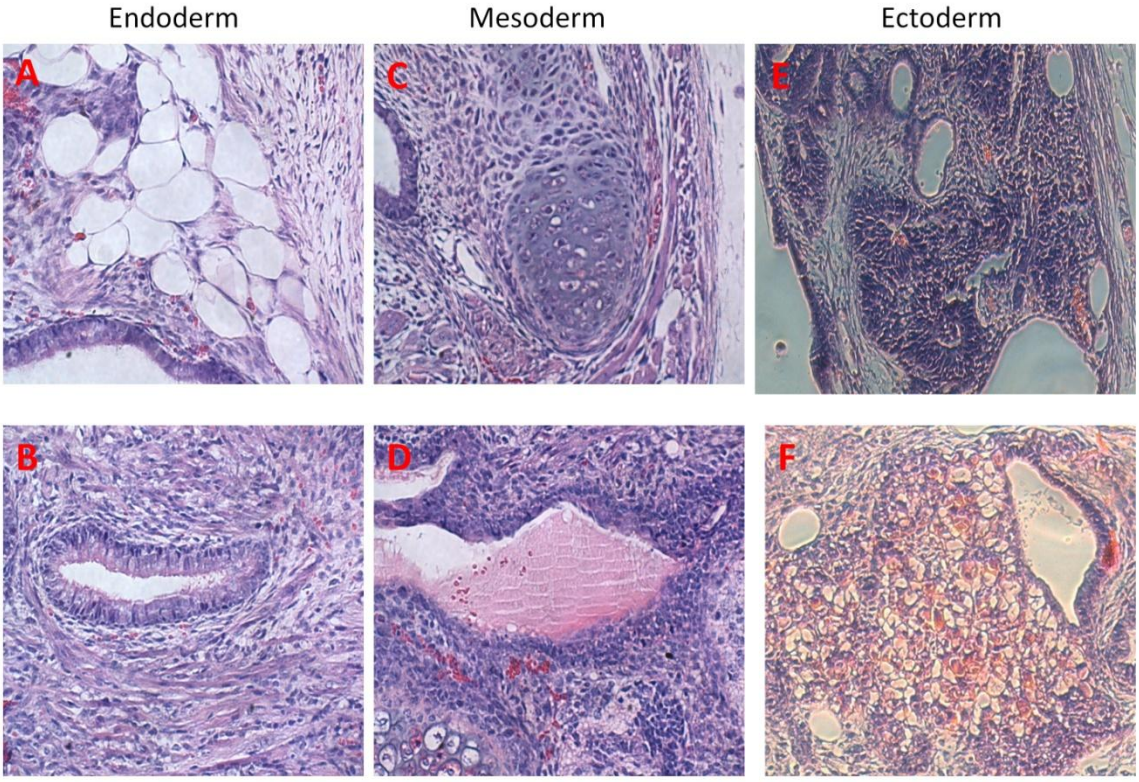


Figure 4.3. *In vivo* characterization of iPS-3 cell line pluripotency. Hematoxylin and eosin staining shows a teratoma from iPS cells (iPS-3 cell line) containing multiple different tissues representative of three germ layers. A: adipose, B: gut epithelium tissue, C: cartilage, D: muscle, E: Neural tissue, F: gland cells.

of mESCs cultured in suspension culture conditions (Cormier, zur Nieden et al. 2006; zur Nieden, Cormier et al. 2007). Doubling times for the RS-2 miPSCs averaged at 21.25 ± 1.86 h, which is slightly longer than 14.5 h reported for mESCs over several passages in suspension bioreactors (zur Nieden, Cormier et al. 2007). The peak density of RS-2 cells after the third passage was a little lower than the first few passages, but growth rates were gradually restored until the miPSCs regained normal peak densities, which were maintained until the end of day 32 (P8). Doubling times for the iPS-3 miPSCs averaged at 16.42 ± 0.52 h, which is more similar to mESCs. The cell density of iPS-3 was more consistent during the expansion period (Fig. 4.5A). On average, the suspension bioreactor for RS-2 line yielded around 24-fold iPSC expansion over each 4 day passage period, ranging from around 15 to over 30 while iPS-3 line expansion was average at 58 per passage, ranging from 48 to 70 (Table 4.1). The cumulative cell fold- expansion of RS-2 and iPS-3 iPSCs in suspension culture through eight passages were 8.8×10^{10} and 1.2×10^{14} respectively (Fig. 4.5B). The cell viability of RS-2 line continued to be greater than 85% during the maintenance experiment except day 13, which was 80%, while the cell viability of iPS-3 was more than 93% throughout the course of maintenance period (Fig. 4.6A). The diameter of aggregates increased after the first inoculation and during subsequent passages reached its maximum size on the final day prior to being passaged. RS-2 iPSCs formed slightly bigger aggregates (Average: 201 μm) than iPS-3 (Average: 181 μm) at the end of 4 day (Fig. 4.6B).

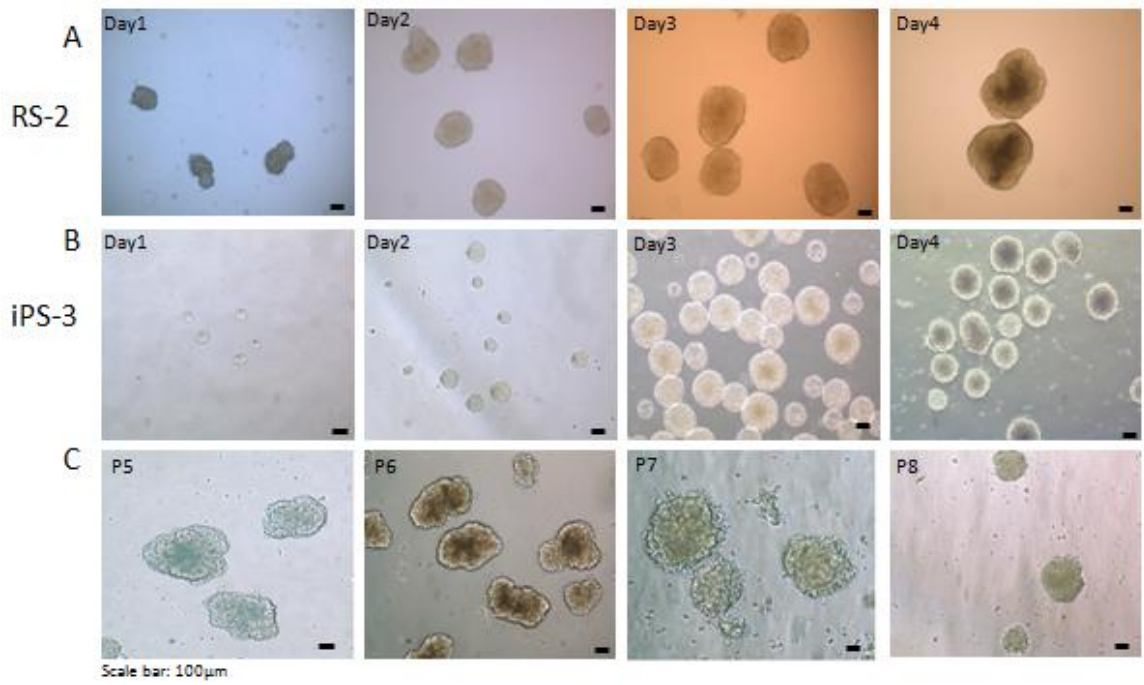


Figure 4.4. Long-term passaging of miPSCs in a SSB. (A) Morphology of RS-2 miPSC aggregates during the first four days culture period. (B) Morphology of iPS-3 miPSC aggregates during the first four days of suspension bioreactor culture. (C) Morphology of the stirred suspension culture aggregates (RS-2) was stable during a 32-day culture period. (Passages 5-8).

	RS-2			iPS-3		
Passage (Day)	**Apparent Growth Rate (d^{-1})**	**Average Doubling Time (h)**	**Passage Cell-fold Expansion (-)**	**Apparent Growth Rate (d^{-1})**	**Average Doubling Time (h)**	**Passage Cell-fold Expansion (-)**
P1 (4)	**0.8536**	**19.49**	**30.4**	**0.9678**	**17.19**	**48.0**
P2 (8)	**0.8486**	**19.6**	**29.8**	**1.0585**	**15.72**	**69.0**
P3 (12)	**0.6868**	**24.22**	**15.6**	**0.9982**	**16.67**	**54.2**
P4 (16)	**0.7112**	**23.39**	**17.2**	**0.9926**	**16.76**	**53.0**
P5 (20)	**0.7382**	**22.53**	**19.2**	**1.0628**	**15.65**	**70.2**
P6 (24)	**0.8457**	**19.67**	**29.5**	**1.0185**	**16.33**	**58.8**
P7 (28)	**0.8081**	**20.59**	**25.3**	**1.0108**	**16.46**	**57.0**
P8 (32)	**0.8086**	**20.57**	**25.4**	**1.0018**	**16.61**	**55.0**

Table 4.1. Cell growth kinetics over 8 serial passages for miPSCs inoculated as single cell suspensions at 5×10^4 cells/mL each passage.

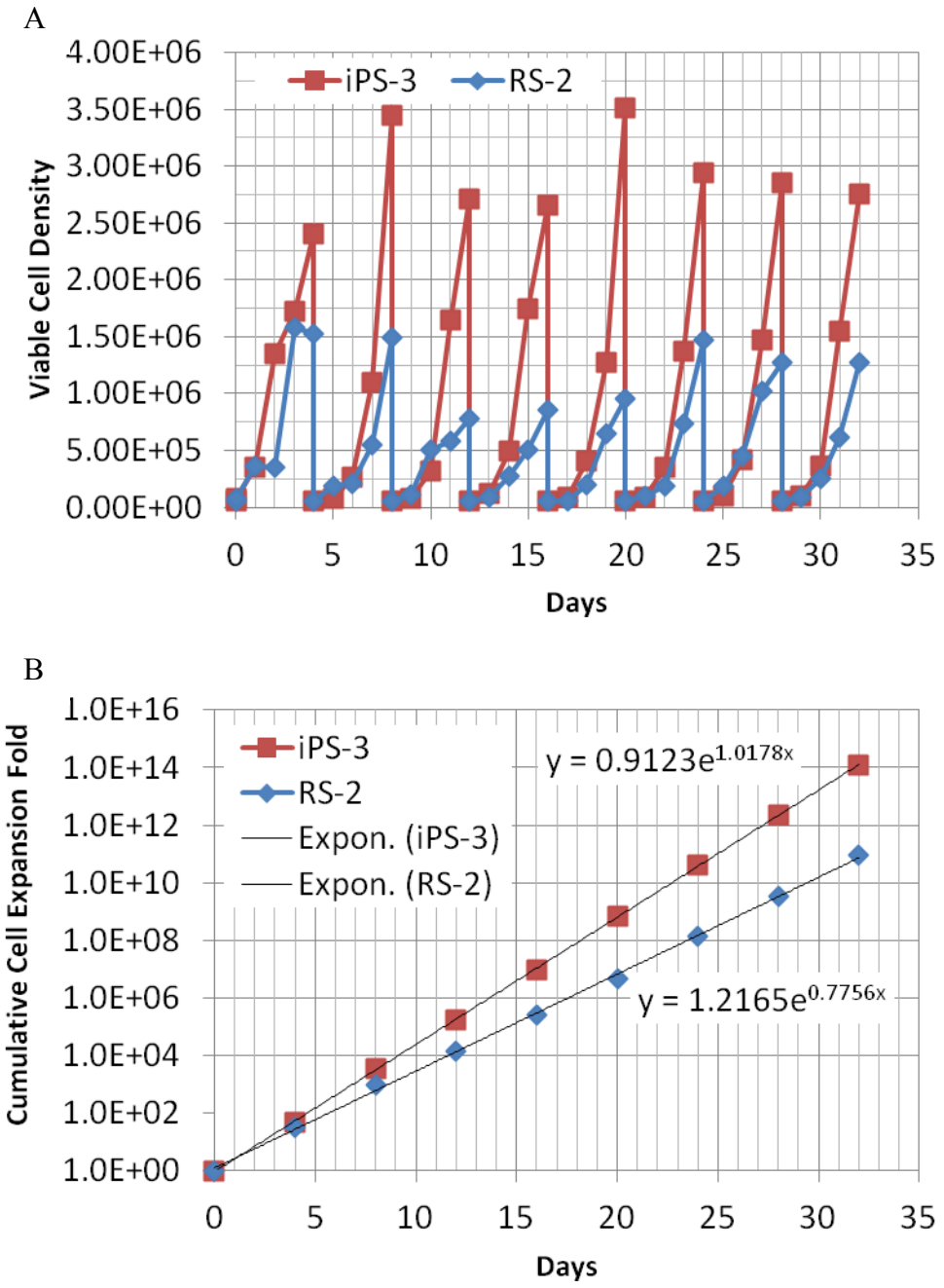


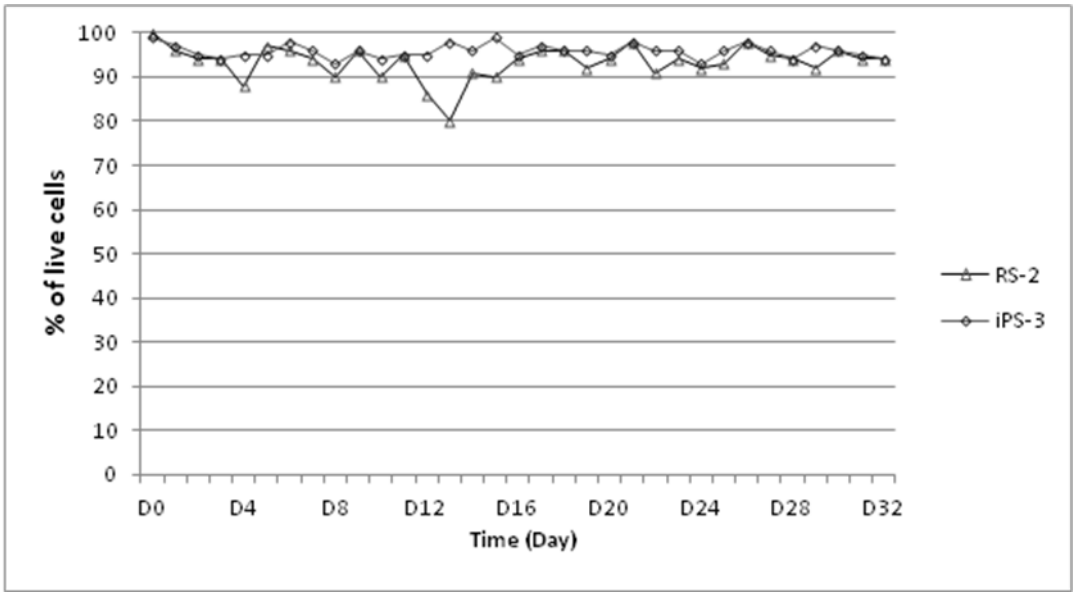
Figure 4.5. Cell density and expansion rate of iPSCs in suspension bioreactors. (A) Live c ell de nsities of mi PSCs during the maintenance pe riod in suspension culture (P1 -P8). C ultures were inoculated at 5×10^6 cells/100ml with agitation rate of 100 rpm. (B) Cumulative c ell e xpansion ove r the c ourse of e ight passages for RS-2 and iPS-3 cell lines.

SSB cultured miPSCs express pluripotency markers

We next assessed the pluripotency of miPSCs cultured in SSBs. Alkaline Phosphatase (ALP) is a recognized marker of pluripotency in miPSCs (Takahashi and Yamanaka 2006; Huangfu, Osafune et al. 2008). We stained SSB miPSCs for ALP prior to each passage. Our results demonstrated that aggregates from both RS-2 and iPS-3 bioreactors maintained their original expression of ALP (Fig. 4.7A, B). The percentage of positive aggregates during suspension culture was over 75% for RS-2 and 89% for iPS-3 at all times (Fig. 4.7C).

In order to confirm the pluripotency of the cells, expression of the three major pluripotency markers was determined by real-time quantitative PCR. miPSCs from day 0 static culture were utilized as a control and compared to passage 4 (day16) and passage 8 (day 32) in the SSB. Q-PCR analysis for RS-2 line showed that there was no major difference between the levels of Oct4 gene expression in miPSCs at the beginning, middle or end of the experiment, whereas the expression of Sox2 was induced gradually from day 0 to day 32. Expression of Nanog was up-regulated on day 16, but showed a down-regulation on day 32. Although the expression of pluripotency genes was down-regulated on day 16 for iPS-3 line, they showed an up-regulation again on day 32. (Fig. 4.8A, B). Immunofluorescence microscopy was also carried out using antibodies directed against various pluripotency markers. At the end of the suspension culture period (P8), SSB miPSCs expressed the major pluripotency markers Rex-1, Oct4 and Nanog (Fig. 4.9A). Collectively, our results suggest that miPSCs maintained their pluripotent state when expanded in SSBs for over one month.

A



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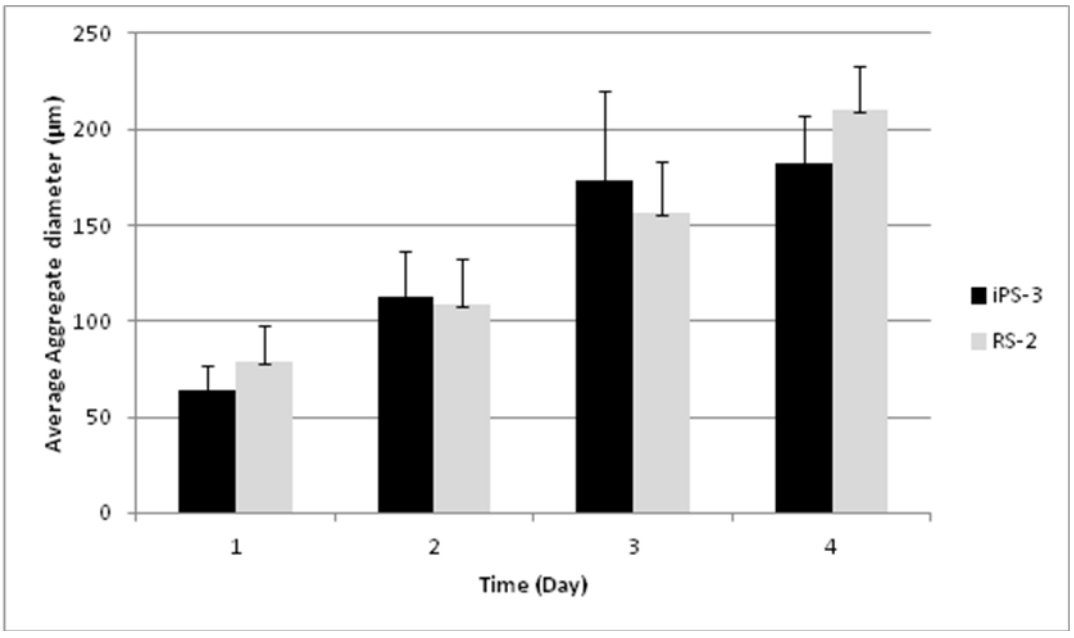


Figure 4.6. Stirred s uspension bioreactors pr ovide a suitable e nvironment for miPSCs maintena nce. (A) Via bility of mi PSCs was very well maint ained in suspension culture system. (B) Values for the average diameter of RS-2 and iPS-3 miPSCs aggregates during different time course of each passage.

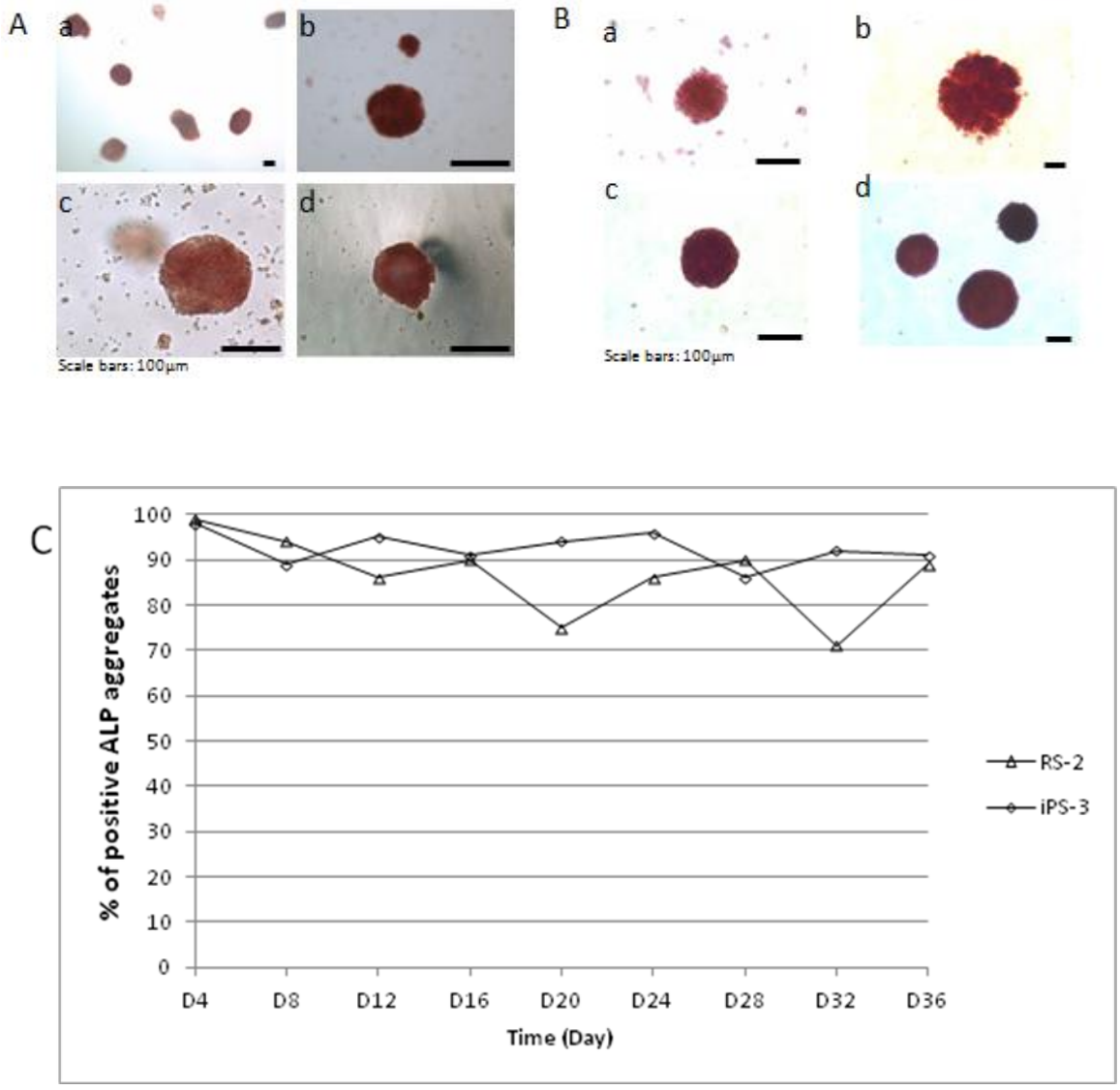


Figure 4.7. Maintenance of RS-2 and iPS-3 lines in SSBs. (A) Alkaline phosphatase (ALP) staining of RS-2 miPSC aggregates during maintenance period (a: Passage1, b: passage 2, c: passage 5 and d: passage 8). (B) ALP staining of iPS-3 aggregates during 32 days of expansion time (a:passage 1, b: passage 2, c : passage 5 and d: passage 8. (C) The percentages of cell aggregates expressing ALP during the time course of suspension culture.

SSB cultured miPSCs are karyotypically normal

In order to monitor the possible effect of culturing miPSCs in suspension bioreactor on the cell chromosomal state, we counted miPSCs chromosomes before starting the experiment, on day 16 (P4) and on day 32 (P8). Cell aggregates were dissociated with trypsin and cultured for 24 hrs in static culture. The percentage of normal karyotype was over 90% during the 32 days of maintenance (Fig. 4.9B). The cells were then harvested and examined for any chromosomal abnormalities. Compared to the starting cell population (Fig. 4.9Ca), miPSCs had a normal chromosomal count on day 16, as well as day 32 (Fig. 4.9Cb).

SSB miPSCs form colonies and embryoid bodies.

In order to assess the pluripotency of d 32 SSB miPSCs, cells were evaluated for their ability to form new colonies in static culture, in addition to their ability to form embryoid bodies. Day 32 SSB miPSCs could form ESC-like ALP positive colonies in static culture, which were morphologically similar to d 0 miPSCs used to initiate the experiment (Fig. 4.9D). Similarly, day 32 SSB miPSCs also retained the ability to form EBs (Fig. 4.10A).

