Parameter Optimisation for Expansion of Pluripotent Stem Cells

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by

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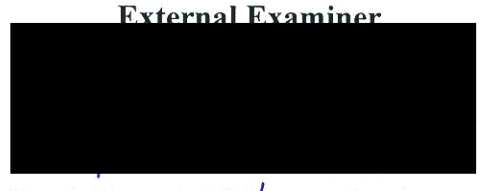

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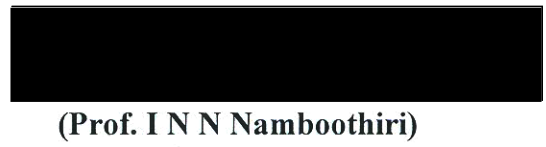

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For Bapi,

my north star

ABSTRACT

Pluripotent stem cells have the unique properties of extensive replication and ability to differentiate into derivatives of all three germ layers. By virtue of this, they have been deemed to be of immense importance in the fields of tissue engineering, regenerative medicine, drug discovery, disease modeling, etc. The field of stem cell culture has undergone huge advancements over the past few years, with extensive studies being carried out for the various intrinsic and extrinsic parameters involved in pluripotent stem cell culture. This thesis looks at the effect of some of the extrinsic parameters involved in large scale expansion of pluripotent stem cells *in vitro*.

The first work looks at the ability of two new materials for viability and pluripotency maintenance of mouse embryonic stem cells. Traditionally, the pluripotent stem cells are grown as co-culture on a feeder layer of inactivated mouse embryonic fibroblasts. Efforts are still being made to replace the feeder cells with appropriate biomaterials to avoid cross contamination of the pluripotent cells. In this work, tropoelastin was tested as a 2D coating material and compared with gelatin and matrigel coating. Cell count, pluripotency analysis by flow cytometry and visual inspection of colony morphology were carried out for mouse ES cells grown on all three materials to test the suitability of tropoelastin in comparison to the other two well known materials. It was observed that although tropoelastin was able to maintain cell growth and pluripotency, the number of viable cells obtained was lower in comparison to gelatin and Matrigel. PMVE- MA + gelatin composite was used for 3D scaffold preparation and compared with pure gelatin scaffold. Cell attachment, viability, and pluripotency maintenance measurement were undertaken for mouse ES cells on both the scaffolds; the novel scaffold on PMVE- MA+ gelatin was found to be suitable for the expansion of mouse ES cells. Preliminary comparison between commercially available Cytodex 3 and Hillex II microcarriers' ability to maintain mouse ES cells was also carried out. Cell attachment and expansion were found to be better on Cytodex 3 in comparison to Hillex II in static culture.

Encouraged by the findings in the previous work, Cytodex 3 microcarrier were used for long term expansion of mouse induced pluripotent stem cells in a spinner flask. Optimization of the spin rate was first carried out for maximum carrier suspension without breaking them. The optimized range was then tested for attachment of iPS cells on the carriers. Long term culture of mouse iPS cells was then carried out followed by characterization of the cells growth, viability, pluripotency and multilineage differentiation capability. The results suggested that long term culture of mouse iPS cells in a spinner flask was possible by using Cytodex 3 without any prior coating of the carriers or prior adaptation of the cells to a feeder free culture at an optimized spin rate of 25 RPM.

The third work looks at the effect of change in pH and increase in lactate concentration on mouse pluripotent stem cells. Cell viability, proliferation rate and pluripotency were investigated to see the effect of pH and externally added lactate on pluripotent stem cells, both feeder dependent and feeder independent. A pH of 7.5 was found to be optimum for feeder independent embryonic stem cells while the optimum range for feeder dependent pluripotent stem cells (both embryonic and induced) was between 7.0 and 7.5. Any change in medium pH from the optimum value had detrimental effects on cell proliferation, metabolic activity and pluripotency. The effect of increased lactate concentration in the medium was also studied. It was seen that cell proliferation decreased with an increase in lactate concentration in the medium while there was no significant change in cell pluripotency. Interestingly, cellular metabolic activity assay and analysis of spent medium suggested that the mouse pluripotent stem cells were able to metabolise the externally added lactate, a feature so far known to exist only in a few cell types eg. CHO cell line.

Conditioned media from MEFs have been used for culturing of pluripotent stem cells for a long time. However, being a spent media, metabolite concentration and pH changes are expected. Hence, an experimental and theoretical analysis of MEF conditioned media was taken up to estimate changes in key metabolites like glucose and lactate. The study utilised the High-Performance Liquid Chromatography (HPLC) method for experimental estimation of glucose uptake and lactate production in the conditioned media at specific time intervals. At the end of 72 hours, glucose concentration decreased from 3.05 mg/ml to 2.03mg/ml. Accumulated lactate

concentration reached a final value of 2.23mg/ml in the same time. Theoretical modeling of the same was performed to extend the study beyond the experimentally studied time points.

All these studies highlight the importance of individual optimization of the various parameters involved in the large scale expansion of pluripotent stem cells. It needs to be kept in mind that it is possible that the effect of some of these parameters may be interlinked and calls for further studies in this field.

ABBREVIATIONS

AP - Alkaline Phosphatase

bFGF – Basic Fibroblast Growth Factor

BHK – Baby Hamster Kidney

BrdU – Bromodeoxyuridine

BSA – Bovine Serum Albumin

CHO – Chinese Hamster Ovary

DMEM – Dulbecco's modified Eagle's medium

DMSO – Dimethyl Sulfoxide

EB – Embryoid Body

ECM – Extra Cellular Matrix

ES Cell – Embryonic Stem Cell

FACS – Flow Associated Cell Sorting

FITC – Fluorescein isothiocyanate

FBS – Fetal Bovine Serum

GFP – Green Fluorescence Protein

HCASMC – Human Coronary Artery Smooth Muscle Cells

HPLC – High Pressure Liquid Chromatography

HUVEC – Human Umbilical Vein Endothelial Cells

ICM – Inner Cell Mass

iPS Cell – Induced Pluripotent Stem Cell

LIF – Leukemia Inhibitory Factor

MEF – Mouse Embryonic Fibroblast

MTT – 3-(4, 5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide

NMR – Nuclear Magnetic Resonance

PBS – Phosphate Buffered Saline

PEG – Poly ethylene glycol

PET – Poly ethylene terepthalate

PGA – Poly glycolic acid

PI – Propidium Iodide

PLA – Poly lactic acid

PLGA - Poly(lactic-*co*-glycolic acid)

PMVE-MA – (Poly Methyl Vinyl Ether) - alt- Maleic Anhydride

PSC – Pluripotent Stem Cell

ROCK – Rho- associated Protein Kinase

RPM – Revolutions per Minute

RT – PCR – Reverse Transcription Polymerase Chain Reaction

SEM – Scanning Electron Microscopy

STLV – Slow Turning Lateral Vessel

2D – Two Dimensional

3D – Three Dimensional

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Chapter 1 INTRODUCTION

1.1 Background:

Life is regenerative by definition but, by and large, most cells in the mammalian body lack the capacity to regenerate, unlike those of hydras and newts. Although some cells in the body have inherent capability to regenerate and repair, most of the body's cells only form specialized cells for which they are destined. As a result of this specificity of cells' chronic diseases, extensive organ injuries are considered to be fatal[1]. The quest for effective treatments of such incurable degenerative diseases has been one of the long lasting challenges for the world of scientific research. The fields of tissue engineering, cellular therapy and regenerative medicine are deemed to be of immense importance in solving these problems. Tissue engineering combines the biological importance and understanding of cells with engineering, along with suitable physicochemical and biochemical factors to replace and/or improve organs and/or their biological functions. Regenerative medicine is an emerging field of research and therapeutics to restore, maintain and improve normal functions of organs by delivering safe and effective therapies composed of cells administered either alone or in conjunction with specifically designed materials[2]. The discovery of stem cells and their potential use for such therapeutic purposes has been established recently. Along with their potential application in cellular therapy, stem cell technology has enormous potential in the fields of regenerative medicine, drug screening and disease modeling. The use of stem cells for such a vast field of application requires it to be readily available *in vitro*, which in turn requires the optimization of various intrinsic as well as extrinsic parameters.