SSB miPSCs retain the ability to differentiate into specific lineages *in vitro*.

In order to confirm that SSB miPSCs were pluripotent and maintained their capacity to differentiate into functional progeny of specific lineages, we carried out *in vitro* differentiation assays to compare day 32 SSB miPSCs with their original starting population. Day 32 suspension aggregates were dissociated with trypsin and subjected to

differentiation protocols to cardiomyocytes and cartilage/bone lineages using hanging drop and micro-mass approaches, respectively. miPSCs were also induced to make embryoid bodies (EBs) in 125 mL suspension bioreactor to evaluate the EB formation capacity of cells in suspension culture (Fig. 4.10A).

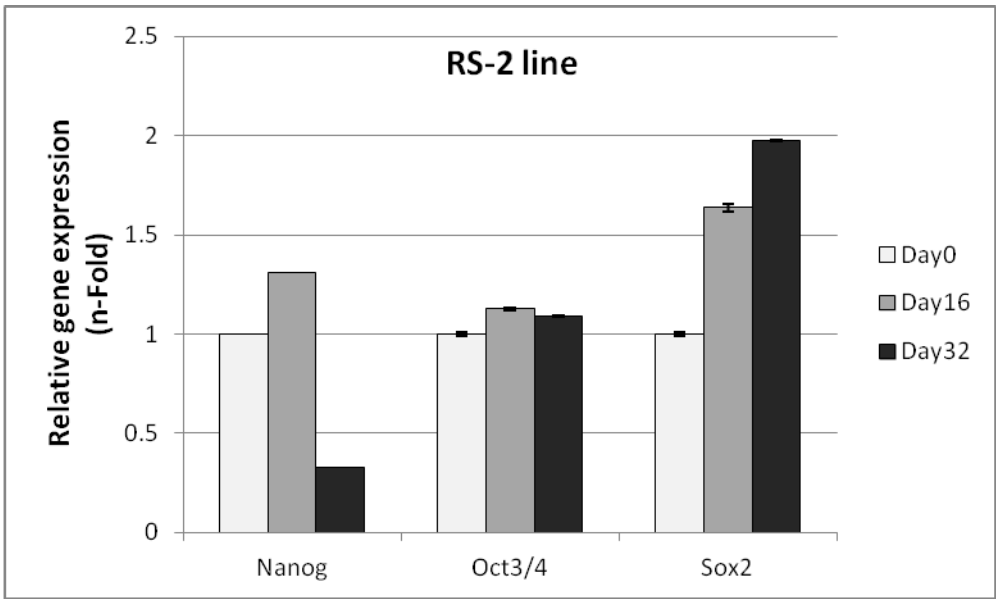
Micromass chondrocyte and osteoblast differentiations were carried out using TGF-β1/BMP-2/AA and AA/β-GP/Dex respectively (Yamashita, Nishikawa et al. 2010; Yamashita, Nishikawa et al. 2010). After 21 days of differentiation culture, Alcian Blue and Alizarin Red S staining, indicative of fully mature chondrocytes and mineralized bone, respectively, demonstrated that miPSCs could differentiate to both lineages. These results were substantiated by immunofluorescence microscopy using the chondrocyte- and osteoblast-specific markers collagen 2 (COL2) and osteocalcin (OC) respectively (Fig. 4.10B).

Cardiomyocyte differentiation was performed on EBs in iPS media (without LIF) using ascorbic acid induction. After 11 days, clusters of contractile cardiac bodies appeared in culture (Fig. 4.10C). Immunofluorescence against α-MHC demonstrated that the cells maintained their capacity to differentiate *in vitro* (Fig. 4.10D). We checked the expression of cardiac markers in both beating and non-beating areas after differentiation. Gene expression profile revealed the expression of cardiomyocyte markers in both areas (Fig. 4.10E). Hence, the miPSCs retained their ability to differentiate into different lineages *in vitro* after extended passaging in suspension bioreactors.

SSB miPSCs differentiate into all three germ layers *in vivo*.

After 32 days of expansion, SSB miPSCs were disaggregated with trypsin whereupon 1×10^6 cells were injected subcutaneously into the inner thigh of a SCID mouse. Teratomas generated by SSB miPSCs were comparable to those generated from the starting population. Teratomas from each population gave rise to differentiated cells representing all three germ lineages (Fig. 4.11). This result confirmed that miPSCs sustained their pluripotency following long-term expansion in the SSB.

A



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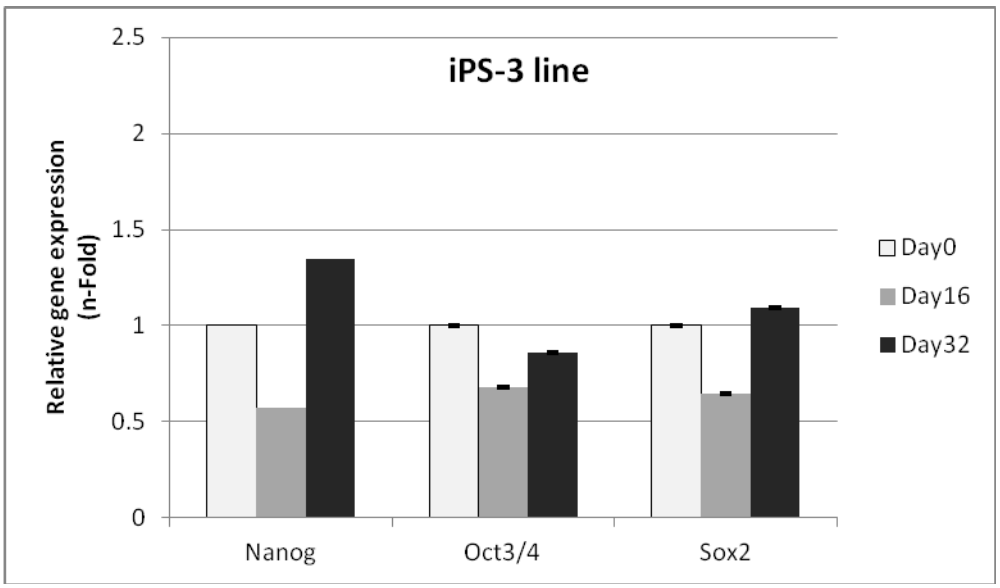

Figure 4.8. Pluripotency markers are preserved in suspension culture aggregates. Quantitative real-time PCR of major pluripotency markers before and during mi PSCs expansion of RS-2 (A) and iPS-3 (B) mouse iPSCs.

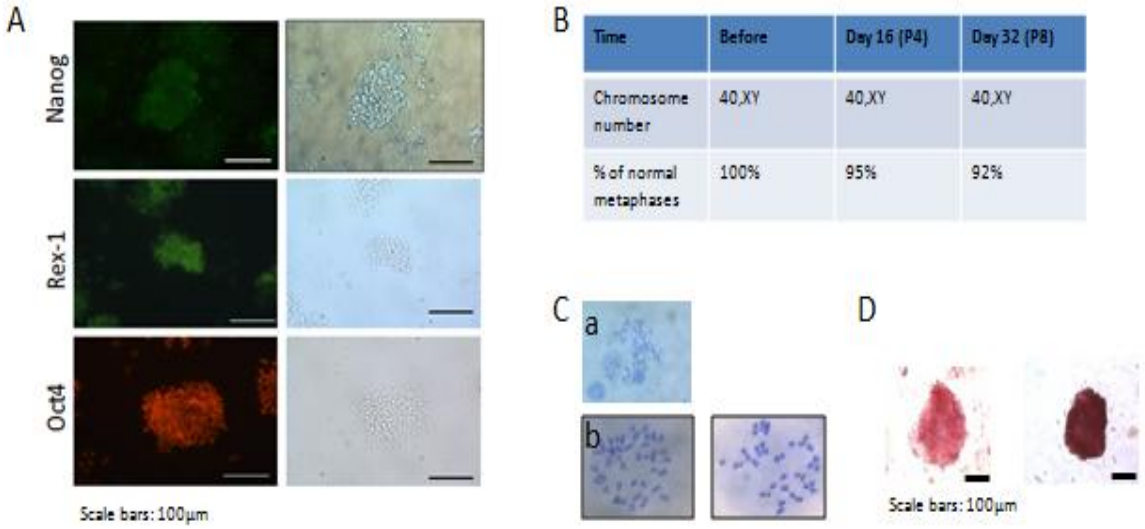

Figure 4.9. iPSCs express pluripotency markers and show normal karyotype at the end of maintenance period. (A) Immunofluorescence staining of aggregates showed the expression of major pluripotency genes in the cell nucleus at the end of expansion period. (B) miPSCs maintained high percentage of normal karyotype during and at the end of maintenance suspension culture similar to the starting cells. (C) Representative of a normal karyotype from each day has been shown. (D) The cells retained their ability to form ESC-like and ALP+ colonies at the end of the maintenance period.

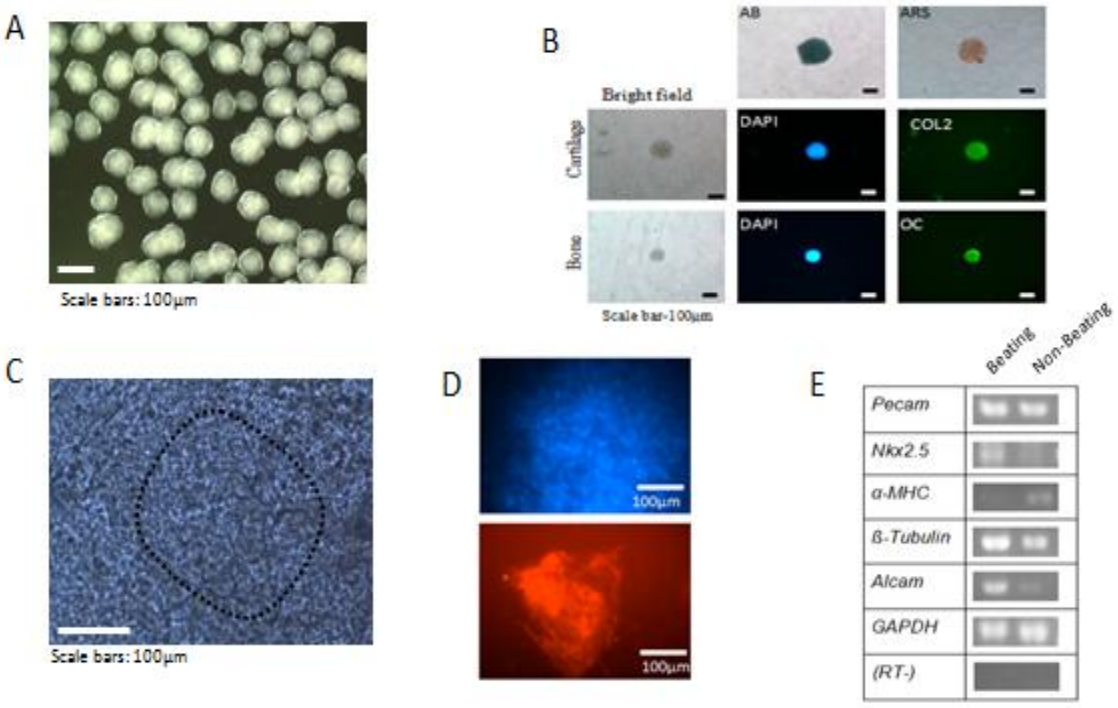

Figure 4.10. Bioreactor-expanded miPSCs retain their ability to differentiate *in vitro*. (A) miPSCs preserve their capacity to form EBs in suspension culture after the removal of LIF. (B) *In vitro* differentiation into cartilage and bone lineages. Differentiation was induced according to established protocols in our lab by Dr. Akihiro Yamashita. Alcian Blue (AB), collagen 2 (COL2), Alizarin Red S (ARS), osteocalcin (OC). (C) Mature cardiomyocytes in the beating area were beating rhythmically for 3 weeks. Dotted line shows the beating area. (D) The beating area stained positive for MF-20 (α-MHC) after 11 days in culture. (E) Differentiated cells in beating and non-beating areas expressed major cardiomyocytes markers. Scale bar: 100μm.

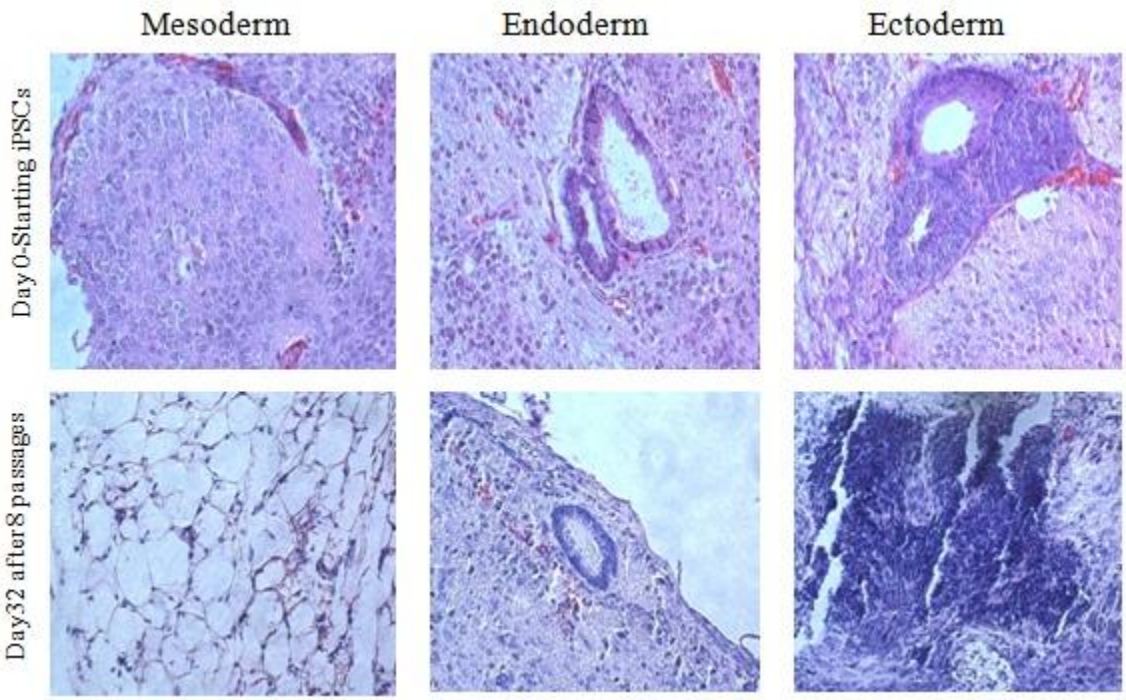

Figure 4.11. Bioreactor-expanded miPSCs retain their ability to differentiate *in vivo*. miPSCs in SSB generate teratomas *in vivo* consisting of three germ layers after the maintenance period similar to their starting cells before expansion.

4.5 Discussion

The similarity between pluripotent miPSCs and mESCs in their global chromatin structure and gene expression profile (Guenther, Frampton et al. 2010), cell morphology, capacity to differentiate into three germ layers, teratoma formation, and tetraploid complementation (Okita, Nakagawa et al. 2008; Boland, Hazen et al. 2009; Kang, Wang et al. 2009; Smith, Luong et al. 2009; Zhao, Li et al. 2009) has suggested that these cells might hold a great potential for future cell therapies in regenerative medicine and also derivation of patient or disease-specific miPSCs (Yamanaka 2007; Saha and Jaenisch 2009). Similar to mESCs, miPSCs have the capacity to self-renew and generate different specific cell types *in vitro* and *in vivo*. iPSCs have enormous promise for the next generation cell therapies, since ethical, technical, and immunological concerns attributed to ESCs are not existing with these cells. One of the essential prerequisites for the clinical application of iPSCs is the large-scale expansion of the cells in a cost-effective and reproducible manner. Despite much progress in this field since the first derivation of iPSCs, all of the studies have used static culture conditions to generate and expand these cells. This might be due to the technical difficulties attributed to derivation and large-scale expansion of iPSCs and/or their related progeny in suspension culture.

Recently, effective suspension bioreactor protocols have been developed for the large-scale production of murine and human ESCs (Fok and Zandstra 2005; Cormier, zur Nieden et al. 2006; zur Nieden, Cormier et al. 2007; Krawetz, Taiani et al. 2010). Such procedures have been also established for neural stem and precursor cells (Kallos and Behie 1999; Kallos, Sen et al. 2003; Gilbertson, Sen et al. 2006), human mesenchymal

progenitor cells (Baksh, Davies et al. 2003) and hematopoetic stem cells (Eridani, Mazza et al. 1998; Kogler, Callejas et al. 1998). Despite these advances in the development of various stem cell expansion protocols, there is still a lack of data regarding the effectiveness of stirred suspension culture for the large-scale maintenance and expansion of undifferentiated pluripotent iPSCs.

In this study, the pluripotency and self-renewal capacity of miPSCs were maintained through long term culture in SSBs. We achieved a 24-fold /58-fold (RS-2 / iPS-3) per passage increase in the viable cell density and expanded the miPSCs by 8.8×10^{10}/ 1.2×10^{14} (RS-2 / iPS-3) in 32 days. Expanding iPSCs as aggregates in suspension bioreactors is a procedure that proposes enormous effectiveness over culturing iPSCs in static tissue culture flasks, which is very arduous and time consuming. Following expansion, miPSCs retained their pluripotency; they expressed pluripotency marker at levels comparable to their starting population, they could be differentiate into bone, cartilage and cardiac lineages *in vitro* as well as to differentiate to three germ layers *in vivo*. Furthermore, our results showed that the suspension bioreactor did not induce any chromosomal abnormalities during long-term maintenance.

Our study presents for the first time, a reproducible and well-controlled environment for large-quantity production of undifferentiated iPSCs. While we employed murine iPSCs as a model system, these protocols will next be translated to expanding human iPSCs in suspension bioreactors. Since the reprogramming of cells to the pluripotent state is a slow and inefficient process especially for human somatic cell reprogramming, the field of iPSCs is moving forward rapidly to establish more efficient and safer methods of reprogramming. Meanwhile, in the next step, it is definitely

important to develop effective and robust methods for human iPSCs expansion to maintain their pluripotent state during long-term culture, which we believe can be facilitated using SSBs. Our expansion and long term maintenance of miPSCs in the SSB has been proven to produce large quantities of karyotypically normal cells that retain the ability to differentiate into all germ cell lineages. However, there remain several objectives for improvement towards the large-scale generation of hiPSCs for therapeutic usage and disease modeling. To achieve the potential benefits of this target, such as patient-specific tissue production and autologous transplantation, issues such as reprogramming quality and efficiency, economic viability, undesired tumorigenicity and decreasing lifetime somatic cell quality must be addressed. Using the mouse model system, we show that if well-maintained in a suspension culture environment, iPSC aggregates can grow exponentially without any major differentiation effects. Accordingly, this process purports great advantages over static tissue culture, since it may fulfill the need for large numbers of iPSCs necessary for regenerative medicine investigations and cell-based therapies in a controlled and cost-effective manner.

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Chapter Five:

Derivation of iPSCs in Stirred Suspension Bioreactors

In the following manuscript, Mehdi Shafa contributed to the experimental design, data collection and analysis (Figures 5.1-5.4, 5.7-5.9, 5.12-5.20), and preparation of the manuscript.

Efficient and expedited derivation of induced pluripotent stem cells in stirred suspension bioreactors

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Keywords: induced pluripotent stem cells; SSB; derivation; pluripotency

5.1 Abstract

Induced pluripotent stem cells (iPSCs) have previously been developed by reprogramming somatic cells following the expression of defined transcription factors (Takahashi and Yamanaka 2006; Yu, Vodyanik et al. 2007; Hanna, Markoulaki et al. 2008; Stadtfeld, Brennand et al. 2008). In order to be clinically feasible for cytotherapeutic application, it will be necessary to generate relevant cell numbers through an economically viable bioprocess. Although routinely used, the derivation of iPSCs in adherent culture can only support pre-clinical research projects. Here, we report that iPSCs can be generated more quickly and efficiently within stirred suspension bioreactors (SSBs). By applying this approach to mouse embryonic fibroblasts, millions of fully reprogrammed iPSCs can be derived in 10 days, making it 100- to 1000-fold more efficient than conventional adherent culture methods. The resulting suspension-derived iPSCs (SiPSCs) resemble embryonic stem cells in their *in vitro* and *in vivo* characteristics, including gene expression and differentiation potential. SiPSCs also display a normal karyotype, form teratomas, produce viable chimeras, and display germ line transmission competency. We propose that liquid shear stress may play an important mechanistic role in bioreactor induced pluripotency. Our results suggest that the suspension culture environment provides a selective advantage for enhancing iPSC generation. Combined with new methods of reprogramming that do not use integrating genetic constructs, SiPSC technology has the potential to accelerate and standardize iPSC research, bringing it to clinical application more quickly.

5.2 Introduction

The clinical use of stem cells depends on the availability of pluripotent cells that are not limited by ethical, immunological, technical, and other considerations. In 2006, a milestone was achieved by artificially creating pluripotent stem cells called "induced pluripotent stem cells (iPSCs)" using the mouse model system (Takahashi and Yamanaka 2006). Later, these embryonic stem cell (ESC)-like cells were generated from adult human somatic cells following viral transduction of four defined pluripotency transcription factor genes. Yamanaka's group used the factors: Oct4, Sox2, Klf4, and c-Myc, while Thomson's group substituted Nanog and Lin28 in place of Klf4 and c-Myc (Takahashi, Tanabe et al. 2007; Yu, Vodyanik et al. 2007). These studies demonstrated that iPSCs resemble ESCs morphologically; they express similar cell surface and pluripotency markers, have a normal karyotype, express telomerase and demonstrate multi-lineage differentiation potential in both embryoid bodies and teratomas. Since 2007, the field has expanded to generate iPSCs using vastly different approaches. Several groups have reported iPSC derivation using other means of transduction: adenoviruses (Stadtfeld, Nagaya et al. 2008), non-integrating plasmids (Okita, Nakagawa et al. 2008), lentiviruses (Soldner, Hockemeyer et al. 2009), plasmids (Kaji, Norrby et al. 2009), transposons (Woltjen, Michael et al. 2009), recombinant proteins (Zhou, Wu et al. 2009), mRNA (Yakubov, Rechavi et al. 2010) and microRNA (Anokye-Danso, Trivedi et al. 2011). Others are exploring the use of small molecules, which modulate reprogramming epigenetically (Shafa, Krawetz et al. 2010). Regardless, there remain several obstacles preventing the application of iPSCs for cytotherapeutics and disease modeling.

Currently, there is a "bioprocess gap" between the promise of iPSCs and their inadequacy, which necessitates an appropriate plan for large-scale production protocols. iPSC generation is an inefficient process, which requires several weeks before a cell line is established. Conventional reprogramming approaches employ adherent culture, which has several disadvantages, such as time-consuming feeding and passaging, and culture-to-culture variability due to non-homogenous culture conditions (Takahashi and Yamanaka 2006; Takahashi, Tanabe et al. 2007; Yu, Vodyanik et al. 2007; Woltjen, Michael et al. 2009). Importantly, the limited number of derived cells is unable to support potential clinical applications.

We have previously demonstrated that SSBs (SSBs) are a promising and scalable environment for the regulated expansion of ESCs and iPSCs (Cormier, zur Nieden et al. 2006; zur Nieden, Cormier et al. 2007; Krawetz, Taiani et al. 2010; Shafa, Sjonnesen et al. 2011) as aggregates. SSBs offer several benefits over the traditional adherent culture, including decreased cost/cell and providing a more homogenous culture environment, making them more suitable for clinical applications. Using lab scale 100 ml SSBs, billions of cells are expanded as ~100 micron, 100 cell aggregates in one week. Stirring plays an important role in maintaining aggregate size, allowing suitable mass transfer while hiving off new aggregates. Stirring also appears to play a role in regulating pluripotency and suppressing differentiation through fluid shear stress (Taiani, Krawetz et al. 2010). This study examined whether SSBs provide a selective advantage to enhance iPSC reprogramming through application of shear stress. These results support the possibility of iPS derivation through chemical approaches, which would accelerate the therapeutic application of pluripotent cells.

5.3 Materials and Methods

Preparation of reprogramming retroviral vectors

Platinum-E retroviral packaging cells (Cell Biolabs, San Diego, CA) were prepared for plasmid transfections by seeding 8×10^6 cells per 100 mm dish (One dish for each reprogramming gene). Plat-E cells were maintained in FP (Fibroblast-Platinum) medium, which consisted of DMEM containing 10% FBS and 50 U of penicillin/streptomycin. The next day, each pMXs retroviral plasmid DNA (Oct4, Sox2, Klf4 and c-Myc; Addgene plasmids 13366, 13367, 13370 and 13375 respectively, Cambridge, MA) (Kitamura, Koshino et al. 2003) were introduced into Plat-E cells using Fugene 6 transfection reagent (Roche, 815075) according to the manufacturer's recommendations. Briefly, 27 µl of Fugene 6 transfection reagent was added to 300µl of Opti-MEM in a 1.5 ml tube. In the next step, 9 µg of each retroviral vector was added into the prepared Fugene/OpiMEM tube drop by drop and incubated for 15 min. Each vector/Fugene 6 complex was added drop wise into the Plat-E dishes and incubated overnight at 37°C, 5% CO_2. The following day, medium was changed and replaced with 10 ml fresh FP medium. Forty eight hours post-transduction virus-containing medium from each transfection was collected, filtered through 0.45µm Acrodisc filter (Pall life Sciences) and supplemented with 4 µg/ml Polybrene.

Generation of SiPSCs

Briefly, for derivation of SiPSCs, mouse embryo fibroblasts (MEFs) were seeded at 8×10^5 cells/100 mm dish. Equal parts of retroviral conditioned media supplemented with polybrene were mixed together and added to MEFs in dishes. Cells

were incubated overnight at 37°C in 5% CO_2. The following day, supernatant was removed and replaced with fresh FP medium. Two days after infection, MEFs were trypsinized and transferred to 100 ml SSB with mESC/iPS medium supplemented with LIF (see below). Resulting ESC-like aggregates were selected and characterized over a 4 week period according to standard procedures (see below). This bioreactor reprogramming method was repeated successfully a total of 9 times using MEFs from different strains: six times using 4 factors on CD1 (once), C57BL/6 (twice), and 129/Sv (thrice) and three times using 3 factors (less c-Myc) on 129/Sv.

Adherent and Stirred Suspension Culture

SiPSCs and ESCs were cultured in 100 ml SSBs (NTS Technologies, Vineland, NJ) in high glucose DMEM (Invitrogen) supplemented with 0.1 mM non-essential amino acids, 50 U/ml penicillin, 50 U/ml streptomycin, 0.1 mM β-mercaptoethanol (Gibco), 1000 U/ml LIF (ESGRO, Chemicon) and 15% FBS (Invitrogen). After 10 days, cells were sub-cultured in suspension bioreactors every four days to prevent their differentiation. Briefly, SiPSCs were inoculated in the bioreactor with 100 ml miPSC medium at a cell density of 5×10^4 cells/ml miPSC medium. Every four days, the miPSC aggregates were dissociated and passaged using 0.25% Trypsin/EDTA and mechanical dissociation. Dissociated cells were reintroduced to the bioreactor at the same concentration (5×10^6 cells/100 ml) as a single-cell suspension and agitated at 100 rpm.

For adherent culture, SiPSCs were trypsinized and grown as a monolayer in the pluripotent state on gelatin-coated tissue culture dishes with inactivated MEF feeder cells in miPSC/ES medium (high glucose DMEM (Invitrogen) supplemented with 0.1 mM

non-essential amino acids, 50 U/ml penicillin, 50 U/ml streptomycin, 0.1 mM β-mercaptoethanol (Gibco), 1000 U/ml LIF (ESGRO, Chemicon) and 15% FBS (Invitrogen). Cells were sub-cultured every second day on inactivated-MEFs feeder cells to prevent their differentiation.

Growth rate

Samples (2.0 ml) were taken from the bioreactors every other day from day 8 to 14 at specific times following inoculation. While SiPSC aggregates were dissociated with 0.25% trypsin/ EDTA, total cell numbers and viabilities were calculated using a hemocytometer combined with Trypan blue staining.

Alkaline Phosphatase Assay

SiPSC aggregates were stained for alkaline phosphatase to monitor the pluripotency state of each aggregate. Briefly, at different times during reprogramming, aggregates were taken from bioreactor and transferred to 15 ml tube. After aspirating excess medium, aggregates were fixed with 4% paraformaldehyde (PFA) in PBS for 2-3 min. The fixative was then aspirated and rinsed with PBS. The staining reagent was prepared according to manufacturer's protocol (Millipore Corporation). The aggregates were stained for 15 min at room temperature. The percentage of positive red aggregates were recorded by counting fifty separate aggregates.

Chromosome counts

After dissociating of SiPSC aggregates on day 14 in the bioreactor, cells were cultured overnight in adherent culture conditions with gelatin and without feeder cells. The following day, cells were incubated with 0.2 µg/ml colcemid in media at 37°C for 1 hour, trypsinized, resuspended, and incubated in 2-3 ml of aqueous 0.068 M KCl for 15 min at 37°C. They were then fixed with fresh 3:1 methanol:glacial acetic acid, followed by three rinses with fixative solution. Single cells were dropped on the precleaned slides to spread chromosomes. The dried slides were stained with Giemsa solution. A minimum number of forty separate metaphase spreads were examined to evaluate cell ploidy.

Immunofluorescence microscopy

Aliquots of SiPSC aggregates, taken from the bioreactor were washed with PBS, and fixed overnight in 4% paraformaldehyde PBS at 4°C. Aggregates were then washed three times with PBS, permeabilized in 0.5% saponin in PBS at 4°C overnight, rinsed three times with PBS, then blocked in 3% BSA in PBS at 4°C overnight. The primary antibodies were diluted 1:50 in 3% BSA and added to the aggregates (overnight at 4°C). Approximately 20-25 aggregates were incubated in 50 µl of antibody solution. The aggregates were then washed 3 times with PBS and blocked again overnight at 4°C. Following the blocking step, the aggregates were incubated overnight at 4°C with Alexa-fluor 488 secondary antibody and TOTO-3 (Molecular Probes). After incubation, the aggregates were washed 3 times with PBS and mounted on slides with mountant (9:1 glycerol:PBS). Spacers (0.25mm) were attached to slides before mounting to avoid aggregate deformation. Slides were analyzed by confocal microscopy (Zeiss 510) using

488, 568 and 633nm filters. LSM image browsing software was used to process the images.

For immunofluorescence staining of SiPSC colonies on adherent culture, cells were washed twice with PBS and then fixed with 4% paraformaldehyde/PBS for 15 min at room temperature. Cells were then washed twice with PBS (10 min each) and permeabilized with 0.1% Triton X-100 (Sigma, T8532) in PBS for 10 min at room temperature. After washing twice with PBS, the cells were treated with blocking solution (3% BSA) for one hour. The primary antibodies (Santa Cruz Biotechnology, CA) including Oct4 and Nanog were diluted to working concentrations in blocking solution (1:200) and incubated overnight at 4°C. The next day, cells were washed three times with PBS and incubated with secondary antibody (1:500) overnight at 4°C. The cells were washed with PBS three times and examined under a fluorescence microscope.

RT-PCR

RNA was isolated from SiPSCs using RNeasy Mini Kit (Qiagen, 74106) according to the manufacturer's instructions. RNA concentrations were determined by spectrophotometer (BioPhotometer, Eppendorf). Reverse transcription polymerase chain reaction (RT-PCR) was carried out to evaluate expression of the pluripotency transcripts. Total RNA (1 µg) was transcribed into cDNA using oligo dT primer and SuperScript III cDNA synthesis kit (18080-51, Invitrogen). PCR amplification was performed in a final volume of 20 µl using Taq DNA polymerase (Invitrogen) consisting of the following steps: 94°C for 3 min, 94°C for 30 sec, 55°C for 45 sec and 72°C for 1 min. Primer sets used in the amplification reactions were designed and blasted (NCBI) for mouse

specificity. GAPDH was used as internal standard. PCR products were separated on 1.5% agarose gels, stained with ethidium bromide, visualized and photographed on a UV trans-illuminator.

In vitro differentiation

For chondrogenic and osteogenic differentiation, we used a micro-mass method as described previously (Yamashita, Krawetz et al. 2009). Briefly, miPSCs were dissociated using 0.25% trypsin/EDTA and seeded in high-density micromass spots (1.0 $\times 10^5$ cells per 10 µl spot) for 2 hrs. Medium was added to each dish without dissociating the spots. For chondrogenic differentiation, we used differentiation media containing DMEM (Gibco), 1% non-essential amino acids (Invitrogen), 50 U/ml penicillin and 50 µg/ml streptomycin (Invitrogen), 0.1 mM 2-mercaptoethanol (Invitrogen), 1% ITS (Invitrogen), 1% FBS (Gibco), 10 ng/ml TGF-β1 (PeproTech), 10 ng/ml BMP-2 (PeproTech), and 50 µg/ml ascorbic acid (Sigma). Medium was changed every 2 days. At day 5, differentiated miPSCs formed aggregates within the micro-mass. These aggregates were transferred to adherent suspension culture in 6 cm petri dishes containing chondrogenic differentiation media 1% insulin, transferrin and selenium (ITS), 1% FBS, 10 ng/ml BMP-2 and 50 µg/ml ascorbic acid (Yamashita, Nishikawa et al. 2010). Medium was changed every 3-5 days. Following 3 weeks differentiation, these aggregates were stained with Alcian Blue or collagen 2 immunofluorescence.

For osteogenic differentiation, we used differentiation media containing DMEM (Gibco), 1% non-essential amino acids (Invitrogen), 50 U/ml penicillin and 50 µg/ml streptomycin (Invitrogen), 0.1 mM 2-mercaptoethanol (Invitrogen), 15% knock-out

serum replacement (KSR), 50 µg/ml ascorbic acid, 10 mM β-glycerophosphate (β-GP) (Sigma) and 100 nM dexamethasone (Dex) (Sigma). After 5 days, the aggregates were transferred to adherent suspension culture. Medium was changed every 3-5 days. Following 3 weeks differentiation, aggregates were stained with Alizarin Red S or osteocalcin immunofluorescence.

Flow cytometry

4F-SiPSCs on day 10 and 16 were harvested from the bioreactor and investigated for expression of pluripotency markers (Oct4, Nanog, SSEA1 and Rex1) by flow cytometry. Accutase (eBioscience) treatment for 15 min followed by pipetting was used to dissociate EBs to single cell. Dispersed cells were washed one time with PBS. The pellet was resuspended in 1 ml of 4% paraformaldehyde in PBS then washed 3×5 min with PBS. The cells were permeabilized in 2 ml of 0.5% saponin for 15 min at room temperature. They were washed once with PBS for 5 min and resuspended in 3%BSA/PBS for 30 min at 37°C. The primary antibody (approximately 1 µg per 1 million cells) was conjugated with 5 µl of Comp.A (Invitrogen) and incubated 5 min at room temperature. Then 5 µl of Comp B was added and incubated 5min. The conjugated primary antibody was then added to cell suspension and incubated for 60 min at 37°C. The cells were washed 3 times with PBS and resuspended in 200 µl FACs tubes. All FACs experiments were performed using a BD FACSVantage SE™ System at the University of Calgary Flow Cytometry Facility.

Teratoma formation assay

Severe combined immune deficient (SCID) mice were purchased from Charles River Company and housed in the animal facility of the Faculty of Medicine, University of Calgary. Animal protocols were performed as approved by the Animal Care Committee of the University of Calgary. Cells were harvested from bioreactor at day 14 of reprogramming using Trypsin/EDTA. About 1.0×10^6 cells in a total volume of 100 µl PBS were injected subcutaneously in the inner thigh of SCID mice. After 28 days, animals were dissected and tissues were examined by histology. Briefly, 4% paraformaldehyde was used to fix the tissue overnight at 4°C. After dehydration, the tissue was embedded in paraffin. Sections were stained with hematoxylin and eosin (H&E) and examined for different types of tissue by light microscope. All of the cell injections were performed by Dr. Rancourt's lab technician, Mrs Shiying Liu.

Chimera formation and germline transmission assays

Media of the bioreactors were changed early in the morning and fresh media were added 1-2 hrs before microinjection. SiPSCs aggregates were trypsinized for 10 min followed by mechanical dissociation to achieve single cell suspension. Fifteen to twenty 3F-SiPSCs were microinjected into 130 E3.5 C57BL/6 mouse embryos or blastocysts. The injected blastocysts were transferred into the uteri of 9 pseudopregnant CD1 recipient females. 36 chimeric (mosaic) mice consisting both of cells originating from the blastocyst and from SiPSCs were born approximately 17 days after transfer. Of those, one litter of 7 pups was cannibalized and one pup that recovered via C-section also died. Working with 28 pups, we had 17 chimeras with different percentages of chimerism.

Germline transmission was detected by the appearance of non-black coat colors after backcrossing to C57BL/6 mice.

5.4 Results

Stirred suspension bioreactor enhances generation of Four-factor iPSCs (4F-iPSCs)

This study examined whether SSBs provide a selective advantage to enhance iPSC reprogramming through application of shear stress. Initially, 129sv mouse embryonic fibroblasts (MEFs) were transduced with Oct4, Sox2, Klf4 and c-Myc expressing retroviral vectors and transferred to a 100 ml, 100 rpm SSB two days post transduction. ESC-like aggregates appeared on day 3 post SSB inoculation (Figure 5.1a), and started to express ALP from day 5 onward (Figure 5.1b-d). By day 12, we obtained 50 million viable cells in SSBs, while adherent culture only gave rise to about 4 million cells within the same period (Figure 5.2A). Flow cytometry revealed that approximately 30% of SiPSCs expressed pluripotency markers on day 10. This cell population increased to about 90% on day 16 for most of the markers tested (Figure 5.2B).

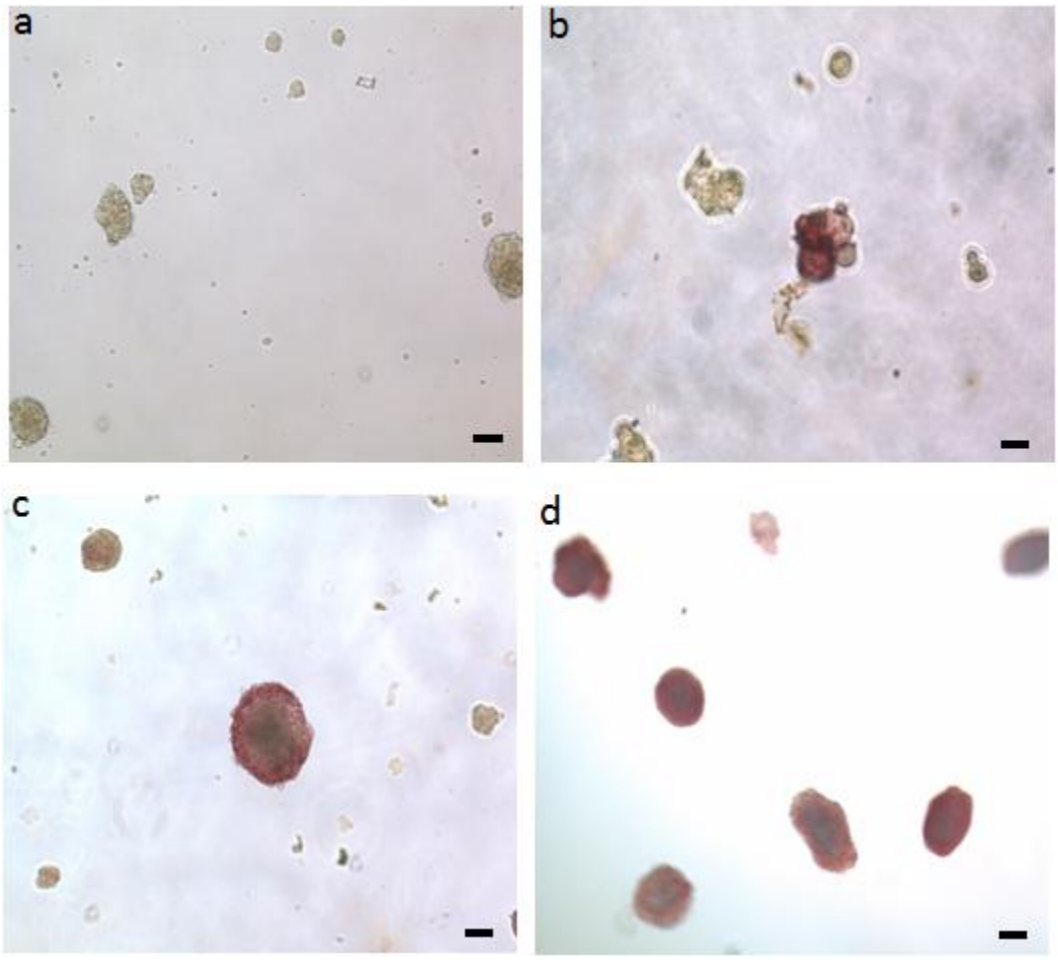


Figure 5.1. Four factor bioreactor-derived iPSCs. A) ESC-like aggregates post inoculation in the SSB (SSB): a) day 5 post transfection. Alkaline phosphatase staining of emerging iPSC aggregates in the SSB: b) day 5, c) day 7, d) day 10 post transfection. Scale bar: 100 μm.

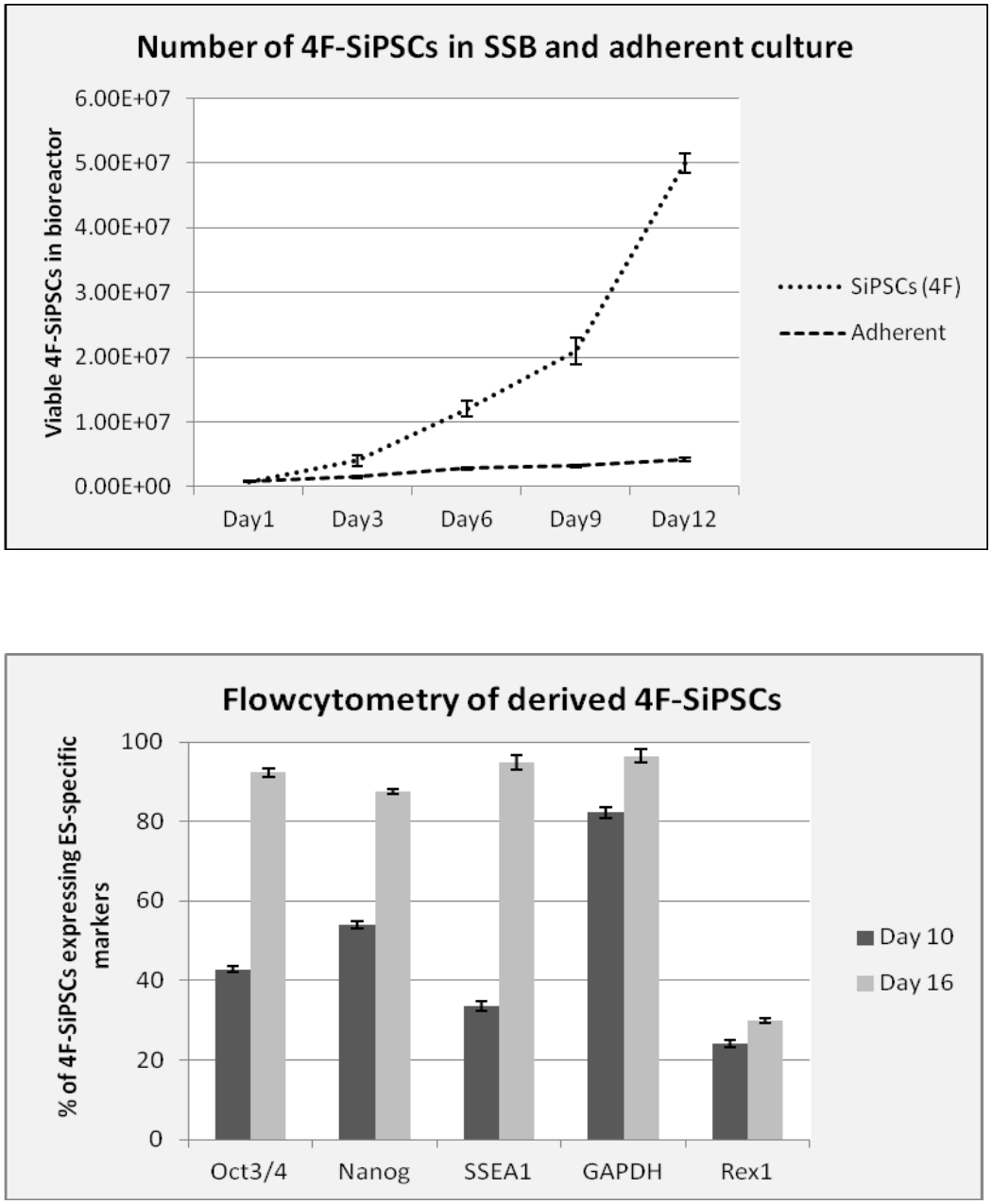


Figure 5.2. Growth rate and pluripotency marker expression of suspension-derived iPSCs. A) Expansion of 4F-SiPSCs in SSB and adherent culture during 12 days post inoculation. In the SSB, cell numbers increased exponentially to 5.0×10^7 on day 12 whereas adherent culture only gave rise to less than 4.0×10^6 during the same period. B) Expression of major pluripotency markers on day 10 and 16 post transfection as shown by FACS. Approximately 30% of cells expressed pluripotency markers on day 10 and this cell population increased to about 90% on day 16 post transfection for most of the markers tested.

4F-SiPSCs are karyotypically normal and express pluripotency genes

Derived aggregates were further characterized on day 10 and displayed the characteristics of ESCs. RT-PCR showed that the suspension-derive induced pluripotent stem cells (SiPSCs) express important markers of pluripotency including Oct4, Sox2, Klf4, Rex1, c-myc, Nanog, etc. The primers for Oct4, Klf4, Sox2 and c-myc have been design to detect only the endogenous transcript. Two separate established lines were characterized (Figure 5.3). Confocal microscopy further confirmed the expression of pluripotency markers Oct4 and Nanog in a subpopulation of cells on day 10 (Figure 5.4A). In order to monitor the possible effect of suspension bioreactor on the cell chromosomal state during iPSC derivation, we karyotyped 4F-SiPSCs chromosomes on day 10 (P0) and on day 33 (P6). Cell aggregates were dissociated with trypsin and cultured for 24h in static culture. The cells were then harvested and examined for any chromosomal abnormalities. The percentage of normal karyotype was over 92% (Figure 5.4B and Table 5.1).

4F-SiPSCs show differentiation capacity *in vitro* and *in vivo.*

In order to confirm that 4F-SiPSCs were pluripotent and maintained their capacity to differentiate into functional progeny of specific lineages, we carried out *in vitro* and *in vivo* differentiation assays. Day 10 suspension aggregates were dissociated with trypsin and subjected to differentiation protocols to three germ layers and also specifically cartilage and bone lineages using suspension culture EB formation and micro-mass approaches, respectively. When 4F-SiPSC aggregates were dissociated into single cells on day 10 post transfection and cultured on agar-coated dishes for 8 days,

they generated cystic embryoid bodies (EBs) (Figure 5.5a). After transferring to gelatin-coated plates for spontaneous differentiation, immunofluorescence revealed cells from all three different germ layers (Figure 5.5b-d).

Chondrocyte and osteoblast differentiations were carried out for 21 days using TGF-β1/BMP-2/AA and AA/β-GP/Dex respectively (Yamashita, Nishikawa et al. 2010; Yamashita, Nishikawa et al. 2010). Alcian Blue and Alizarin Red S staining, indicative of fully mature chondrocytes and mineralized bone, demonstrated that 4F-SiPSCs could differentiate to both lineages. These results were substantiated by immunofluorescence microscopy using the chondrocyte- and osteoblast-specific markers collagen 2 (COL2) and osteocalcin (OC) respectively (Fig. 5.6A,B). In order to examine the pluripotency of derived 4F-SiPSCs, one million cells were implanted subcutaneously into SCID mice. Teratomas formed from 4F-SiPSCs after 28 days contained tissues representative of all three germ layers (Figure 5.7).