1.1.1 Thesis Objective

This thesis looks at the effect and optimization of some of the important extrinsic parameters involved in large scale expansion of pluripotent stem cells *in vitro.* The overall objective of the thesis is to study the effect of some of the extrinsic parameters involved in large scale *in vitro* expansion of pluripotent stem cells. The parameters included in the study are growth surface/ material, pH as a physicochemical factor, lactate and dynamic motion or shear stress.

1.2 Organization of the Thesis:

The thesis is organized into seven chapters presented sequentially followed by an appendix and the reference section.

Chapter 1, entitled "*Introduction",* gives a brief background for the research followed by an extensive but concise literature review of the field.

Chapter 2 is the "*General Methods and Materials"* chapter and records the common materials and methods used throughout the work.

Chapter 3, entitled "*Expansion of Embryonic Stem Cells on Different Biomaterials in 2D and 3D Culture Systems",* reports the expansion of mouse embryonic stem cells on different artificial biomaterials in static 2D and 3D culture systems. Both commercially available as well as novel biomaterials have been used for these studies.

Chapter 4, " Expansion of Mouse induced Pluripotent Stem Cells in a Spinner Flask System: Effect of RPM on Microcarriers and Cells", focuses on the expansion of induced pluripotent stem cells in a dynamic spinner flask.

The study of the effect of change in medium pH and externally added lactate in medium on pluripotent stem cells (both embryonic and induced) forms the subject of *Chapter 5, "Effect of Lactate and pH on Expansion of Pluripotent Stem Cells".*

Chapter 6, entitled "*Fibroblast Conditioned Media Analysis and Comparison with Theoretical Modeling",* discusses glucose and lactate metabolism analysis of spent medium from mouse embryonic fibroblast. Apart from giving an estimation of cellular metabolism of the fibroblast, the study also provides us with an estimation of lactate concentration limit for future studies involving only lactate or fibroblast conditioned media.

The last and final chapter of this thesis, *Chapter 7,* is the *"Conclusion",* which includes a general conclusion and future prospects of the work along with possible extensions of this thesis.

The *Appendix* includes optimization experimental details for some of the methodologies used in this work.

1.3 Literature Review:

The first step to any research is an in-depth literature review to acquire knowledge on the status of research in the field and to identify the gaps in the same, along with necessary questions to be answered. The following paragraphs focus on an in-depth but concise literature review on stem cells, specifically focusing on pluripotent stem cells, stem cell niche and some of the extrinsic parameters involved in large scale *in vitro* expansion of the same.

1.3.1 Stem Cells:

Stem cells are a unique type of cells defined and identified by their properties of self-renewal and capability of differentiating in to mature cells. Specifically, it is well accepted that stem cells must fulfill the following criteria[3]:

1. The cells must be capable of self-renewal and undergo symmetric or asymmetric divisions for population maintenance. Symmetric division implies that both daughter cells retain full stem cell characteristics while asymmetric division refers to the process whereby one of the two daughter cells remains a stem cell while the other goes on to a differentiation pathway.

2. A single cell is capable of multilineage differentiation. That is, it is able to differentiate into multiple lineages that are not due to the presence of a mixture of cells having different differentiation potential, but due to only one cell.

3. The cells must be capable of *in vivo* functional reconstitution of a given tissue.

1.3.2 Stem Cell Classification:

Stem Cells can be classified according to their origin, i.e., their place of derivation, natural or artificial as well as according to their plasticity or their developmental versatility. Figure 1.1 shows a schematic diagram of the different types of stem cells according to the above two broad classification umbrellas.

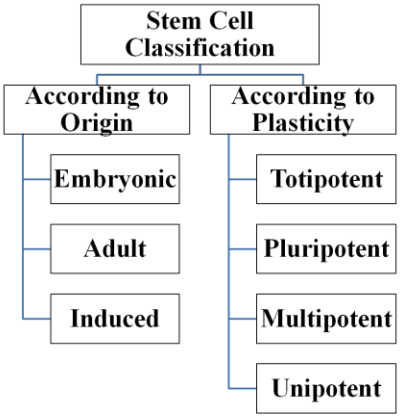

Figure 1.1 Classifications of stem cells according to their origin and plasticity

❖ **According to Origin**:

Stem cells are classified into three types according to their origin.

a. Embryonic: Typically derived from the inner cell mass of the blastocyst, an early stage embryo.
b. Adult: Undifferentiated stem cells found within the body after embryonic development.
c. Induced: Artificially produced stem cells by de-differentiation of adult somatic cells by specific factors.