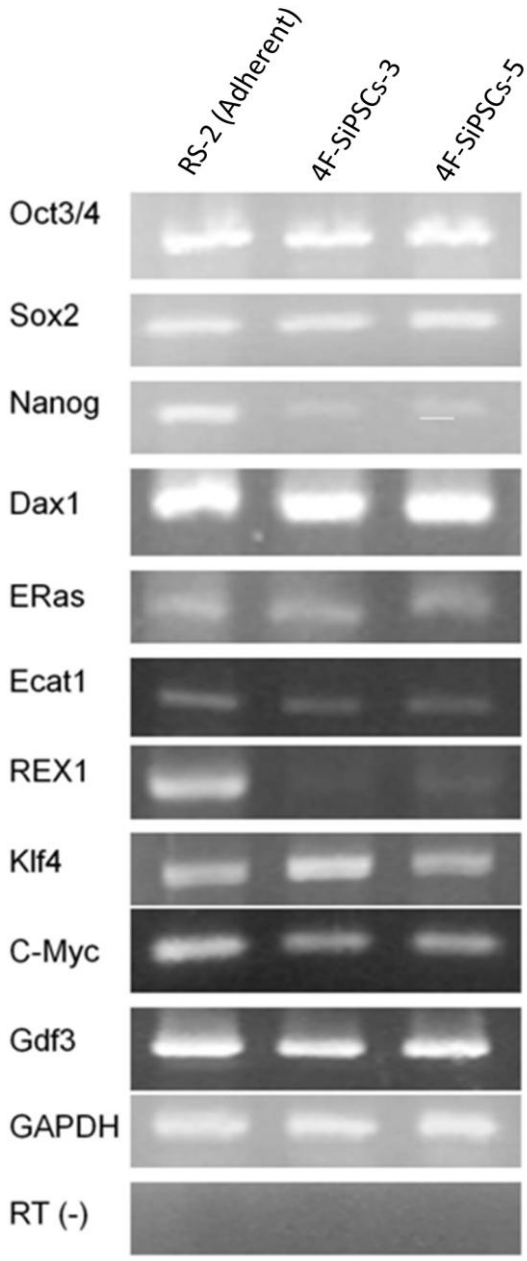


Figure 5.3. 4F-SiPSCs express multiple pluripotency specific markers. Expression of ESC specific markers on day 10 in 4F-SiPSCs as shown by RT-PCR. Two derived lines, SiPSCs-3 and SiPSCs-5 compared to a mouse iPSC line (RS-2), which was previously established in our lab using adherent culture conditions. Primers specific to transcripts from endogenous genes were used. GAPDH was used as internal control. P1=passage 1, P3=passage 3.

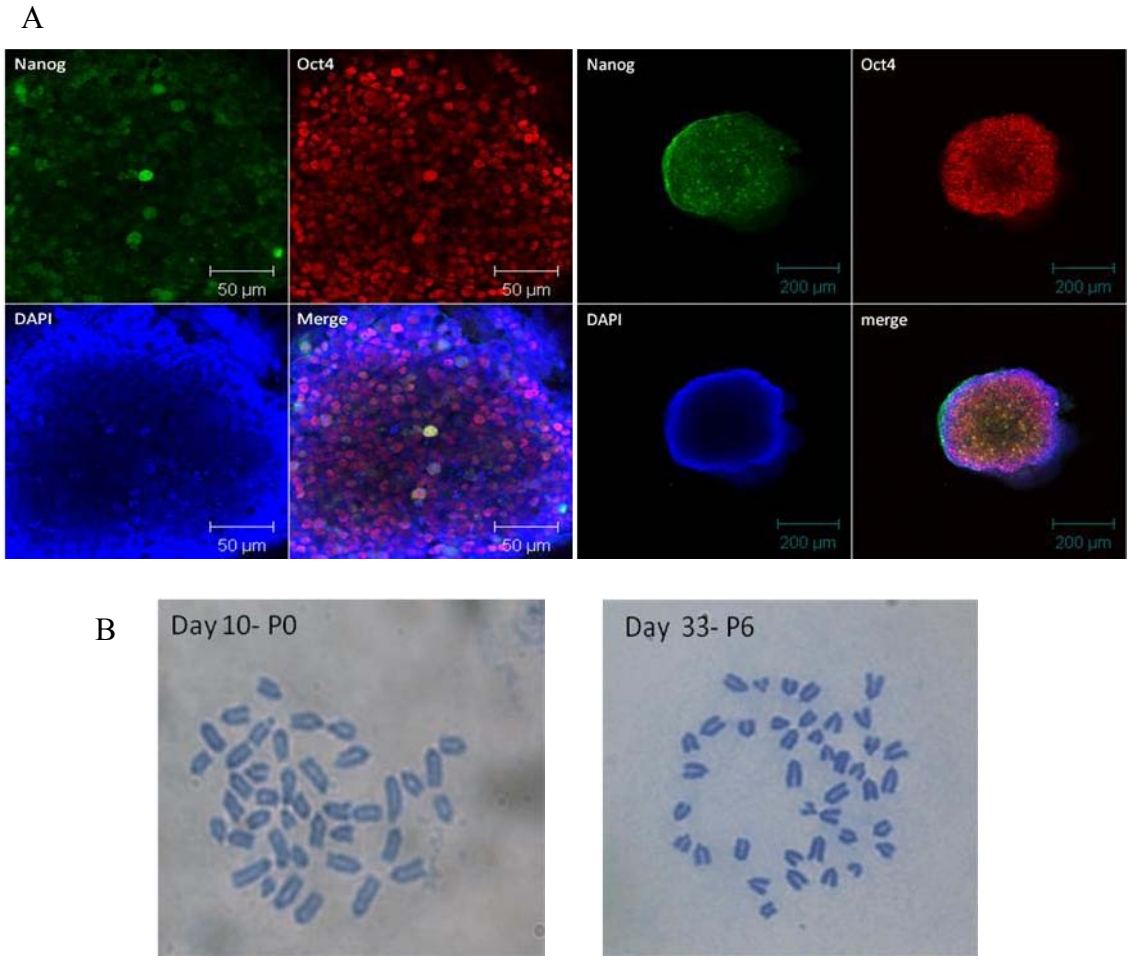


Figure 5.4. 4F-SiPSCs express specific pluripotency markers and showed normal karyotype. A) Confocal microscopy confirmed the expression of Oct4 and Nanog in a sub-population of cells within aggregates, 10 days post transfection in the SSB. B) 4F-SiPSCs maintained high percentage of normal karyotype on day passage 0 (10) and after 6 passages (day33) in bioreactor. Representative of a normal karyotype from each day has been shown.

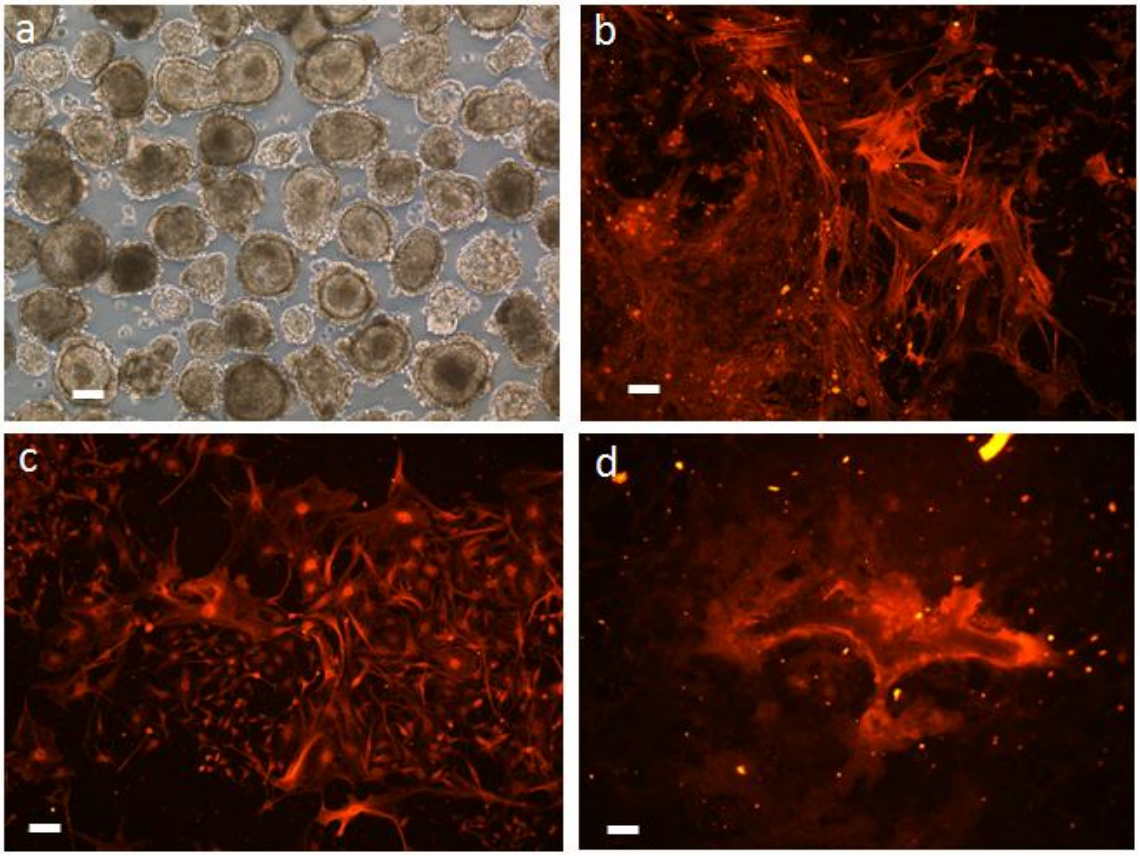


Figure 5.5. Spontaneous *in vitro* differentiation of 4F-SiPSCs to cells from three germ layers. Immunostaining of the differentiated 4F-SiPSCs reveals cells from all three different germ layers. After EB formation, cells were transferred to gelatin-coated plates for spontaneous differentiation. Differentiated cells were then stained with antibodies specific for three germ layers: a) *In vitro* differentiation into cystic embryoid bodies (EBs). b) α-actin (mesoderm), c) β-Tubulin III (ectoderm) and d) AFP (endoderm). Scale bar: 100 µm. Thanks to Dr. Guoliang Meng for performing spontaneous *in vitro* differentiation of 4F-SiPSCs.

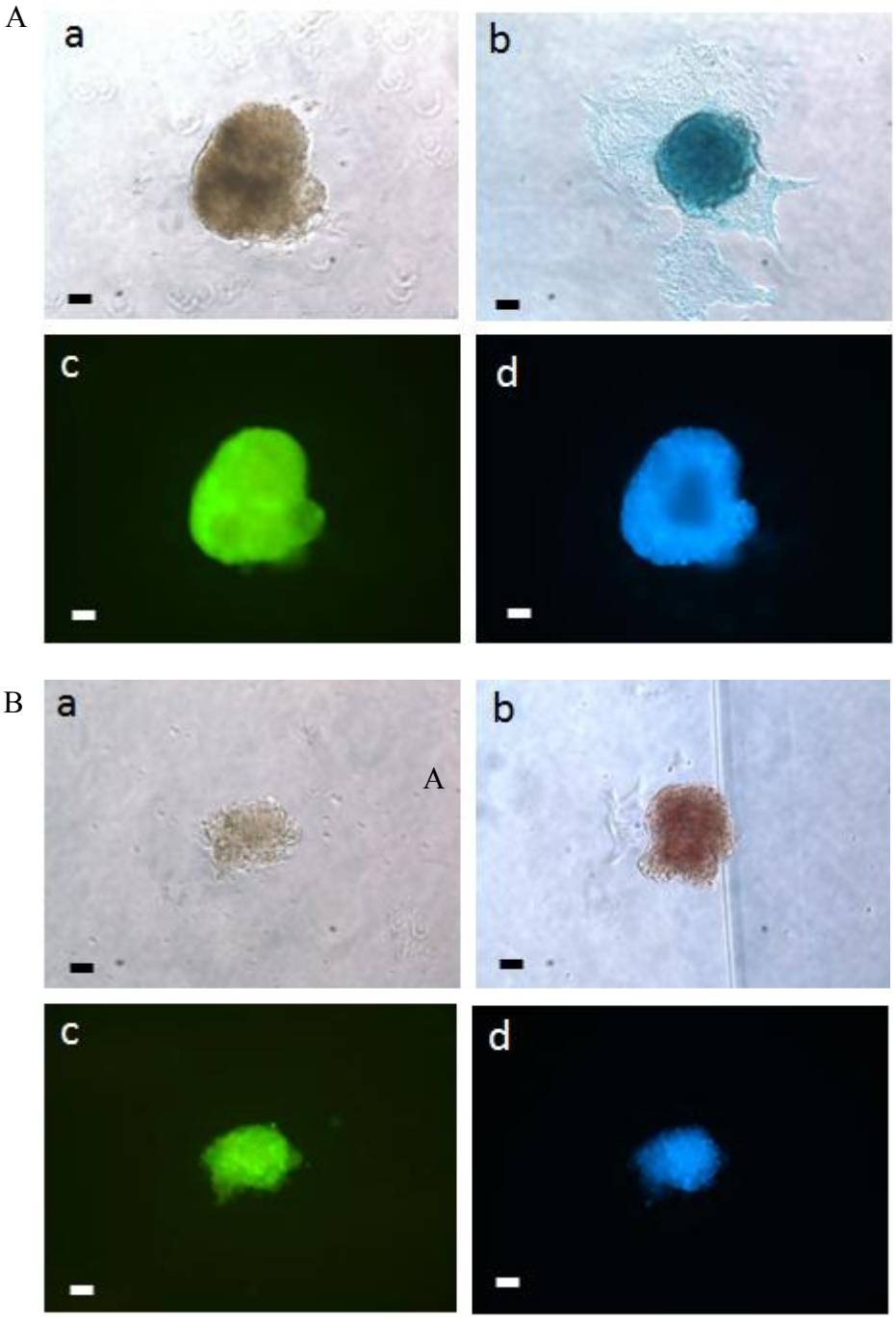


Figure 5.6. Bone and cartilage differentiation of 4F-SiPSCs. A) *In vitro* cartilage differentiation of 4F-SiPSCs: a) Brightfield, b) Alcian Blue staining c) Col 2 immunostaining d) DAPI staining B) *In vitro* bone differentiation of 4F-SiPSCs: a) Brightfield, b) Alizarin Red S staining c) Osteocalcin immunostaining, d) c) DAPI staining. Scale bar: 100 μm. Thanks to Dr. Akihiro Yamashita for performing bone and cartilage differentiation of 4F-SiPSCs.

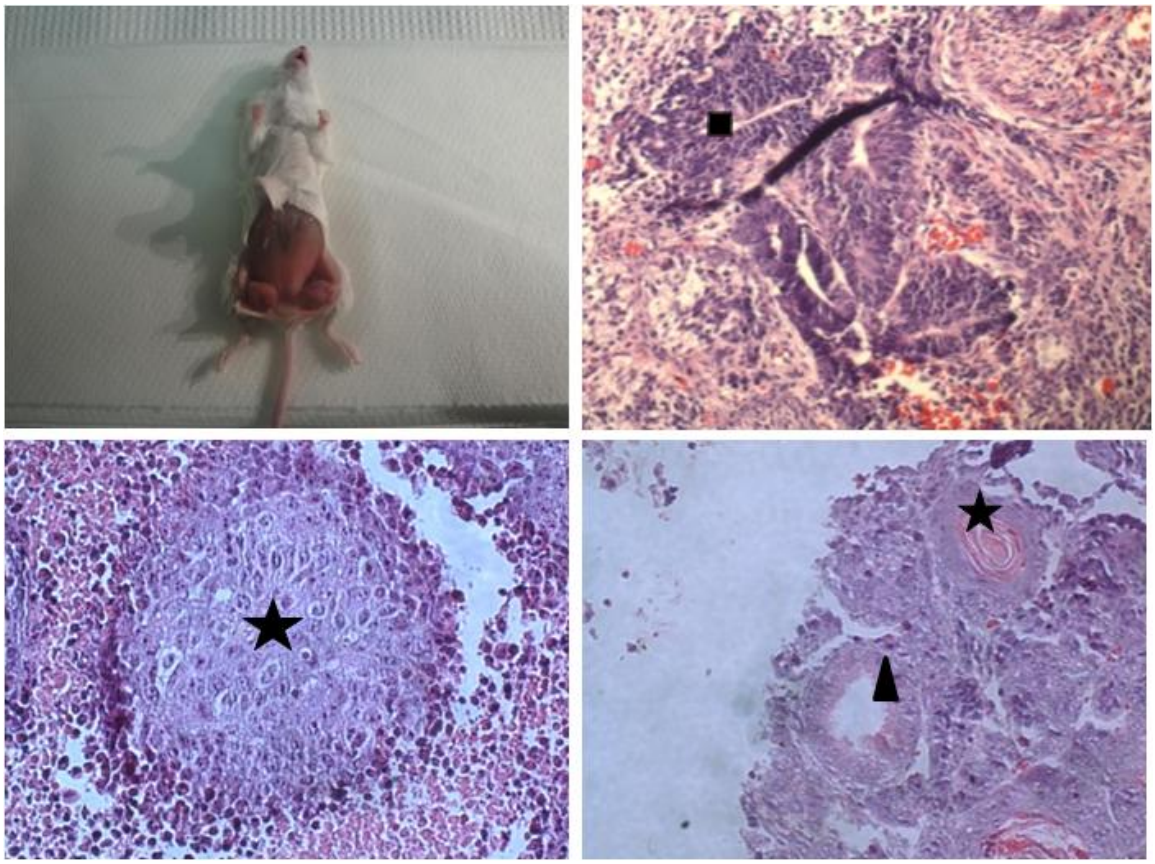


Figure 5.7. 4F-SiPSCs showed *in vivo* differentiation capacity. Teratomas derived from 4F-SiPSCs containing cells representative of the three germ layers. 1.0 $\times 10^6$ cells in a total volume of 100 µl PBS were injected subcutaneously into the inner thigh of a SCID mouse. After four weeks, animals were dissected and tissues were examined histologically via haematoxylin and eosin staining. Shown is a teratoma with three germ layers. (★) Mesoderm; (■)Ectoderm; (▲) Endoderm.

Expedited derivation of 3F-SiPSCs (OKS) in suspension bioreactor

Bioreactor reprogramming was next performed with three factors: Oct4, Sox2 and Klf4, where c-Myc was eliminated as a reprogramming factor. Previous studies have reported that the increased tumorigenicity of certain iPSC lines in both chimeric mice and their germline-transmitted progeny is the result of c-Myc reactivation (Okita, Ichisaka et al. 2007; Nakagawa, Takizawa et al. 2010). Although c-Myc can be removed, conventional reprogramming efficiency drops approximately 100-fold (Nakagawa, Koyanagi et al. 2008). Because the derivation of SiPSCs was such an efficient procedure, we investigated whether this approach could be used to eliminate c-Myc and still derive SiPSCs at high efficiency.

MEFs were transduced with Oct4, Sox2, and Klf4 retroviral vectors in adherent culture and transferred to a 100 ml, 100 rpm SSB two days post transfection. We obtained aggregates three days post SSB inoculation (Figure 5.8Aa-b), which were morphologically similar to ESC aggregates cultured under SSB conditions (Cormier, zur Nieden et al. 2006; zur Nieden, Cormier et al. 2007). Current adherent culture protocols (without the c-Myc retrovirus) require 30 days after transduction for iPSC colonies to appear (Nakagawa, Koyanagi et al. 2008). ALP staining revealed that these SSB aggregates started to express ALP (Day 5) whereas ALP expression was completely absent in the parallel control adherent culture (Figure 5.8Ac-g). SSB-derived iPSCs (SiPSCs) continued to express ALP both in suspension and when transferred to adherent culture. Importantly, these SiPSC aggregates remained stable after 18 passages in suspension culture, as determined by morphology, percentage of ALP^+ aggregates and ploidy. (Figure 5.8Ah-j; Table 5.1).

Compared to four factor SiPSCs, the kinetics of expansion was considerably retarded when only three reprogramming factors were used. By day 12, only 2 million cells were derived, compared to 50 million using four factors; however this number quickly increased to 10 million by day 14 (Fig. 5.8B). On both days 12 and 14, over 90% of the aggregates stained positively for ALP, suggesting that the majority of cells were pluripotent (Fig. 5.8C). This significant boost in reprogramming efficiency that is afforded by the SSB may make it possible to remove additional reprogramming factors in the future.

Characterization of 3F-SiPSCs (OKS) *in vitro*

When day 10 SiPSCs were trypsinized and transferred to adherent culture on inactivated MEF feeders, they formed ESC-like colonies (Figure 5.9a). Immunostaining of SiPSC colonies validated that they expressed Oct4, Nanog, SSEA1 and E-Cadherin protein (Figure 5.9). SiPSCs also maintained multi-differentiation potential by differentiating into all three germ layers *in vitro*. When SiPSC colonies were dissociated into single cells and cultured on agar-coated dishes for 8 days, they generated cystic embryoid bodies (EBs) (Figure 5.10a). After transferring to gelatin-coated plates for spontaneous differentiation, immunofluorescence revealed cells from all three different germ layers (Figure 5.10b-d). *In vitro* differentiation of SiPSCs into bone and cartilage lineages further confirmed the developmental potential of cells (Figure 5.11 A, B).

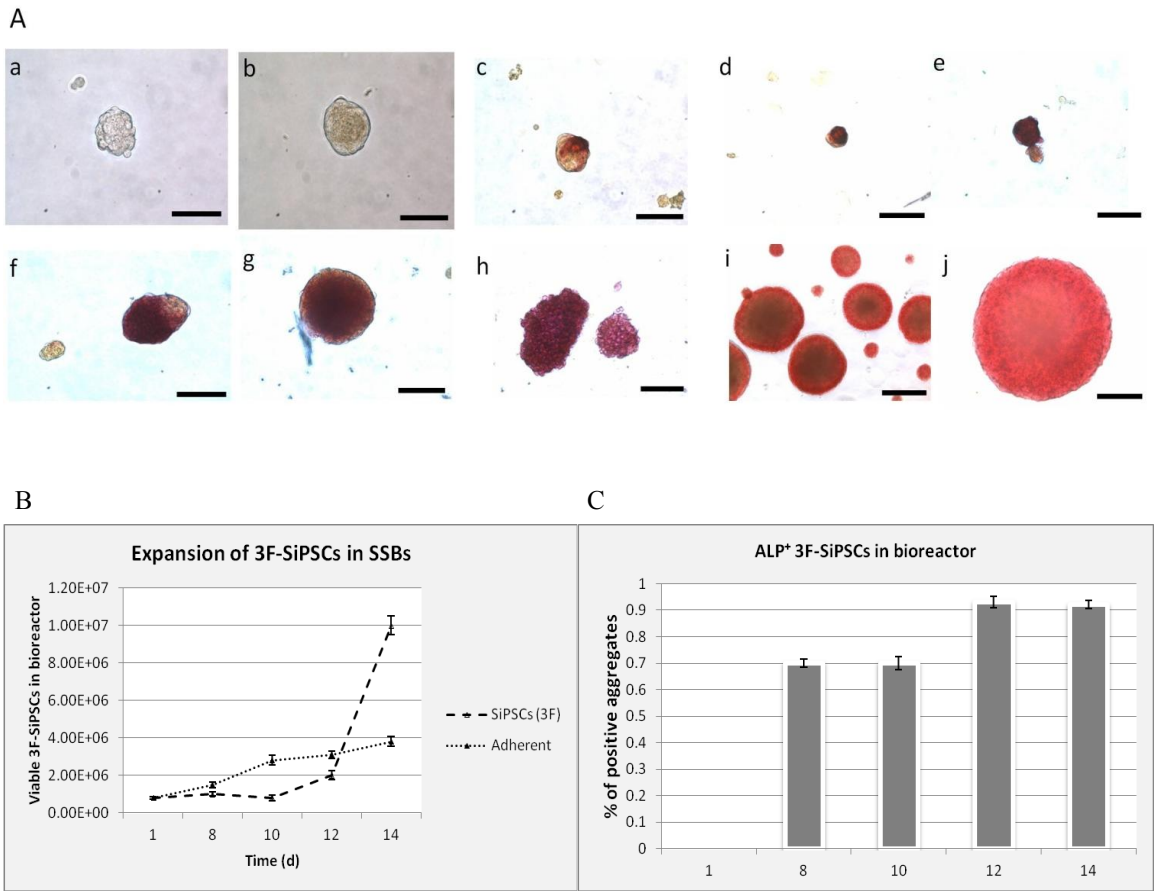


Figure 5.8. Generation of three factor suspension-derived SiPSCs (OKS). A) ESC-like aggregates post inoculation in the SSB: a) day 6, b) day 8 post transfection. ALP staining of emerging SiPSC aggregates in SSB: c) day 6, d) day 8, e) day 10, f) day 14, and g) day 19 post transfection. Long term passaging of derived 3F-SiPSCs showed that ALP expression persisted: h) SiPS colonies on their passage 8 on adherent culture, i) SiPSC aggregates cultured in suspension bioreactor up to passage 11, j) single SiPSC aggregate cultured after 11 passages in SSB. B) Expansion of SiPSCs in the SSB and adherent culture. During the derivation of SiPSCs, cell numbers exponentially increased to 1.0×10^{7}, whereas adherent culture gave rise to less than 3.8×10^{6} in the same period. C) Percentage of ALP^{+} aggregates grown in suspension bioreactor. 70% of aggregates were positive on day 8 post transfection and this percentage reached to 93% by day 12. Scale bar: 100 µm

Time (Post transduction)	**4F-SiPSCs (Day 10-Passage 0)**	**4F-SiPSCs (Day 33-Passage 6)**	**3F-SiPSCs (Day 10-Passage 0)**	**3F-SiPSCs (Day 33-Passage 6)**	**3F-SiPSCs (Day 54-Passage 11)**	**3F-SiPSCs (Day 85-Passage 18)**
Chromosome number	40,XY	40,XY	40,XY	40,XY	40,XY	40,XY
% of normal metaphases	95%	92%	90%	89%	91%	92%

Table 5.1. 4F-SiPSCs (OKSM) and 3F-SiPSCs (OKS) showed normal karyotype. After dissociating of 4F and 3F-SiPSC aggregates on day 10 and 33, cells were cultured overnight in static culture condition without feeder cells. Cells were then treated with colcemid and stained with Giemsa solution. A minimum number of forty chromosomal metaphases were counted for each day.