❖ **According to Plasticity:**

Stem cells are also classified based on their potency or plasticity, i.e., their differentiation capabilities.

a. Totipotent: A totipotent cell has the ability to divide and produce all types of cells in the body including the extraembryonic tissues. Essentially, a totipotent stem cell has the capability to form a complete organism. The zygote and blastomeres of the two and four cell stage in mouse are totipotent in nature.

b. Pluripotent: A pluripotent cell is capable of differentiating into all types of cells in the body except the extraembryonic tissues or the placenta.

c. Multipotent: A multipotent stem cell is able to differentiate into different cell types but only of closely related cell family or in to cells of a single germ layer, e.g., Hematopoietic stem cells can give rise to the different blood cells.

d. Unipotent: A unipotent stem cell can self renew and differentiate into only one specific cell lineage. For example, muscle stem cells can give rise to only mature muscle cells.

Each of the different classes of stem cells has its own advantages, uses, limitations and bioprocess challenges. Figure 1.2 gives a figurative outline of these aspects for all stem cell types currently studied. The pluripotent stem cells have the highest expansion and differentiation potential but at the same time, the complexity of its *in vitro* culture is also the highest along with ethical complications. The ease of cell availability along with their *in vitro* culture process increases as the potency of the cells decrease. Due to their unique ability to differentiate into any cell type, pluripotent cells are most extensively studied for their potential use in the field of biology and medicine