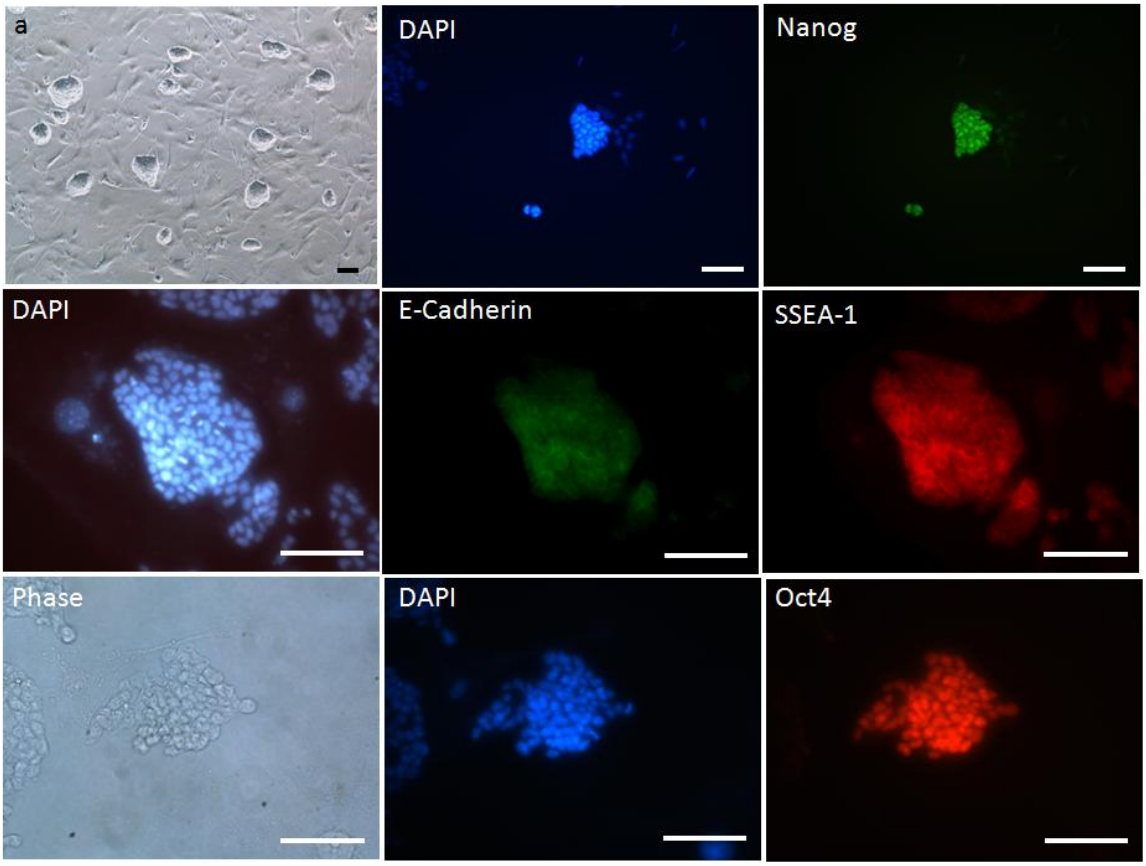


Figure 5.9. Morphology and pluripotency of three-factor SiPSCs (OKS). SiPSCs formed ESC-like colonies in adherent culture, which expressed pluripotency-associated markers: a) 3F-SiPSCs passaged onto inactivated MEF feeder cells; 3F-SiPSCs colony stained with DAPI and immunostained for Oct4, Nanog, SSEA1 and E-cadherin. Scale bar: 100µm.

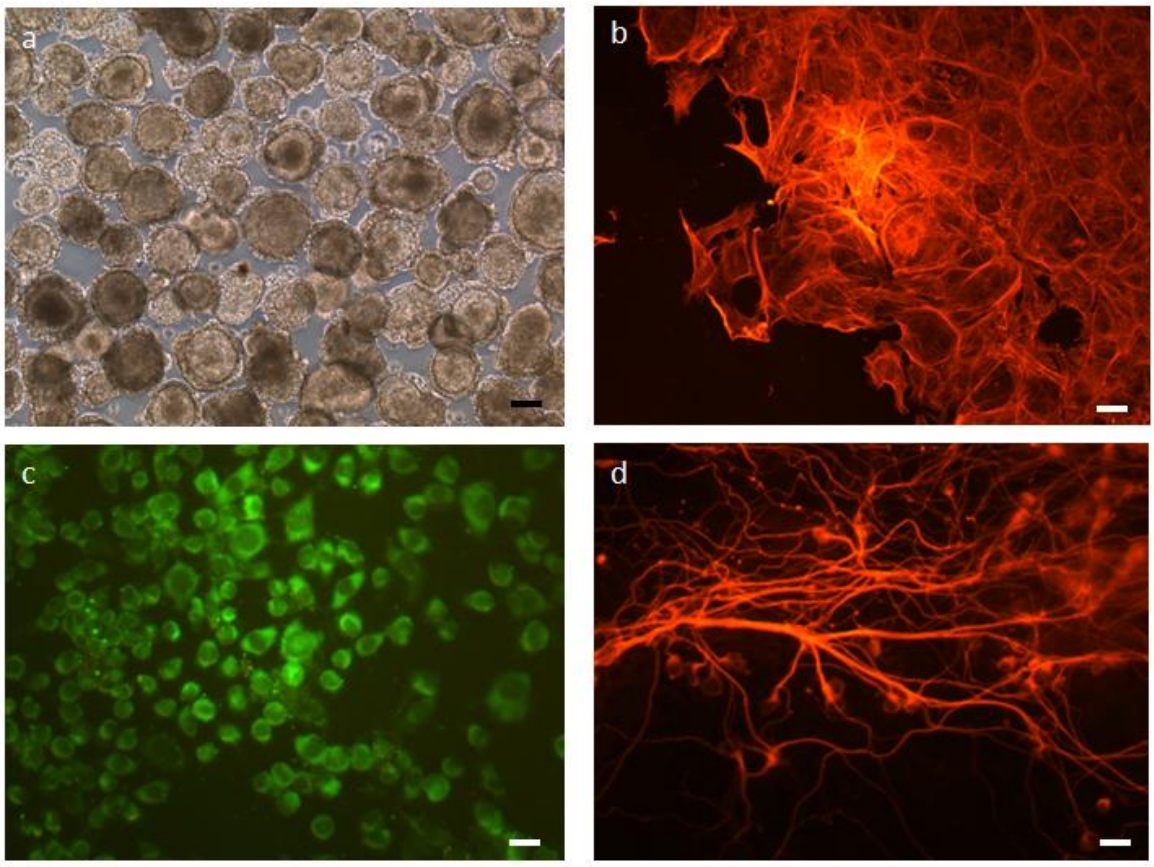


Figure 5.10. Spontaneous *in vitro* differentiation of 3F-SiPSCs (OKS). Immunostaining of the differentiated SiPSCs reveals cells from all three different germ layers. After EB formation, cells were transferred to gelatin-coated plates for spontaneous differentiation. Differentiated cells were then stained with antibodies specific for three germ layers: a) *In vitro* differentiation into cystic embryoid bodies (EBs). b) α-actin (mesoderm), c) AFP (endoderm) and d) β-Tubulin III (ectoderm). Scale bar: 100μm. Thanks to Dr. Guoliang Meng for performing iPSC spontaneous differentiation. Thanks to Dr. Guoliang Meng for performing spontaneous *in vitro* differentiation of 3F-SiPSCs (OKS).

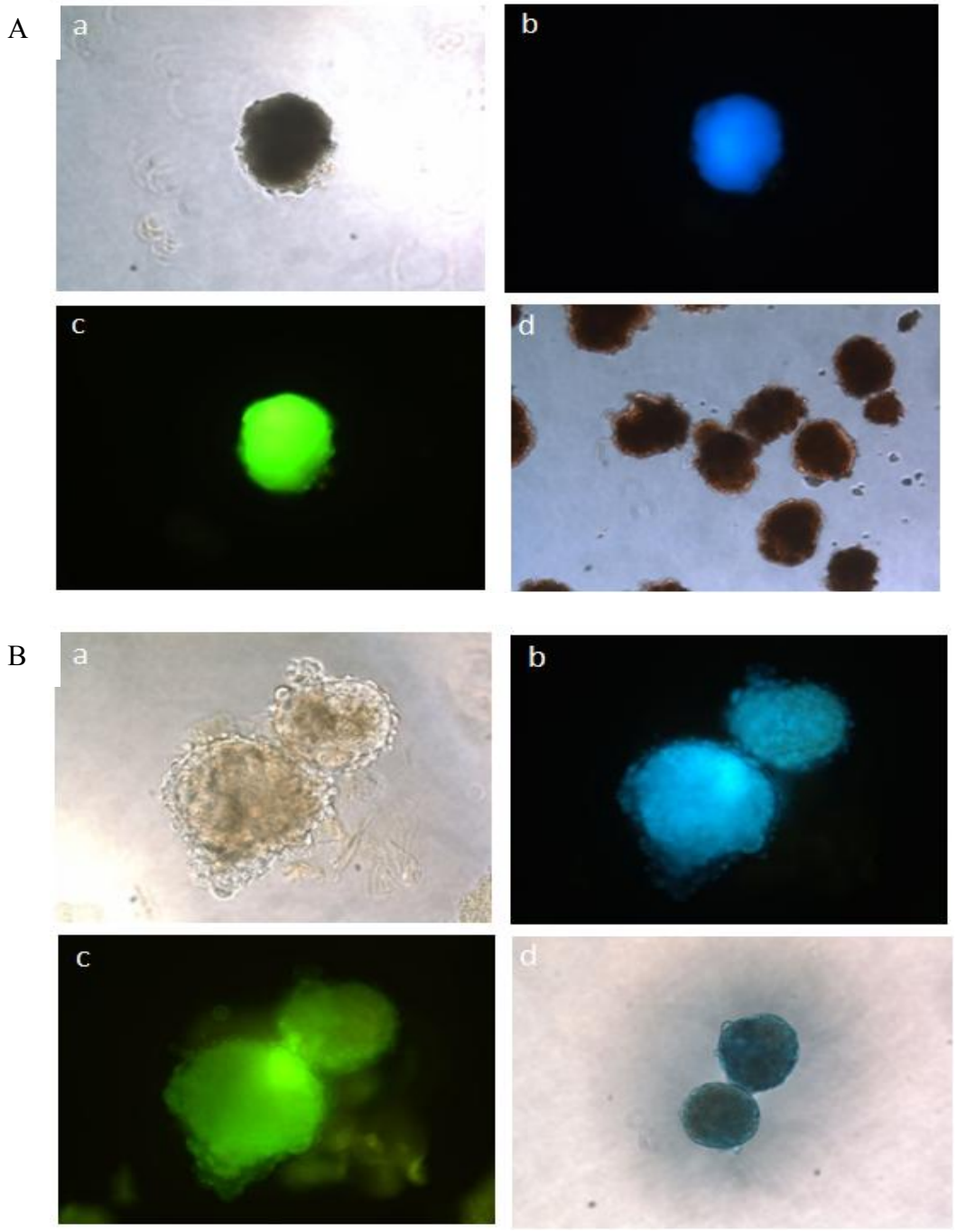


Figure 5.11. *In vitro* differentiation of three-factor SiPSCs (OKS) to bone and cartilage. A) *In vitro* cartilage differentiation of SiPSCs: a) brightfield, b) DAPI staining, c) collagen 2 immunostaining d) alcian Blue staining. B) *In vitro* bone differentiation of SiPSCs: a)brightfield, b) DAPI staining, c) osteocalcin immunostaining, d) alizarin Red S staining. X200. Thanks to Dr. Akihiro Yamashita for performing bone and cartilage differentiation of 3F-SiPSCs.

We also analyzed these 3F-SiPSCs (OKS) for *de novo* expression of major pluripotency markers such as Oct4, Nanog, SSEA1, Rex1, Dax1 and E-Ras on day 10 using RT-PCR (Figure 5.12). These SiPSCs expressed hallmark of pluripotency markers on passage 1 (day 10), 3 and 9. Confocal microscopy of day 10 3F-SiPSCs showed the expression of Oct4 and Nanog (Figure 5.13). In order to monitor the possible effect of suspension bioreactor on the cell chromosomal state during iPSC derivation, we karyotyped 3F-SiPSCs chromosomes on days 10 (P0), 33 (P6), 54 (P11) and 85 (P18) post transfection. Cell aggregates were dissociated with trypsin and cultured for 24 h in static culture. The cells were then harvested and examined for any chromosomal abnormalities. The percentage of normal karyotype was around 90% and more over the course of bioreactor maintenance. (Table 5.1).

3F-SiPSCs (OKS) showed full developmental potential through teratoma formation and germline transmission

In order to examine the pluripotency of derived SiPSC, one million cells were implanted subcutaneously into SCID mice. Teratomas formed from SiPSCs after 28 days contained tissues representative of all three germ layers (Figure 5.14A). Furthermore, 129/Sv 3F-SiPSCs generated from three independent experiments were microinjected into C57BL/6 blastocysts and transferred into pseudopregnant CD1 females. Thirty six chimeric (mosaic) mice consisting both of cells originating from the blastocyst and from 3F-SiPSCs were born approximately 17 days after transfer. Of those, one litter of 7 pups was cannibalized right away and one pup, which was recovered via C-section also died. Working with 28 pups, we had 17 chimeras with different percentages of chimerism

(Figure 5.14Ba and Table 5.2). After breeding the resulting chimeras to C57BL/6 mice, germline transmissions were detected by the appearance of non-black coat colors (Figure 5.14Bb).

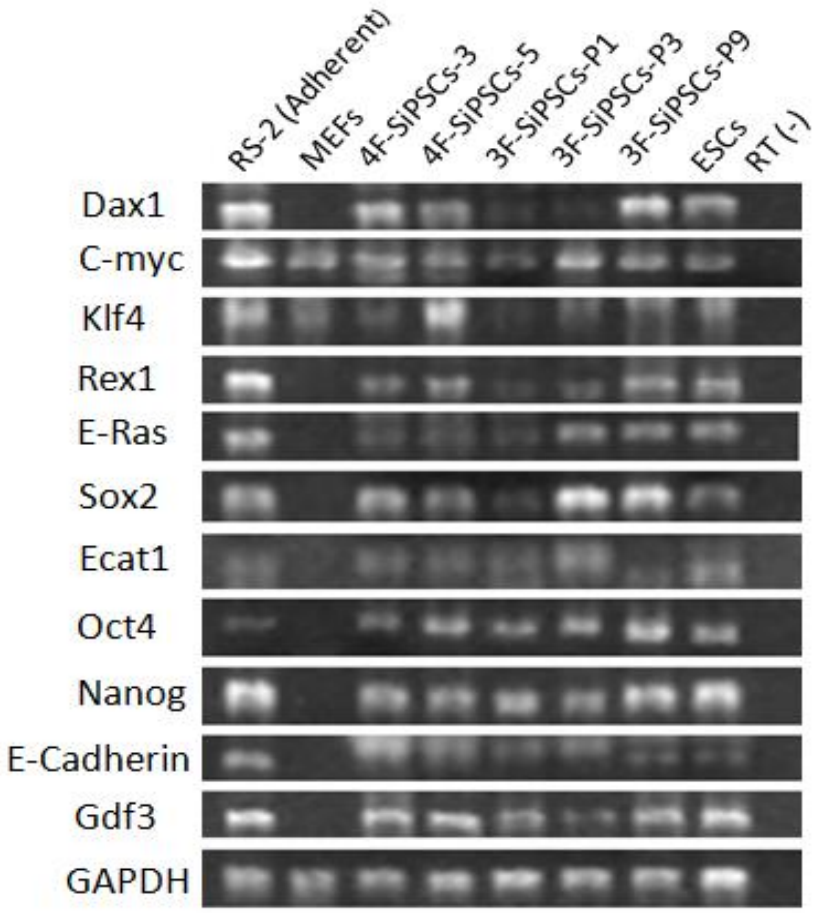


Figure 5.12. *De novo* expression of pluripotency markers in SiPSCs. 3F-SiPSCs express multiple pluripotency specific markers. Expression of ESC specific markers on day 10 in 3F-SiPSCs as shown by RT-PCR (3F-SiPSCs-P1). 3F-SiPSCs in three different passages compared to a mouse ESC line (D3), starting MEFs and RS-2 iPS (Adherent) line. Primers specific to transcripts from endogenous genes were used. GAPDH was used as internal control. P1=passage 1, P3=passage, P9=passage 9. Two already established 4F-SiPSC lines have also shown in this figure.

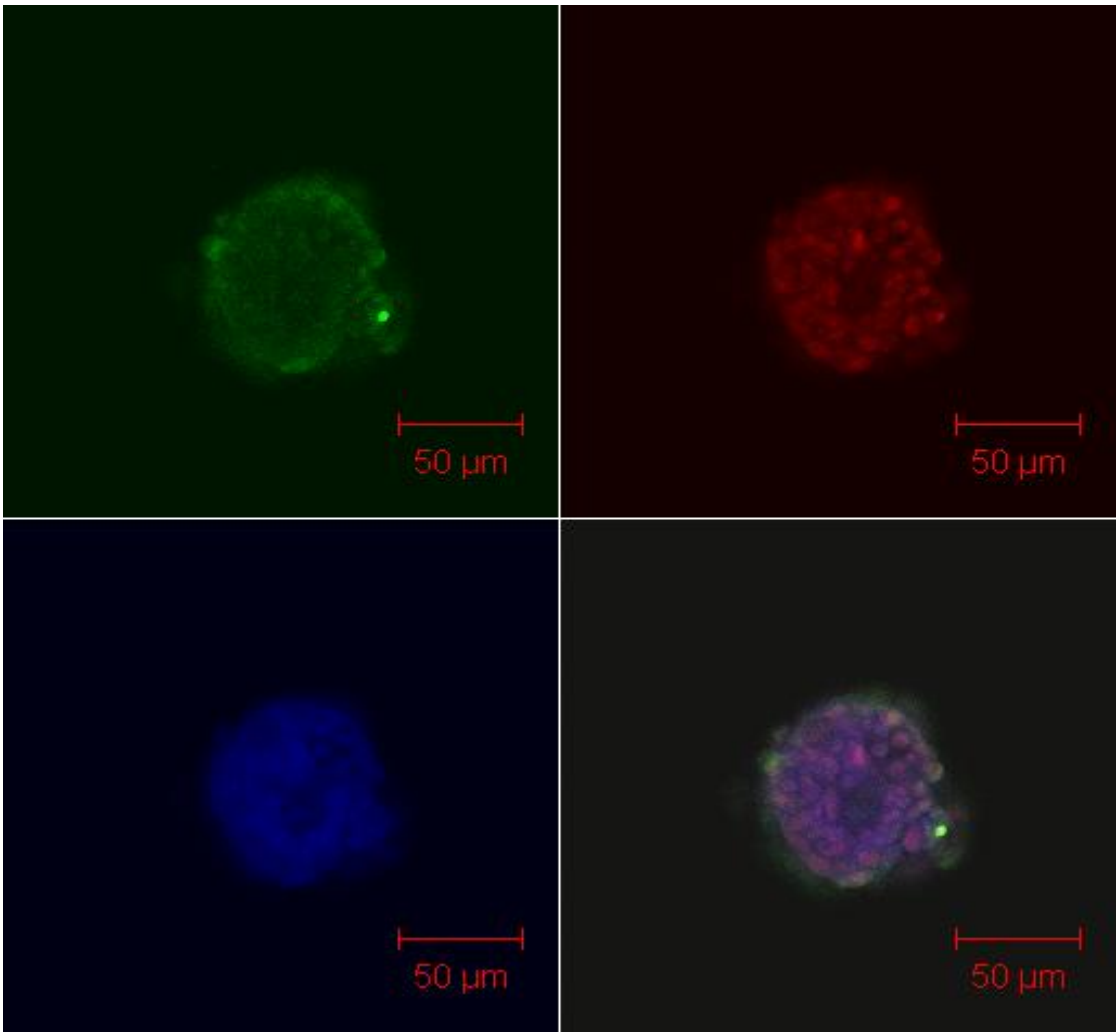


Figure 5.13. Confocal microscopy confirmed the expression of Oct4 and Nanog in a sub-population of 3F-SiPSCs (OKS) within aggregates, 10 days post transfection in the SSB.

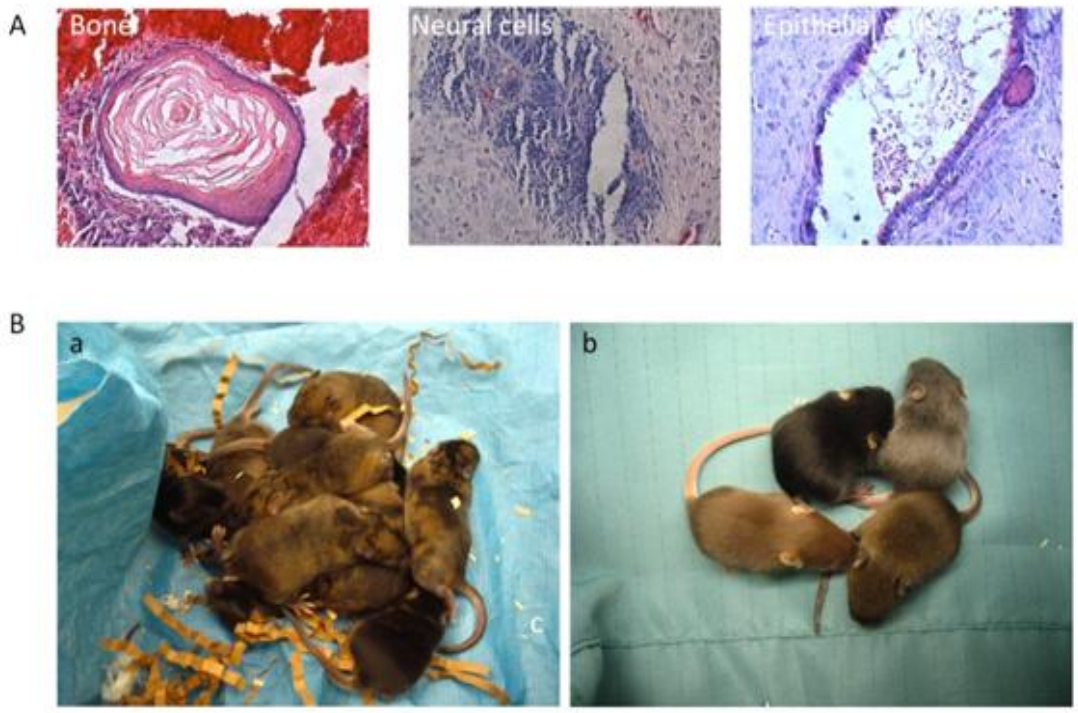


Figure 5.14. *In vivo* developmental potential of 3F-SiPSCs (OKS). A) Teratoma derived from 3F-SiPSCs containing cells representative of the three germ layers. 1.0×10^6 cells in a total volume of 100 µl PBS were injected subcutaneously into the inner thigh of a SCID mouse. After four weeks, animals were dissected and tissues were examined histologically via haematoxylin and eosin staining. Shown is a teratoma with bone (mesoderm), neural cells (ectoderm) and epithelium (endoderm). B) Chimeric mice generated by 3F-SiPSCs and germline transmission of these cells. a) 3F-SiPSCs contribute to chimeric mice with different percentages of brown ranging from 5%-100%. b) SiPSCs showed germline transmission competency. There are 4 different coat colors, Black (non germline), grey, brown and agouti (germline pups). Thanks to Clara Christie Centre for Mouse Genomic (CCCMG) at the University of Calgary for performing the chimera and germline transmission experiments.

Mouse chimera	Sex	% of chimerism
1	M	100
2	F	20
3	M	<5
4	F	60
5	F	<5
6	F	<5
7	M	90
8	M	85
9	M	85
10	M	85
11	M	85
12	M	<5
13	F	80
14	F	70
15	F	<5
16	F	95
17	F	20

Table 5.2. 3F-SiPSC (OKS) gave rise to several high percentage chimeras. Chimeric (mosaic) mice consisting both of cells originating from the host blastocyst and from SiPSCs were born approximately 17 days after transfer. Working with 28 pups, we had 17 chimeras with different percentages of chimerism. Percentage key: VL=0-19%, L=20-39%, M=40-59%, H=60-79%, VH=80-99%

Suspension bioreactors enhance kinetics of 3F-SiPSCs generation (removing Klf4, Sox2 and Oct)

In order to evaluate the role of SSBs in the generation of iPSCs, we examined the efficiency of reprogramming with the other three-factor combinations including Oct4/Klf4/c-Myc (OKM), Oct4/Sox2/c-Myc (OSM) and Klf4/Sox2/c-Myc (KSM). In summary, 129/Sv MEFs were transduced with each of the mentioned three factor mixes. Two days after infection, MEFs were trypsinized and transferred to 100 mL, 100 rpm SSB with mESC/iPS medium supplemented with LIF. Viable cell density and ALP^+ aggregates were scored on 5, 7, 10 and 12 days post transduction. ESC-like aggregates appeared on day 5 and three days post SSB inoculation for all three reprogramming. The size of aggregates grew gradually for all of reprogramming experiments from day 5 to 10 (OKM, OSM and KSM) (Figure 5.15A-C). When stained, some of these aggregates express ALP from day 5 onward (Figure 5.16A-C). The percentage of ALP+ aggregates reached 90% for the OKM experiment whereas this percentage was only 4% and 2% for OSM and KSM respectively (Figure 5.17A). OKM had the highest number of viable cells during the first 10 days after transduction, reaching to about 4.4×10^7, while viable cell number was considerably lower for OSM and KSM (Figure 5.17B). Based on the percentage of ALP^+ aggregates and the number of growing viable cells in bioreactor, we achieved the highest efficiency of SiPSC generation with OKSM transduction whereas the efficiency of OKM, OKS, OSM and KSM stand next respectively (Table 5.3).

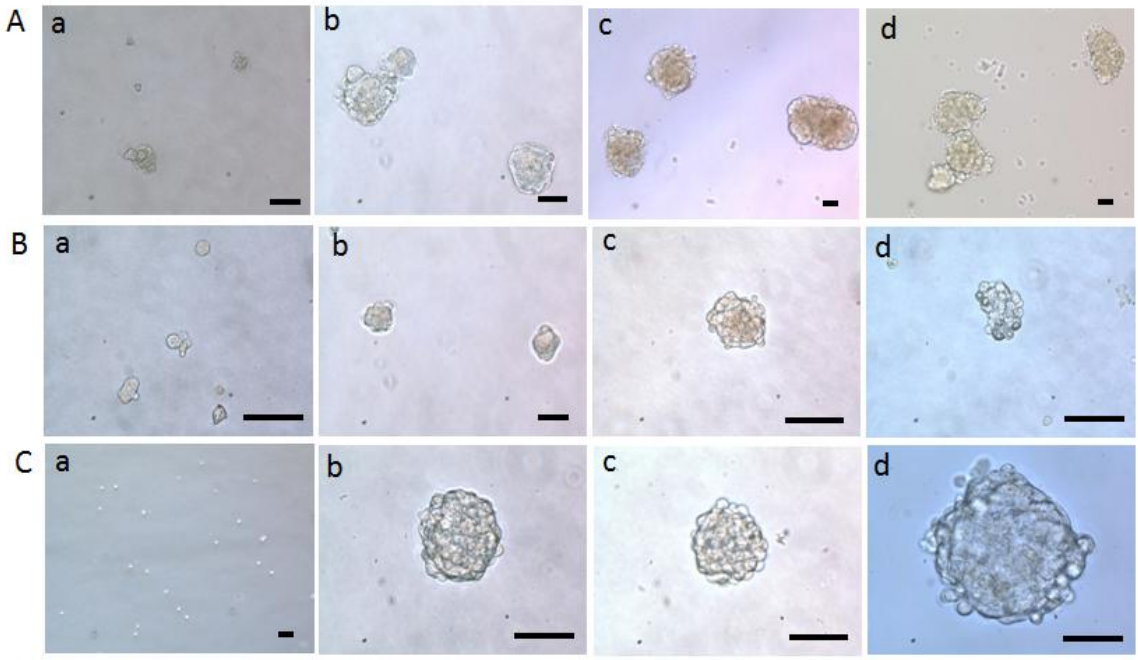


Figure 5.15. Aggregates of three factor suspension-derived SiPSCs. ESC-like aggregates post inoculation in the SSB using (A) OKM, (B) OSM and (C) KSM gene mix respectively. a) day 5, b) day 7 c) day 10 and d) day12 post transfection. Scale bar: 100 μm.

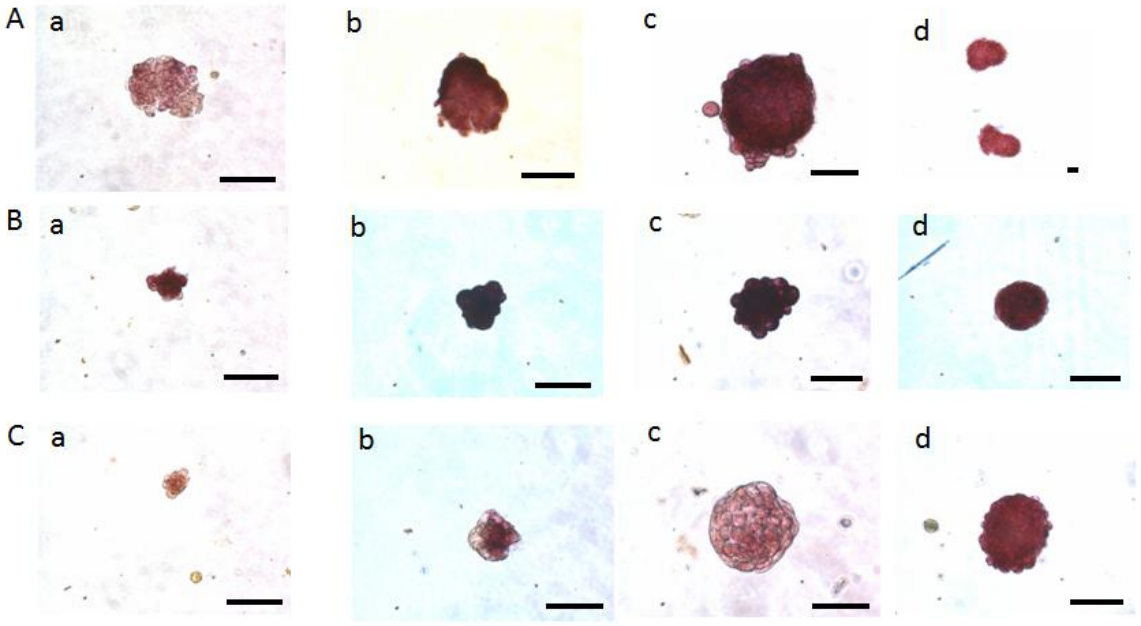
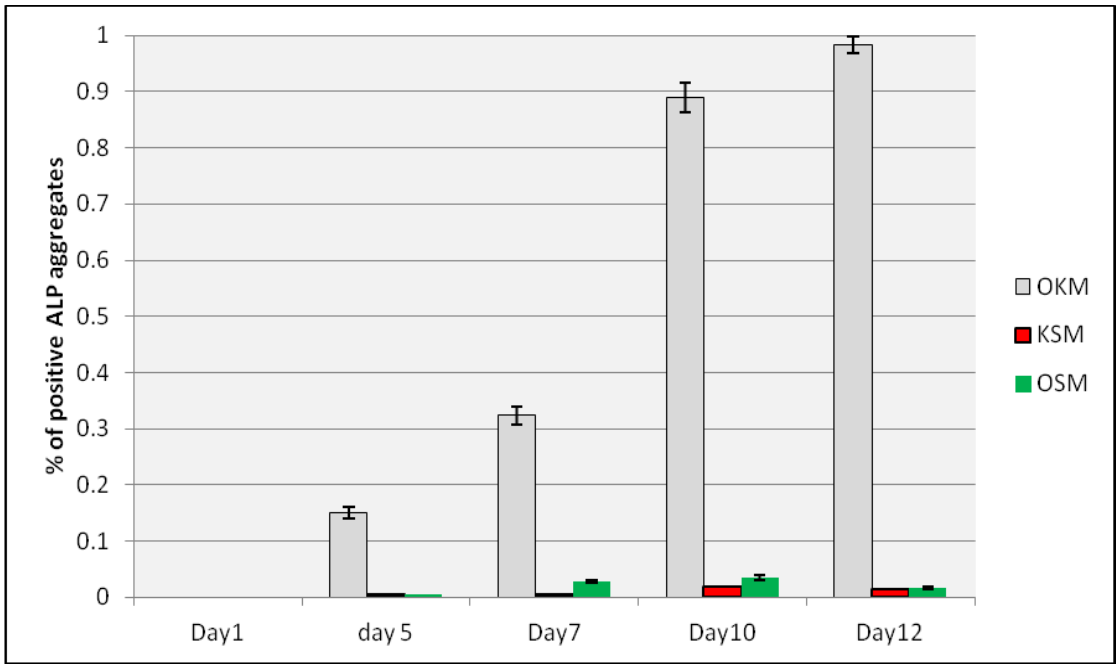


Figure 5.16. ALP staining of emerging SiPSC aggregates in suspension bioreactor using OKM, OSM and KSM gene reprogramming: a) day 5, b) day 7, c) day 10, d) day 12. The first ESC-like ALP^{+} aggregate was seen on day 5 post transfection in all grous. Scale bar: 100 μm.

A



B

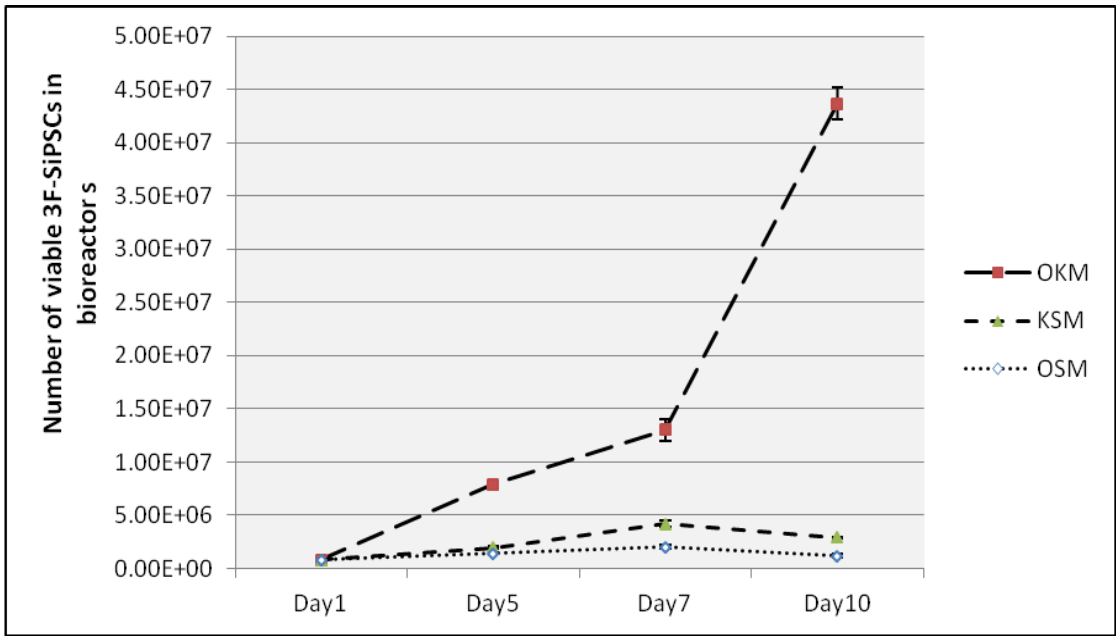


Figure 5.17. Growth rate of 3F-SiPSCs (OKM, OSM and KSM). A) Percentage of ALP^+ aggregates grown in suspension bioreactor. About 90% of aggregates were positive on day 10 post transfection for OKM and this percentage reached to 98% by day 12. This percentage was considerably lower for OSM and KSM. B) Expansion of 3F-SiPSCs in the SSB. During the derivation of 3F-SiPSCs, cell numbers exponentially increased to 4.4×10^7 for OKM, whereas OSM and KSM gave rise to less than 1.2×10^6 and 3.0×10^6 respectively in the same period.

	Starting number of MEFs	Viable cells on day 5	Viable cells on day 10	ALP+ aggregates on day 5	ALP+ aggregates on day 10
4F-SiPSCs (OKSM)	8.60E+05	1.45E+07	5.10E+07	24%	92%
3F-SiPSCs (OKS)	8.60E+05	1.20E+06	1.10E+06	18%	73%
3F-SiPSCs (OKM)	8.60E+05	7.90E+06	4.37E+07	15%	89%
3F-SiPSCs (OSM)	8.60E+05	1.42E+06	1.17E+06	0.5%	4%
3F-SiPSCs (KSM)	8.60E+05	1.97E+06	2.90E+06	0.5%	2%

Table 5.3. Efficiency comparison of different combination of reprogramming factors. 4F-SiPSCs showed the highest efficiency by giving rise to highest viable cells and percentage of ALP^{+} aggregates on day 5 and 10. 3F-SiPSCs (OKM) stand next for the efficiency by generating %89 ALP+ aggregates on day 10. 3F-SiPSCs (OKS), 3F-SiPSCs (OSM) and 3F-SiPSCs (KSM) are next. KSM (without Oct4) had the lowest efficiency. O:Oct4, K: Klf4, S: Sox2 and C: c-Myc.

Confocal microscopy confirmed expression of pluripotency markers in 3F-SiPSCs (OKM) aggregates.

In order to evaluate the pluripotency of derived 3F-SiPSCs (OKM), the cells were characterized on day 10 using immunostaining approaches. Whole-mount immunofluorescence and confocal microscopy revealed nuclear expression of Oct4 and Nanog in a sub-population of cells within SiPSC aggregates at 10 days post transfection in the SSB (Figure 5.18). It is noteworthy that the localization of the cells expressing Oct-4 and Nanog within the aggregates tended to localize peripherally in the aggregates. 3F-SiPSCs (OKM) were further characterized through immunostaining using confocal microscopy. SSEA1 and β-catenin protein expression was localized to the cell membrane whereas the Rex1 was observed within the nucleus (Figure 5.19). The expression of E-Cadherin was also examined during various days post transduction using Immunostaining approaches. Our results showed that expression within SiPSC aggregates started as early as day 7, 5 days post inoculation in the suspension bioreactor (Figure 5.20). In order to examine the pluripotency of derived SiPSCs (OKM) *in vivo*, one million cells were implanted subcutaneously into SCID mice. Teratomas formed from SiPSCs after 28 days contained tissues representative of all three germ layers (Figure 5.21).

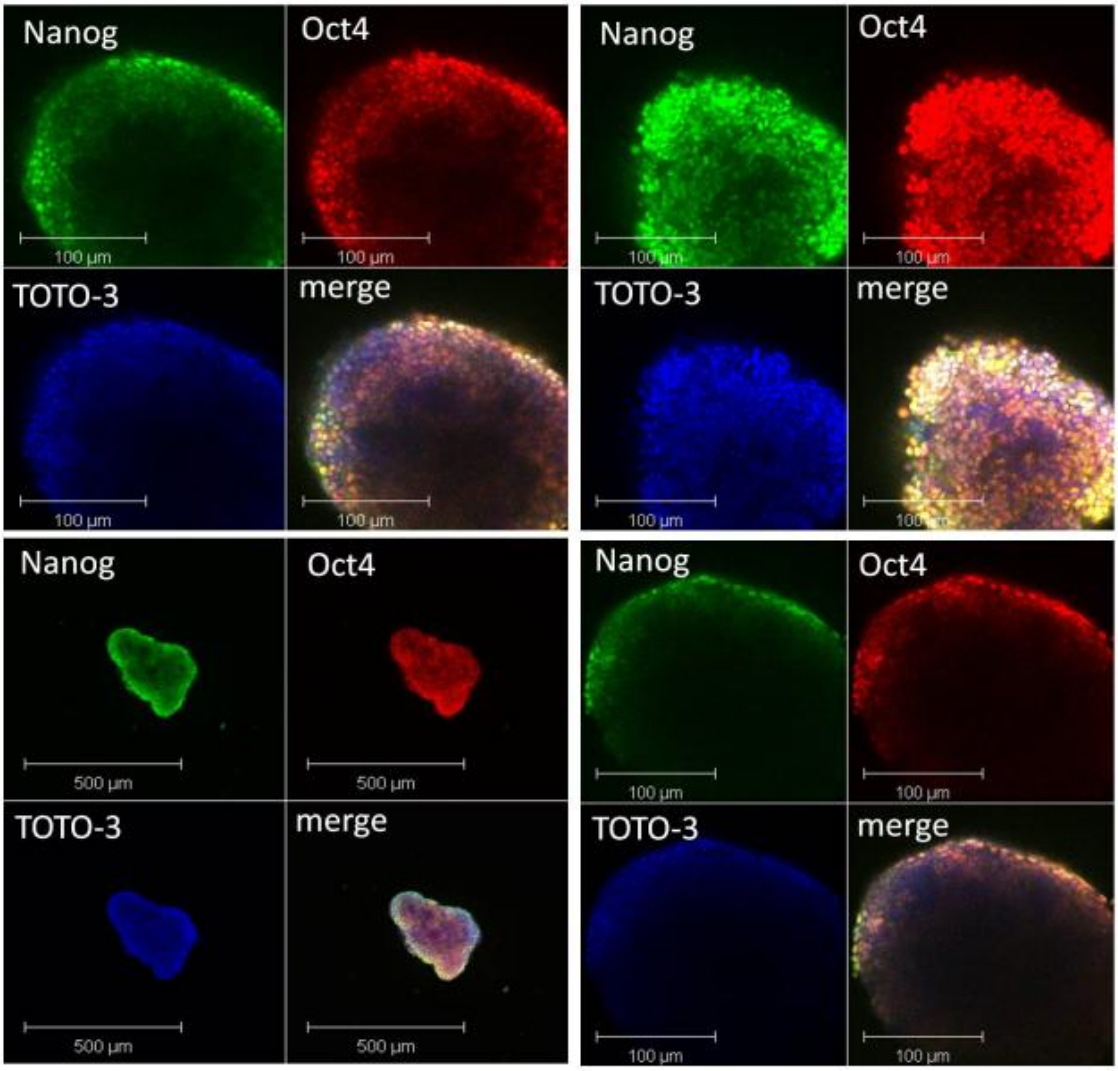


Figure 5.18. Pluripotency marker expression of derived 3F-SiPSCs (OKM). A) Confocal microscopy revealed the expression of Oct4 and Nanog in the nucleus of a sub-population of cells within aggregates 10 days post transfection within SSB. The expression was localized mostly to the edges of aggregates. TOTO-3 was used as nuclear marker.

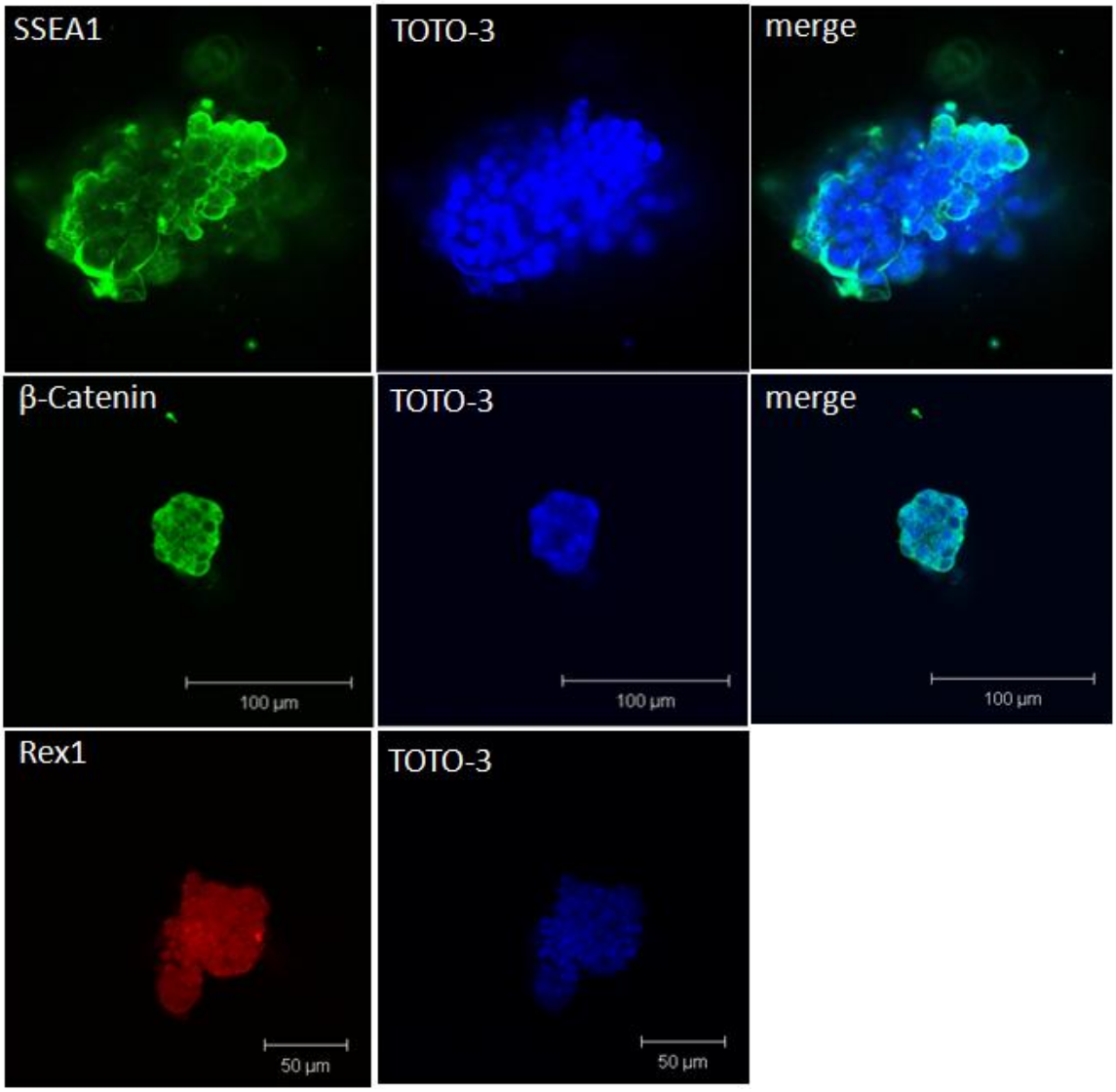


Figure 5.19. 3F-SiPSCs (OKM) were further characterized through immunostaining using confocal microscopy. SSEA1, Rex1 and β-catenin expression within aggregates on day 10. SSEA1 and β-catenin protein expression was localized to the cell membrane whereas the Rex1 was observed within the nucleus. TOTO-3 was used as nuclear probe for nucleic acid detection.

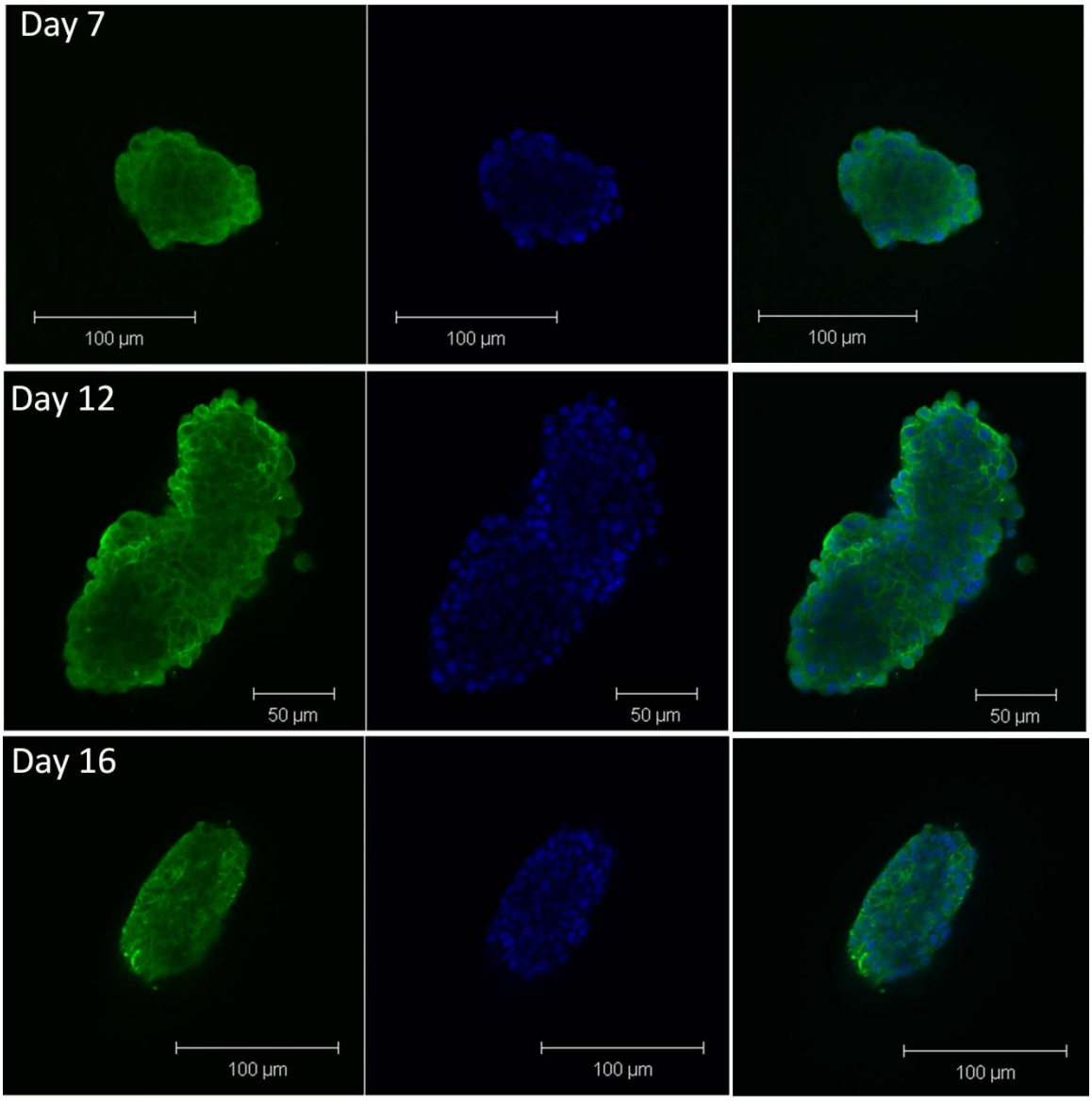


Figure 5.20. Z-stack projection analysis of derived 3F-SiPSCs (OKM) on different days post transfection. Confocal microscopy confirmed the expression of E-Cadherin within the cytoplasmic membrane of aggregates. TOTO-3 was used as nuclear probe for nucleic acid detection.

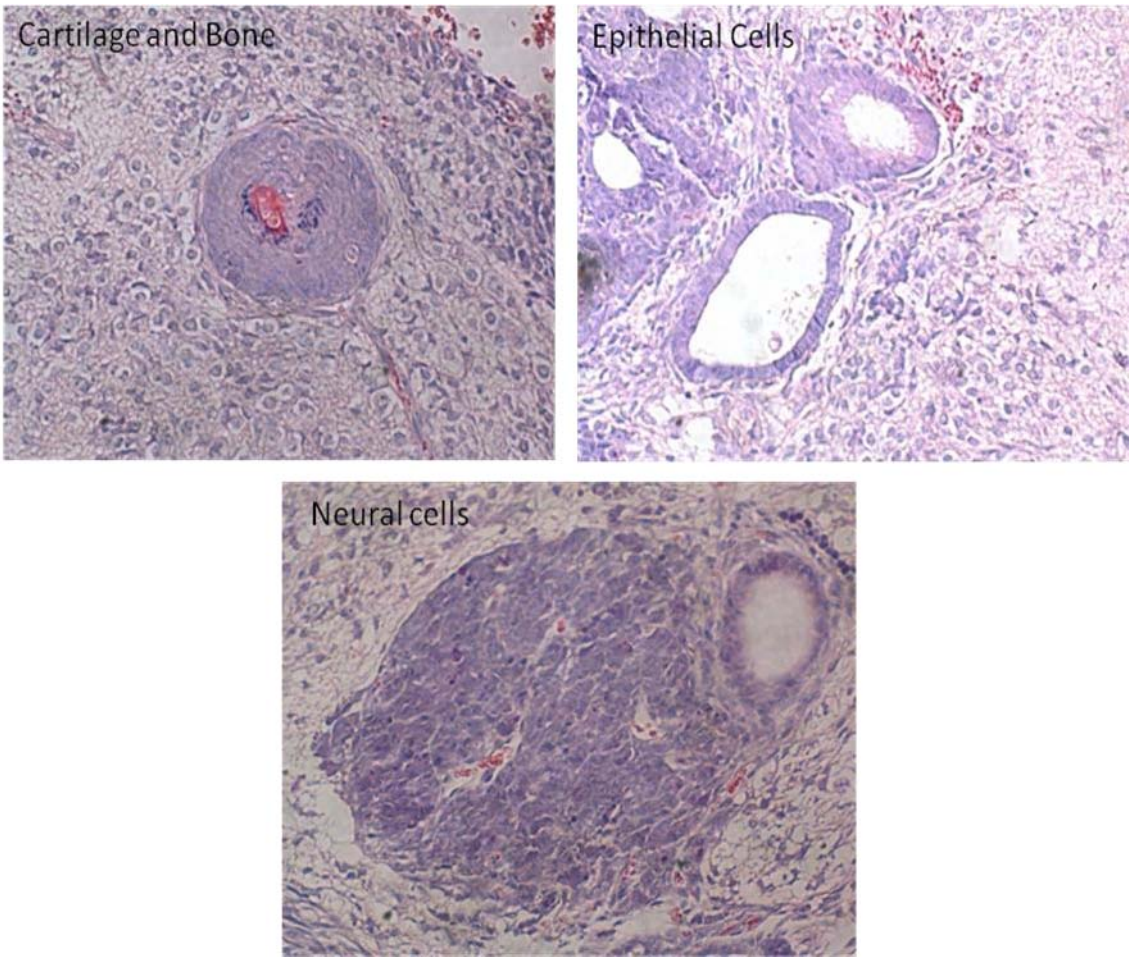


Figure 5.21. *In vivo* developmental potential of 3F-SiPSCs (OKM). Teratoma derived from 3F-SiPSCs (OKM) containing cells representative of the three germ layers. 1.0 $\times 10^6$ cells in a total volume of 100 µl PBS were injected subcutaneously into the inner thigh of a SCID mouse. After three weeks, animals were dissected and tissues were examined histologically via haematoxylin and eosin staining. Shown is a teratoma with cartilage and bone (mesoderm), neural cells (ectoderm) and epithelium (endoderm). Thanks to Mrs. Shiying Liu for performing the teratoma assay.

5.5 Discussion

The recent advances in the derivation of induced pluripotent stem cells (iPSCs) from different mouse and human somatic cells are improving our knowledge about pluripotent cells. There are several notable benefits of iPSCs. Most importantly, from an ethical viewpoint, these cells do not have the obvious ethical considerations that currently impose a hurdle in hESCs investigations. Another critical advantage of iPSC technology is that it allows for the creation of cell lines that are genetically customized to a patient. The issue of immune rejection, in which the body's immune system recognizes implanted cells or tissues as foreign and attacks them, is another obstacle to the therapeutic application of cell-based therapies. If cells for therapy can be generated using a patient's own cells, the issue can be potentially rendered. Despite major improvements in the methods of iPSC generation, the process still remains inefficient and poorly characterized. Development of a scalable and well-controlled system for the consistent and expedited derivation and expansion of iPSCs is a critical hurdle that needs to be overcome. The robustness of controlled suspension bioreactors has the potential to produce large amounts of iPSCs on a clinical scale.

Our study demonstrates the successful derivation of iPSC from MEFs in stirred suspension bioreactors without the application of supporting feeder-cells or tissue culture substrates. Suspension culture methods for expansion of ESCs and iPSCs normally involve repeated dissociation and re-aggregation of cells (zur Nieden, Cormier et al. 2007; Shafa, Sjonnesen et al. 2011). In this study, we observed the first ESC-like aggregate only 5 days post transfection for all of the reprogramming experiments, including four factor or three factor reprogramming. Formed aggregates appeared to be

fully reprogrammed to the pluripotent state based on the *in vitro* and *in vivo* characterization. Induction of reprogramming by four or three factors led to high cellular growth rates compared to adherent culture systems. Based on the expression of ALP and pluripotency markers, we observed an enhancement of MEF reprogramming compared to the adherent culture environment both in 3F or 4F reprogramming system.

The established bioreactor reprogramming method is not only efficient, it is also robust and reproducible. We have validated the method nine times using MEFs from three different mouse lines: CD1, C57BL/6, and 129/Sv using four or three factors. Although there was a slight fluctuation in the number of viable ALP^+ cells, ploidy, and pluripotency marker gene expression between experiments, the ultimate test for pluripotency was confirmed by germline transmission using SiPSCs generated from three independent bioreactor reprogramming experiments.

Our results suggest that the SSB culture environment plays an important mechanistic role in bioreactor-induced pluripotency. Shear stress can modulate gene expression through mechanotransduction, where physical signals are sensed at the cell periphery, transduced into biochemical signals within the cell, ultimately resulting in cell responses, including changes in gene expression (Vogel and Sheetz 2009). Previous studies in other cells have demonstrated that shear stress can induce the nuclear translocation of ß-catenin into the nucleus (Avvisato, Yang et al. 2007). As ß-catenin is an important regulator of pluripotency (Dreesen and Brivanlou 2007), we are interested in the role of the canonical Wnt signaling pathway in this induced pluripotency process. While β-catenin can be sequestered by E-cadherin within the adherens junction complex,

recent studies suggest that this complex is mechano-responsive and subject to rearrangement upon exposure to shear stress (le Duc, Shi et al. 2010).

Although it is difficult to compare adherent and suspension culture methods, we estimate our process to be 100- to 1000- fold more efficient than conventional methods. We believe that both inductive and selective events are occurring with bioreactor induced reprogramming. Since we are able to isolate germline competent iPSCs as early as day 10 in the SSB, we are increasing the kinetics of reprogramming. However, because fibroblasts are substrate dependent for growth and viability and cannot grow in suspension culture, SSB culture may also be selecting for aggregate formation via mesenchymal-epithelial transition (MET), and allowing reprogrammed cells to flourish in an open environment.

In summary, our study demonstrates that suspension cultures provide a selective advantage for enhancing iPSC generation. Our results show for the first time that fibroblasts can be efficiently reprogrammed in SSBs without using c-Myc as a reprogramming gene. Combined with new methods of reprogramming that do not use integrating genetic constructs, our SiPSC technology has the potential to accelerate and standardize iPSC research, bringing it to clinical application more quickly.

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Chapter Six:

Discussion

The direct reprogramming of adult cells to a pluripotent state represented a crucial discovery that has the potential to revolutionize the fields of stem cell biology and regenerative medicine. The work presented in this thesis describes a novel approach for the expansion and derivation of iPSCs. It also demonstrates the impact of suspension bioreactor culture on the maintenance of pluripotency during cardiomyocyte differentiation.

Our initial studies suggested that the SSB is not a suitable environment for differentiating ESCs toward cardiomyocytes, as it inhibits complete differentiation by promoting pluripotency. This effect is probably the result of fluid shear stress that cells experience in this culture system. Possible explanations for this induced pluripotency, as well as known mechanotransduction pathways, will be discussed later in this chapter. We found that the SSB represented a satisfactory and efficient culture system for the expansion and long term maintenance of iPSCs. Further, our study demonstrated that the SSB is a superior feeder-free culture system for reprogramming fibroblasts into iPSCs. The application of SSBs in ESC differentiation, as well as its utilization for the derivation and expansion of iPSCs, has raised several remarkable questions regarding the effects of fluid shear stress on the pluripotency of cells, which need further elucidation.

The discussion section of the each manuscript was focused on addressing some of the questions raised during the course of each study. Although further investigations are required to give conclusive answers to some of these questions, the following

discussion chapter is meant to address some of the questions more comprehensively and provide insight into the potential applications of SSBs in regenerative medicine.

6.1 Cardiomyocyte differentiation, pluripotency and tumorigenicity of ESCs in SSBs

ESC-derived cardiomyocytes are believed to be one of the most favorable cell-types for clinical-based cellular therapies. One of the ultimate goals of stem cell-mediated cardiomyocyte differentiation is the cellular transplantation in patients affected by cardiac disease and ischemic disorders (Klug, Soonpaa et al. 1996). This requires the ability for large scale production of donor cardiomyocytes, which present no risk of producing teratomas or tumors. In order to use ESC- or iPSC-derived cardiomyocytes in clinical therapeutic applications, it will be essential to have a pure population of cardiomyocytes. The ability to differentiate and enrich for viable cardiomyocytes makes these cells an attainable source for cardiac repair through cell transplantation.

Mouse ESCs have already been used to generate cardiomyocytes *in vitro* using various chemicals, extracellular matrix, co-culture methods and physical stimuli (Doetschman, Eistetter et al. 1985; Wobus, Wallukat et al. 1991; Heng, Haider et al. 2004). The structural and functional analysis of these cardiomyocytes has been extensively investigated. This has led to new insights into cardiac differentiation pathways during early development. Human ESCs have also been induced to differentiate into cardiomyocytes, making the *in vitro* studies of human heart cells achievable (Schuldiner, Yanuka et al. 2000; Kehat, Kenyagin-Karsenti et al. 2001).

Thus far, most of the established methods of cell-specific differentiation of pluripotent stem cells have employed adherent culture. The average number of cells needed for cell therapy and tissue engineering varies between a few million to a few billion. For example, cardiac transplantation of differentiated ESCs would required the successful seeding of as many as 1×10^9 to 2×10^9 donor cardiomyocytes per patient after myocardial infarction (Al-Radi, Rao et al. 2003; Jing, Parikh et al. 2008). In contrast, an extracorporeal tissue engineered device with about 1×10^{10} hepatocytes is required to support a patient with acute liver failure (Tzanakakis, Hess et al. 2000). The generation of such quantities of differentiated cells is feasible through the application of SSBs.

We and others have previously established protocols for the scalable expansion of undifferentiated murine and human ESCs in SSBs as aggregates, on microcarriers and after encapsulation (Cormier, zur Nieden et al. 2006; zur Nieden, Cormier et al. 2007; Kehoe, Lock et al. 2008; Krawetz, Taiani et al. 2010; Alfred, Radford et al. 2011). Furthermore, the bioreactor has been employed by several groups including ours for the differentiation of ESCs into a variety of cell types including osteoblasts, chondrocytes and hematopoietic progenitor cells (Zandstra, Bauwens et al. 2003; Dang, Gerecht-Nir et al. 2004; Alfred, Gareau et al. 2010; Taiani, Krawetz et al. 2010; Alfred, Taiani et al. 2011). As a follow-up to these studies, we endeavored to establish a scalable bioprocess to differentiate ESCs into cardiomyocytes. This study was also conducted to evaluate the tumorgenicity of bioreactor-derived cardiomyocytes, since we had previously observed that ESCs differentiated in stirred suspension culture maintained their pluripotency compared to those differentiated in adherent culture. The manuscript presented in Chapter III of this thesis describes the findings from this study.

In this study we observed that following the removal of LIF in suspension, ESCs underwent efficient differentiation into cardiomyocytes in the presence of ascorbic acid and DMSO. This was confirmed through cardiomyocyte gene expression profiling, transmission electron microscopy and chronotropical responses of cardiac bodies. Similarly, in adherent culture, cells efficiently differentiated to cardiac bodies with characteristics of cardiomyocytes. Large scale differentiation of ESCs into cardiomyocytes has already been demonstrated in SSBs using retinoic acid (RA) (Zandstra, Bauwens et al. 2003). Although Zandstra's study developed a robust and scalable lineage selection system for cardiomyocyte differentiation, the tumorigenicity of drug treated or non-treated cells were ignored. Surprisingly, our results showed that even after differentiation toward cardiomyocytes, a sub-population of cells (54%) still expressed the pluripotency marker Oct4 demonstrating some link between Oct4 and the suspension bioreactor system. In the absence of G418 selection, bioreactor-differentiated cells retained their carcinogenic capacity *in vivo* by forming teratomas containing cells from all three germ layers. These findings were in contrast to the results from control adherent cultures, where pluripotency marker expression and tumourigenic capacity were lost in the ESC-derived cardiomyocytes. Cells from adherent culture only formed an unstructured cell mass in SCID mice. These observations are consisted with other studies that have shown the down-regulation of Oct4 expression following induced or spontaneous differentiation in adherent culture environments (Palmqvist, Glover et al. 2005).

Importantly, no tumors were derived after cardiomyocyte lineage selection using G418. Based on these experiments, we assume that functional cardiomyocytes had

been produced that did not pose a risk of tumor formation *in vivo*. However, even in the presence of drug selection, ESCs differentiated in stirred suspension culture, still maintained the expression of pluripotency markers. This trend was even more obvious in cultures not undergoing drug selection pressure, where a sub-population (about 10%) expressed both Oct4 and α-MHC simultaneously in the same cell. Furthermore, through confocal microscopy, we found the expression of Oct4 and Nanog in a sub-population of cells 20 days after differentiation in the bioreactor. From this, we hypothesized that liquid shear stress in the SSB plays an important mechanistic role in bioreactor induced pluripotency. Some of the signaling pathways that seem to be involved in this induction will be discussed in the following section.

Our observation that bioreactor-derived cardiomyocytes maintain characteristics of pluripotency has significant implications. Before the large scale generation of cardiomyocytes in suspension bioreactors will be possible, it will be necessary to eliminate bioreactor-induced pluripotency, especially since lineage selection may not be suitable for human cell therapy application.

6.1.1 Fluid shear stress and the regulation of pluripotency/differentiation in SSBs

Our group has recently evaluated the application of suspension bioreactor culture for the generation of cartilage and bone tissue (Taiani, Krawetz et al. 2010). Their observations showed that bioreactor-differentiated cells form teratomas, whereas adherent differentiation cells do not. These results also revealed that the cells located along the periphery of the aggregate expressed the highest amount of Oct4 due to exposure to a greater FSS in the SSB.

The findings from the current study also showed that bioreactor-differentiated cells retained the expression of the pluripotency markers and carcinogenic potential *in vivo* even after 25 days of induced differentiation. Unlike adherent culture-derived cells, bioreactor-differentiated cells derived teratomas containing cells from three germ layers. These findings suggested the existence of a relationship between the fluid shear stress and maintenance of pluripotency within bioreactor.

The impact of mechanical forces induced by FSS on the activation of various signaling pathways has been extensively investigated in various cell types including endothelial cells and osteoblasts (Resnick and Gimbrone 1995; Norvell, Alvarez et al. 2004). Despite the increasing employment of suspension culture systems in stem cell research, there is still lack of data regarding the influence of FSS on the regulation of pluripotency in ESCs (Kehoe, Jing et al. 2010). If any, the majority of reports investigating the FSS effects have been conducted in the context of the differentiation capacity of stem cells. Specifically, biomechanical forces have been applied to induce the differentiation of pluripotent cells into vascular endothelial cells and more recently, hematopoietic cell precursors (Yamamoto, Sokabe et al. 2005; Metallo, Vodyanik et al. 2008; Adamo, Naveiras et al. 2009).

The mechanisms by which mechanical forces influence ESC maintenance are yet to be clarified. Some of the important questions that need to be addressed include: how ESCs sense mechanical strain, the corresponding mechano-transduction signalling pathways, interaction of various pathways in controlling gene expression and the mechanism of differentiation inhibition in SSBs. In order to find out how FSS may

influence the self-renewal status of ESCs, a variety of shear sensitive signaling pathways that involve in the regulation of pluripotency are reviewed.

Shear stress can modulate gene expression through mechano-transduction, where physical signals are sensed at the cell periphery (at adhesion points), transduced into biochemical signals within the cell, ultimately resulting in cell responses, including changes in gene expression (Vogel and Sheetz 2006; Vogel and Sheetz 2009).

Numerous genetic and mechano-transduction responses have been reported for different cell types cultured under mechanical forces, such as up-regulation of certain surface receptors (Hu, Bock et al. 1998), alterations in gene expression (Cui, Bryant et al. 2004), transcriptional and post-transcriptional mechanisms (Matsuda, Morita et al. 1998; Kumar, Murphy et al. 2004), ion channel activation (Rawlinson, Pitsillides et al. 1996) and rearrangement of cytoskeletal structure (Vlahakis, Schroeder et al. 2001).

The wingless type protein (Wnt) signaling pathway is one of the key regulators of pluripotency in ESCs that can be responsive to fluid shear stress. Wnt signaling is among the most conserved evolutionary pathways and plays widespread roles in embryonic development, cell fate decision, cellular proliferation, tissue polarity, as well as stem cell maintenance and differentiation (Boiani and Scholer 2005; van Amerongen and Nusse 2009). Wnt signaling occurs either through canonical or non-canonical pathways. The canonical branch of the pathway has been shown to be involved in a variety of tumours including breast and colon cancers and the maintenance of stem cell self-renewal (Dreesen and Brivanlou 2007). In the absence of Wnt ligands, free β-catenin/Adenomatous polyposis coli (APC) complex is phosphorylated by glycogen-synthase kinase-3 (GSK-3β) and targeted for ubiquitination and proteolytic degradation.

Engagement of Wnt ligands with receptors of the frizzled (Fz) family triggers a series of events by activation of Dishevelled (Dvl) protein. Dvl inhibits GSK-3β and stabilise β-catenin in the cytoplasm. Following nuclear translocation, β-catenin forms a complex with DNA binding proteins of the T-cell Factor / Lymphocyte Enhancer binding Factor (TCF/LEF) family (Dreesen and Brivanlou 2007). This complex interacts with its consensus sequences in regulatory regions to activate transcription of certain Wnt target genes such as c-Myc, cyclin D1 (Tetsu and McCormick 1999). This signaling cascade also induces the expression of important pluripotency factors such as Oct4, Nanog and Rex1 (Sato, Meijer et al. 2004; Anton, Kestler et al. 2007).

Previous studies have demonstrated that shear stress can induce the nuclear translocation of ß-catenin into the nucleus in a variety of cell types including colon cancer cells, osteoblasts (Norvell, Alvarez et al. 2004; Avvisato, Yang et al. 2007) and endothelial cells (Estrada, Giridharan et al. 2011). As ß-catenin is an important regulator of pluripotency (Dreesen and Brivanlou 2007) and enhances Oct4 activity (Kelly, Ng et al. 2011), our research group has recently started to elucidate the role of the canonical Wnt signaling pathway in this induced pluripotency process.

Using a LEF/TCF-GFP (Lymphoid enhancer factor/T cell factor) reporter system, Brad Day in our lab has confirmed that ß-catenin nuclear occupancy is considerably increased over controls when cells are exposed to 6.1 dynes/cm^2 shear stress (100 RPM) compared to lower levels of shear generated by lower velocities (3 dynes/cm^2 or 80 RPM) of stirring and also adherent culture (Brad Day, unpublished data).

It is believed that β-catenin is a bifunctional molecule, whereby its presence in the nucleus allows the cells to remain pluripotent. They are only capable of differentiation

when lower levels of β-catenin are present in the nucleus (Anton, Kestler et al. 2007). An increase in nuclear β-catenin signaling allows for embryonic stem cells to maintain pluripotency in SSBs even without the presence of LIF. The differentiation of ESCs in adherent culture can even be rescued by culturing cells in SSBs for four days in the absence of LIF (personal communication with Brad Day, unpublished data).

Through our cardiomyocyte differentiation experiment, we found that the pluripotency of differentiated cells is maintained to a degree: EBs from a day 25 bioreactor can form ESC-like colonies and express Oct4 and Nanog. Confocal microscopy of day 25 EBs showed the simultaneous expression of α-MHC and Oct4 within the bioreactor-derived aggregates. Mechanical forces appear to act to regulate the differentiation of mouse ESCs, as indicated by the fact that after differentiation toward cardiomyocytes, the majority of cells (54%) still express Oct4. The existence of a "bipotent" Oct4/α-MHC^{+} cell population follows from our lab's recent observation that anchorage dependent cells, such as human foreskin fibroblasts, are temporarily reprogrammed in suspension culture, and begin to express pluripotency markers including Oct4, Nanog and SSEA4 (Dr. Roman Krawetz, unpublished data). These cells preserve their ESC-like morphology while in bioreactor, but lose this characteristic quickly upon culturing in adherent system. This may imply that FSS pushes cells toward a metastable pluripotent state by inducing pluripotency gene expression.

β-catenin, α-catenin and p120 also form an adherens junction complex together with cell adhesion molecule E-cadherin (Troyanovsky, Klingelhofer et al. 2011). There might be a possible mechanism whereby E-cadherin on the cell periphery responds to mechanical rotational shear stress caused by the FSS and this conformational changes

recruits vinculin to the adherens junction of cells. Indeed, the recruitment alters cadherin-catenin interaction resulting in changes to cell signaling, which in turn, is mediated by β-catenin. This hypothesis needs further investigation to reveal how E-cadherin/β-catenin complex responds to shear stress. It has been recently demonstrated that E-cadherin complex acts as a mechanosensor and vinculin localization to tension bearing sites enhances this mechanosensing response in a myosin-II dependent manner (le Duc, Shi et al. 2010). It has been also reported that induction of E-cadherin has a crucial role during cellular reprogramming, which will be discussed later in this chapter.

Although there is some evidence suggesting the involvement of the Wnt signaling pathway in the regulation of pluripotency gene expression in SSBs, the integrins are another key regulator of ESC pluripotency and these surface molecules can also be activated through fluid shear. Integrins function as mechanosensors by themselves or in cooperation with cytoskeletal proteins (Katsumi, Orr et al. 2004; Schwartz 2010). Integrins are membrane-associated adhesion glycoproteins receptors consisting of α and β subunits (Berman and Kozlova 2000). Each subunit has a short intraellular domain, a transmembrane spanning region, and a large extracellular domain. The extracellular domain binds to ECM molecules such as collagen, fibronectin and laminin. The cytoplasmic domain interacts with cytoskeleton components and signaling pathways to control cellular functions such as cytoskeletal organization and signal transduction. By attachment to the ECM, integrins physically connect focal adhesions (FAs) proteins, such as vinculin and fibronectin, to the actin cytoskeleton (Geiger and Bershadsky 2002). In the next step, actin microfilaments transmit this mechanical stress to the nucleus. It has been proposed that mechanical forces enhance the aggregation of FAs (Sawada and

Sheetz 2002) and cause integrin-dependent activation of MAPKs. Mechanical forces may also induce the conformational changes of particular FA molecules. Shear stress also causes reorientation of the actin cytoskeleton through accumulation of stress fibers by signaling molecules such as Rac GTPase, RhoA and its downstream effector, Rho-associated kinase (ROCK) (McCue, Noria et al. 2004; Noria, Xu et al. 2004). These shear-induced conformational rearrangements of the integrins are normally inhibited by Arg-Gly-Asp (RGD) peptide. It has been shown that a local force on murine ESCs via RGD coated magnetic beads increases cell spreading and leads to Oct4 gene downregulation (Chowdhury, Na et al. 2010). Application of the same force to integrins via laminin or fibronectin receptors causes the same phenomena in ESCs. In contrast, the cyclic force applied via E-cadherin molecule showed no effect on cell spreading and Oct4 expression (Uda, Poh et al. 2011). This implies that the ESCs response to mechanical stress is sensed differently via E-cadherin or integrins. Recently, it has been suggested that simultaneous signaling of integrins α5β1, αvβ5, α6β1 and α9β1 plays an important role in maintenance of pluripotency in undifferentiated mouse ESCs by inducing SSEA1, Oct4 and Nanog expression (Lee, Yun et al. 2010).

Due to the different reports regarding the role of integrins in the induction pluripotency, we conducted experiments to evaluate if the inhibition of integrins by RGD peptide can enhance cellular reprogramming of MEFs. We transduced the cells with four reprogramming factors in adherent culture and treated them with RGD peptide the day after. Although, the MEFs detached from their ECM 24 hrs post RGD treatment and formed cell clumps, we could not observe any ALP+ aggregates or the enhancement of reprogramming (unpublished data not shown, Mehdi Shafa).. It seems that the inhibition

of integrin signaling in cells growing on adherent culture is detrimental to them. In the next step, the application of RGD peptide may be examined in SSBs.

The transforming growth factor (TGF)-β signaling pathway is another factor that has been reported to be involved in both stem cell pluripotency and fluid shear stress responses. Different animal models have demonstrated the crucial role of TGF-β signaling in early cell fate commitment during embryogenesis (Munoz-Sanjuan and Brivanlou 2002) as well as regulation of ESC identity and self-renewal (James, Levine et al. 2005; Watabe and Miyazono 2009). It has been also shown that this pathway regulates many cellular events including growth, differentiation, apoptosis, tumorigenicity and migration (Yang, Song et al. 2008).

The (TGF)-β superfamily signals through two major pathways including the BMP and TGFβ/activin/nodal branches (Watabe and Miyazono 2009). Upon activation of its surface receptor though ligand binding, SMAD2 is phosphorylated at its C-terminal and associates with SMAD4 to form a complex. Subsequently, this complex translocates to nucleus where it controls the expression of certain transcription factors and co-regulators involve in ESC self-renewal. It has been known for a long time that fluid shear stress increases TGF-β transcription and production in endothelial cells through potassium channel blockade (Ohno, Cooke et al. 1995). Recent studies on endothelial cells have demonstrated that fluid shear stress causes the phosphorylation of Smad2 in its linker region. Similar to ligand activation, Smad2 binds to Smad4 and is then localized to the nucleus in a flow-dependent manner to control gene expression (Shepherd, Kos et al. 2011). Saha et al. have also shown that biaxial cyclic strain above a certain threshold inhibits human ESC differentiation and enhances their self-renewal (Saha, Lin et al.

2006). This group later suggested that strain may induce autocrine or paracrine signaling through TGFβ superfamily ligands in human ESCs (Palecek, Saha et al. 2008).

In our study, pluripotency markers such as Oct4, Sox2 and Nanog were observed to be induced in cells cultured under shear stress. In contrast, a rapid loss of Nanog on day 5 and subsequent loss of Oct4 and Sox2 on day 10 was observed in adherent culture under differentiation. Real-time PCR results in the non-drug selected bioreactors also confirmed that Oct4, Sox2, and Nanog expression persisted during differentiation in the bioreactor culture compared to adherent culture. According to these results, we may hypothesize that the activation of TGF-β receptor or Wnt by shear stress possibly induces the up-regulation of pluripotency genes in SSBs. The exact corresponding mechanism needs further investigation.

6.2 Stirred suspension bioreactor as a suitable environment for expansion and maintenance of iPSCs

Following our observations that SSB culture suppresses ESC differentiation into cardiomyocytes, we hypothesized that fluid shear stress may act as an inducing factor for the expansion and derivation of iPSCs. Similar to mESCs, miPSCs have the ability to self-renew and generate cell types from three germ layers *in vitro* and *in vivo*. One of the essential prerequisites for the clinical application of iPSCs is the large-scale expansion of cells in a cost-effective and reproducible manner. Based on adherent culture methods, lots of time and energy would be required to generate enough cells needed for clinical studies. There are several disadvantages of adherent culture including heterogeneity between plates, higher risk of contamination and increased cost due to large quantities of

material. Despite much progress in this field since the first derivation of iPSCs, all of the studies have used adherent culture conditions to generate and expand these cells. This might be due to the technical difficulties attributed to derivation and large-scale expansion of iPSCs and/or their related progeny in suspension culture. More effective and robust techniques are necessary to expand and maintain large quantities of viable, undifferentiated iPSCs for subsequent differentiation protocols.

Recently, effective suspension bioreactor techniques have been established for the large-scale production of murine and human ESCs (Fok and Zandstra 2005; Cormier, zur Nieden et al. 2006; zur Nieden, Cormier et al. 2007; Krawetz, Taiani et al. 2010). Such procedures have been also developed for neural stem cells (Kallos and Behie 1999; Kallos, Sen et al. 2003; Gilbertson, Sen et al. 2006), human mesenchymal progenitor cells (Baksh, Davies et al. 2003) and hematopoetic stem cells (Eridani, Mazza et al. 1998; Kogler, Callejas et al. 1998). Despite improvements in the development of stem cell expansion protocols, there is still a lack of data regarding the effectiveness of SSB for the large-scale maintenance and expansion of undifferentiated iPSCs.

In the study described in Chapter IV, the pluripotency and self-renewal capacity of miPSCs were well maintained through long term expansion in SSBs in the absence of tissue culture substrate and feeder cells. Maintenance of iPSCs as aggregates in suspension bioreactors is a procedure that proposes enormous effectiveness over culturing iPSCs in adherent tissue culture flasks, which is time consuming and costly. Our study demonstrates that following expansion in the presence of LIF, miPSCs retained their pluripotency; they expressed hallmark pluripotency markers at levels comparable to their starting population and could be functionally differentiated into different lineages

including cardiomyocytes, chondrocytes and osteoblasts *in vitro,* as well as to differentiate to three germ layers *in vivo* through teratoma formation. Importantly, our results showed that the SSB did not induce any chromosomal aberrations during their maintenance period.

Our study presents for the first time, a reproducible and well-controlled environment for large-quantity production of undifferentiated iPSCs. Thus far, there have been no prior reports of iPSC expansion in bioreactors, so it is hard to compare our expansion-fold-increase and growth rates with other studies. Our study demonstrates a 24-fold expansion of the highly viable and undifferentiated RS-2 iPSC line and a 58-fold expansion for iPS-3 per passage. Growth rates were lower in tested iPSCs, but consistent with those characteristic of mESCs cultured in suspension culture conditions (Cormier, zur Nieden et al. 2006; zur Nieden, Cormier et al. 2007). Doubling times for the RS-2 miPSCs averaged at 21.2 ± 1.9 hr, which is slightly longer than 14.5 hr reported for mESCs over several passages in suspension bioreactors (zur Nieden, Cormier et al. 2007), but was more closely approximated to R1 ESC line expansion on microcarriers (Fok and Zandstra 2005). Doubling times for the iPS-3 miPSCs was faster, averaging 16.4 ± 0.5 hr, which is more similar to mESCs. Our results showed a difference between two lines regarding their growth rate and doubling time. Several factors may explain the differences between two cell lines. The RS-2 line was generated using Oct4, Sox2, Klf4 and c-Myc retroviral vectors while the iPS-3 line was produced using equivalent lentiviral vectors. It is possible that the integration sites are different between two lines giving the iPS-3 line faster growth characteristics by possible activation of genes involve

in growth and cell division. Our results necessitate comparing more cell lines with different reprogramming protocols.

The physiological characteristics of ESCs are largely dependent upon the availability of oxygen, nutrients and growth factors. The unique stirring feature of SSBs provides a homogenous but dynamic system for the maintenance of cells. The role of agitation induced fluid shear stress has been discussed earlier. The optimum agitation speed helps to preserve aggregate size and reduces cell damage (Cormier J, zur Nieden et al. 2006). The agitation is important to ensure that all of the cells growing in the SSB are exposed to the same medium components and to keep the aggregates suspended. The role of dissolved oxygen and the accessibility of nutrient and growth factors are the other crucial factors that may change ESC fate decision. As the aggregate size increases in the SSB, cells within the clumps may experience lower oxygen transfer (hypoxia). Several lines of evidence suggest that different stem cells are physiologically lodged in low oxygen pressure niches (Parmar, Mauch et al. 2007; Lin, Lee et al. 2006). It has been demonstrated that upon exposure to hypoxia, expression of the Oct-4, Rex-1 and SSEA-4 is up-regulated (D'Ippolito, Diabira et al. 2006). Recently, the effect of hypoxia has been evaluated in promoting iPSC generation. Page *et al.* found that in human dermal fibroblasts, hypoxia increases the expression of Rex1 and translation of key pluripotent genes including Nanog, Oct-4 and Sox2 and their subsequent translocation to nucleus (Page, Ambady et al. 2009). Recently, it has been demonstrated that cultivation of both human and mouse fibroblasts under 5% oxygen favors the generation of iPS cells (Yoshida, Takahashi et al. 2009). The markers of pluripotency may be upregulated in the

cells residing within the cell aggregates (under hypoxic condition) in SSBs. This hypothesis can be investigated in future studies.

While we employed murine iPSCs as a model system, these protocols can be translated for the expansion of human iPSCs in suspension bioreactors. Meanwhile, in the next step, it is definitely important to develop effective and robust methods for human iPSCs maintenance to preserve their pluripotent state during long-term culture. We believe that the expansion can be facilitated through the application of SSBs.

There remain several roadblocks for improvement towards the large-scale generation of hiPSCs for therapeutic usage and disease modeling. It has been shown that culture conditions differ greatly between mouse and human ESCs. Human ESCs need a ROCK inhibitor to survive in non-adherent conditions, whereas mouse cells readily attached to each other to form cell clumps. Similar to human ESC requirements, several factors should be considered for successful human iPSC expansion in suspension bioreactors including growth factors, agitation speed, chemical additives, and inoculation density. Our group has recently started to expand several human iPSCs lines in SSBs using ROCK inhibitor. Similar to mouse iPSCs, growth rate and doubling time vary among the different cell lines, probably due to their reprogramming method (unpublished data, Dr. Guoliang Meng). It can be hypothesized that suspension bioreactor may also act to select fully reprogrammed iPSCs from semi- or non-reprogrammed cells allowing only good iPSCs to grow. Recently, one group has also reported the effect of agitation on the growth, viability and pluripotency of hESCs and iPSCs in SSB and demonstrated that the influence of FSS is cell line specific (Leung, Chen et al. 2011).

In conclusion, using the mouse model system, we showed that if well-maintained in a suspension culture environment, iPSC aggregates can grow exponentially without any major differentiation effects. Accordingly, this process purports great advantages over adherent tissue culture, since it may fulfill the need for large numbers of iPSCs necessary for regenerative medicine investigations and cell-based therapies in a controlled and cost-effective manner.

6.3 Stirred suspension bioreactors provide a selective advantage and efficient system for enhancing iPSC generation

Despite major advancements in the iPSC derivation protocols during the last few years, the reprogramming process still remains inefficient and poorly characterized. Development of a scalable and well-controllable system for the consistent and expedited derivation and expansion of iPSCs is a critical challenge that needs to be overcome. The robustness of controlled suspension bioreactor offers the potential production of large amounts of clinical grade iPSCs.

Thus far, all of the established protocols for the derivation of mouse and human iPSCs employ anchorage-dependent systems using supporting feeder cells or extracellular matrix (ECM) such as matrigel, fibronection or collagen. In order to produce large quantities of normal cells with clinical importance, it would be necessary to develop scalable and reproducible bioprocesses. Our study demonstrates the expedited derivation of iPSCs from MEFs in stirred suspension cultures in a feeder and ECM-free culture system. We were able to achieve the emergence of ~100,000 ALP^+ aggregates representing more than 10 million fully reprogrammed iPSCs occurring by day 16. iPSC

aggregates appeared to be fully reprogrammed to pluripotency based on the expression of specific markers, morphology, and *in vitro* and *in vivo* differentiation assays as well as germline transmission. Based on the expression of ALP and pluripotency markers, we observed a significant enhancement of MEF reprogramming kinetics compared to adherent culture environment.

We observed cell aggregates on day 5 post transfection and 3 days after inoculation into bioreactors for both four (OKSM) and three factor (OKM) reprogramming. The cultures in bioreactors consisted of small aggregates and single cells on day 5 with more compact aggregation observed by day 10. This aggregate compaction depends upon the up-regulation of several cell adhesion pathways such as cell-cell junction (E-cadherin), gap junctions and tight junctions. Recently, several studies have revealed the crucial role of E-cadherin for ESC pluripotency and also during somatic cell reprogramming (Chen, Yuan et al. 2010; Redmer, Diecke et al. 2011). It has been reported that E-cadherin is the major cadherin molecule in ESCs that controls cellular growth through both signal transduction activity and adhesive binding activity.

The OKSM reprogramming experiment had the highest efficiency based on the percentage of ALP^+ aggregates and viable cells. The lowest efficiency was observed for the OSM and KSM reprogramming, which reveals the important role of Oct4 and Klf4 in cellular reprogramming. Although some ALP^+ aggregates (about 2% on day 5) appeared in KSM experiments, we failed to expand them and the cells did not enter into a self-renewal state. The OKSM and OKS SiPSCs were thoroughly characterized *in vitro* and *in vivo*. Among the other 3F-SiPSCs, OKM has been selected to be fully characterized. The pluripotency of OKM-SiPSCs was shown by immunostaining for major pluripotency

markers and teratoma formation assay. We are currently conducting experiments to confirm the pluripotency of OKM SiPSCs by chimera formation and germline transmission assays.

Our results demonstrated that the cell aggregates express E-cadherin around day 10 post transfection, but the formation of aggregates as early as day 5 shows the existence of cell-cell adhesion, which could be mediated by E-cadherin. As mentioned earlier, there has been increasing evidence that shear stress can modify gene expression through a mechanotransduction phenomenon. There is a possibility that upon up-regulation of E-cadherin during reprogramming in SSBs, physical signals are sensed at the transformed MEF surface by E-cadherin, which are transduced into biochemical signals within the cell. This effect can further reinforce pluripotency in cells undergoing cellular reprogramming. Some of the possible mechanisms of shear-induced pluripotency have been discussed earlier in this chapter.

In order to ensure that each aggregate resulted from one induced cell, our lab has performed the clonal analysis of bioreactor expanded stem cells using SYTO-13 dye. Our observations suggest that individual aggregates were clonally derived in SSB (performed by Dr. Roman Krawetz). Accordingly, the efficiency of the method was calculated based on the ratio between the number of ALP^+ aggregates and the number of starting fibroblasts. On day 12 of suspension culture, we observed ~4.0×105 ALP^+ aggregates from an input for 8.0×10^5 original fibroblasts, which suggests an efficiency of 0.5 for 4F-SiPSCs. Based on this result, each cell clump can be isolated and expanded as a separate iPSC line either in adherent or suspension culture. Additionally, by passaging

the cell aggregates using more SSBs, the number of fully reprogrammed iPSCs can be expanded to more than 1×10^8 by day 16.

Upon the induction of reprogramming factors, transformed cells initiate the process of mesenchymal to epithelial transition (MET). During the transition, anchorage dependent spindle-shaped cells transit to polarized epithelial cells with tight cell-cell contacts. Epithelial-associated genes such as E-cadherin are upregulated while mesenchymal genes such as Snail, Slug and N-cadherin are downregulated within the first few days (Samavarchi-Tehrani, Golipour et al. 2010). Recently, it has been demonstrated that during mouse iPSC derivation, Oct4/Sox2 suppress Snail (a epithelial to mesenchymal transition (EMT) mediator), while Klf4 induces epithelial-related genes including E-cadherin (Li, Liang et al. 2010). Furthermore, the promoter region of E-cadherin gene (*CDH1*) contains Oct4 binding elements (Huang, Chen et al. 2009). Our lab has recently showed that the fluid shear stress causes the up-regulation of Oct4 and reinforces E-cadherin expression in SSBs (Brad Day, unpublished data).

During the course of this study, we tried to culture MEFs in SSBs without any transfection to evaluate the impact of fluid shear stress on their survival and aggregation state. This idea follows our lab's previous observation that anchorage dependent cells, such as human foreskin fibroblasts begin to express Oct4 and other markers of pluripotency upon culture in SSBs (unpublished data). Our results showed that unlike human fibroblasts, MEFs could not survive in suspension and die after a few days. Although we observed some small MEF clumps, they quickly disappeared. We hypothesized that the inoculation of MEFs into the bioreactor after transfection would rescue their death and increase their growth and survival rate. In this scenario, it is

conceivable that MEFs that have not initiated the cellular reprogramming and so could not survive in the bioreactor. Because fibroblasts are substrate dependent and cannot grow in suspension culture, SSB culture may also be selecting for aggregate formation (reprogrammed cells) during the course of reprogramming, allowing transformed cells to divide in an open environment.

We tried to extend our derivation protocols to the derivation of human iPSCs in suspension bioreactors (performed by Mehdi Shafa). Despite several attempts, we could not generate fully pluripotent human iPSCs. Following retroviral transduction with four Yamanka factors (OKSM), we inoculated the SSBs with human foreskin and dermal fibroblasts in separate experiments. We observed small cell aggregates and the expression of ALP as one of the early markers of reprogramming on day 10 post transfection in both cell types. However, the cell aggregates could not survive and were eliminated after a few days. This result shows the different requirements that mouse and human pluripotent cells need to grow and divide under fluid shear stress. Indeed, the idea is reflected by the fact that hESCs are not easily expanded in SSBs and required additives such as ROCK inhibitor to facilitate aggregation and rapamycin to suppress the spontaneous differentiation of human ESC-derived fibroblasts which otherwise cause aggregates to fall apart (Krawetz, Taiani et al. 2010). The recent recognition of similarities between mouse EpiSCs and human ESCs raise challenging questions regarding the true origin, ground state and *in vivo* equivalence of human ESCs during development (Lovell-Badge 2007). Until recently, hESCs were considered to be the human counterpart of mouse ESCs; however, it seems they are intrinsically closer to mouse EpiSC with regard to their *in vitro* characteristics (discussed in Chapter II) and

need the same culture requirements. Since chimera formation and tetraploid complementation tests cannot be performed using human ESCs, there are some arguments that mouse ESCs and EpiSCs/human ESCs are two distinct pluripotent states. Currently, the lab is attempting to derive true hESCs and to develop a method for expanding these cells in SSBs. After these conditions are determined, we may successfully derive human SiPSCs.

In summary, our study demonstrates that suspension cultures provide a selective advantage for enhancing iPSC generation. We believe that both inductive and selective events are occurring with bioreactor-induced reprogramming. Our results show for the first time that fibroblasts can be efficiently reprogrammed in SSBs without using c-Myc or Sox2 as reprogramming genes. Combined with new and safer methods of reprogramming, such as protein/microRNA or small molecules that do not use integrating genetic constructs, our SiPSC technology has the potential to accelerate and standardize iPSC research, bringing it to clinical application more quickly.

Chapter Seven:

Conclusions and Future Directions

iPSC technology has received considerable attention in tissue engineering and regenerative medicine since it potentially offers an unprecedented source of patient-derived pluripotent cells for disease modeling and cell therapies. The ground-breaking study by Yamanaka and colleagues to generate pluripotent cells using transcription factors lessened the ethical arguments surrounding human ESCs. Over the past five years, there have been enormous advancements in the iPSC field. Now, different types of somatic cells from various animals have been successfully reprogrammed to the iPSC state with the aid of viral or non-viral vectors. Since the first generation of iPSCs, experimental studies have concentrated on establishing better tools to improve the kinetics of this process. Despite huge progress, iPSC derivation is still inefficient and several weeks are required for an iPSC line to be established. In turn, the clinical application of these cells necessitates the development of an economical, scalable, and reproducible approach for iPSC production.

The current study was initially undertaken for one major purpose: to develop an effective bioprocess environment for scalable differentiation of ESCs into cardiomyocytes in SSBs. However, during the course of this study, our observations with the suspension bioreactor and its impact on cardiomyocyte differentiation caused the direction of this study to change in order to evaluate the effectiveness of SSBs for the expansion and derivation of mouse iPSCs. The main conclusions from the studies presented in this thesis are:

1. The stirred suspension bioreactor suppresses ESC differentiation in favour of pluripotency possibly through a fluid shear stress mechanotransduction phenomenon. Addition of medium enhancers is not adequate to force complete differentiation of the cell population.
2. Stirred suspension culture provides a suitable environment for the expansion and maintenance of mouse iPSCs without loss of pluripotency over a long-term period.
3. The stirred suspension culture environment enhances the kinetics of cellular reprogramming providing a selective advantage for iPSC generation.

The results of this thesis raise some important questions regarding the role of fluid shear stress in ESC pluripotency and differentiation that need to be addressed.

First, to better establish an efficient bioprocess for the differentiation of ESCs into cardiomyocytes, different shear blocking agents should be evaluated to uncouple the effect of shear stress in SSBs. These may include the application of chemicals or peptides that inhibit various shear responsive signalling pathways including integrins, E-Cadherin and Wnt or protect the cell membrane from the effects of shear forces (such as pluronic F68 and polyethylene glycol).

Second, the SiPSC technology should be carried out to derive human iPSCs. Different culture media with various chemicals that increase the survival of human iPSCs must be used during the reprogramming process.

Third, due to the effect of shear stress in enhancing iPSC generation with four and three factors, the application of fewer transcription factors along with DNA

demethylating agents, chromatin modifying drugs and key developmental signalling pathway molecules should be tested in suspension bioreactors.

Finally, SSB derivation experiments must be conducted to evaluate the mechanism of fluid shear–induced pluripotency and the role of stress responsive genes during reprogramming. Microarray, epigenetic mapping or chromatin immunopercipitation (ChIP) experiments can be useful. By elucidating the exact mechanism(s) by which liquid shear stress may contribute to promoting pluripotency and preventing differentiation, we will be able to create an efficient environment for both the production of large quantities of pluripotent stem cells, and their differentiated progeny.

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