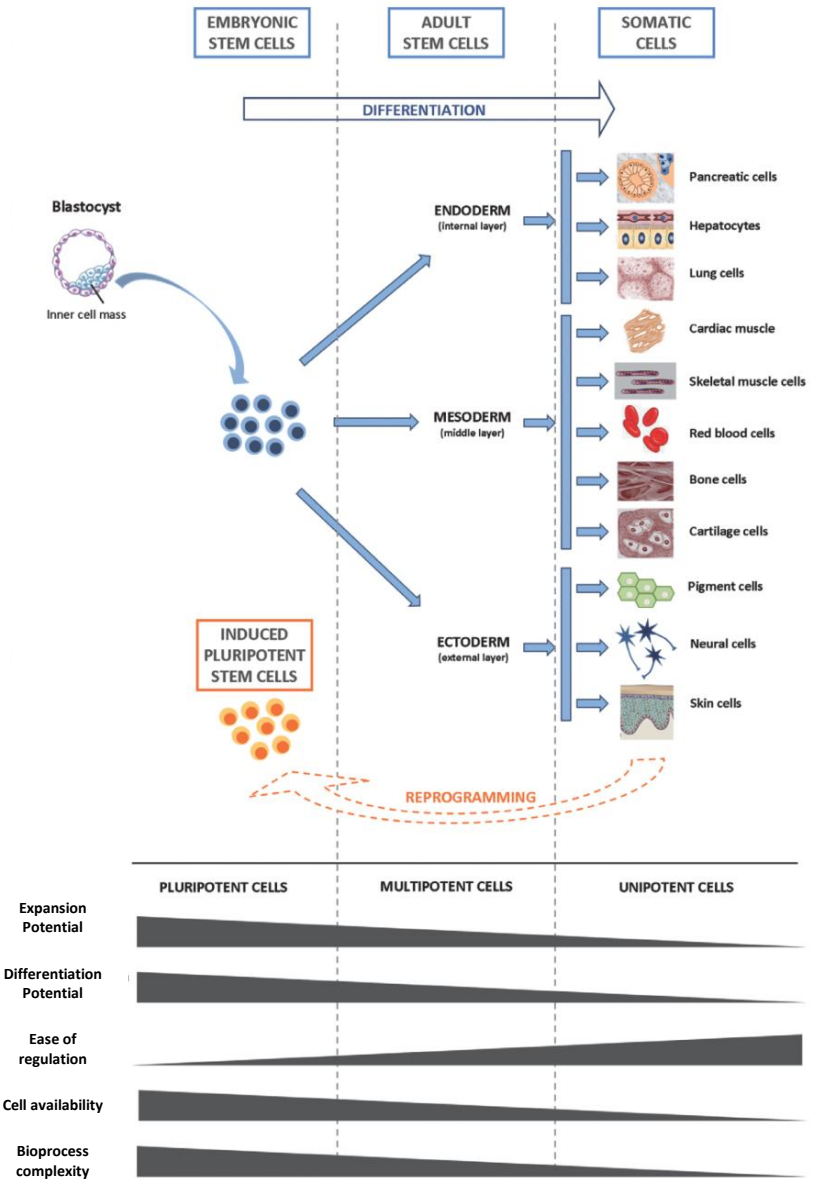

Figure 1.2 Stem cell sources and their characteristics [4]

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1.3.3 Pluripotent Stem Cells:

Jackson and Brues first voiced the idea of cellular pluripotency in the year 1941 during their study of mouse embryonal tumors. Repeated transplantation of tumor fragments led to the formation of a large number of tumors containing various tissues, leading to speculation about the pluripotent nature of the embryonal cells in the tumor[5]. Pluripotent stem cells are identified by their unique ability to give rise to cells of all three germ layers while having unlimited expansion potential. They can be naturally obtained from the inner cell mass (ICM) of an embryo

or artificially generated by nuclear transfer or cell fusion between an adult cell and a naturally obtained pluripotent one. Pluripotent cell can also be generated by 'reprogramming' adult cells, and are known as induced pluripotent stem cells (Figure 1.3). Pluripotent stem cells are characterized by the presence of pluripotent markers like Oct4, Nanog, SOX2, SSEA 1, SSEA 4, etc. The cells show positive staining to alkaline phosphatase and are able to form teratoma *in vivo*.

Pluripotent stem cells have a number of uses including broad uses in the field of basic research as well as use in clinical therapy. They are known to be an excellent tool to study human development and organogenesis. They are critical in investigating safe and new therapeutic techniques as well as for disease modeling and novel drug testing. Apart from these, pluripotent stem cells also have immense potential usage in the field of tissue engineering, regenerative medicine and clinical therapy[6]. Pluripotent stem cells derived tissues have been used to treat diseases like diabetes and cardiac problems[7, 8].

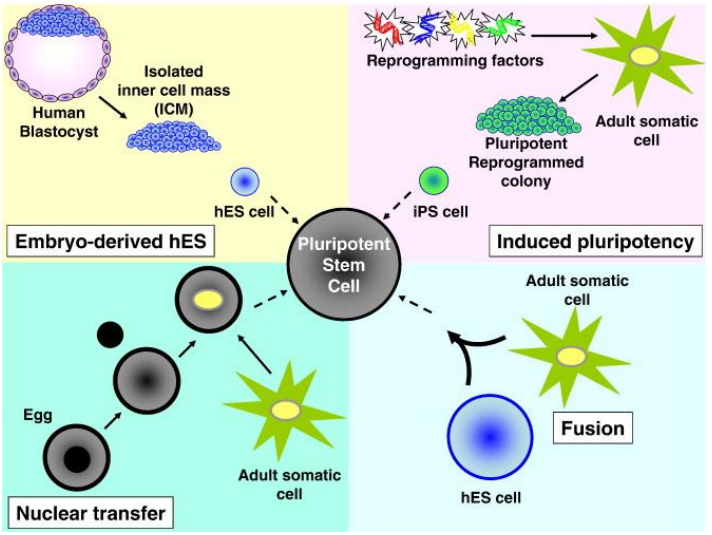


Figure 1.3 Generation and classification of pluripotent stem cells[9]

❖ **Embryonic Stem Cell**:

Embryonic stem (ES) cells are naturally occurring stem cells originating from the inner cell mass (ICM) of the pre implantation blastocyte, an early stage embryo at around 5- 6 days post fertilization in humans and at around 3 days in mouse. They are pluripotent in nature and when *in vivo*, gives rise to a proper embryo. They were first derived from mouse in 1981 and almost

two decades later from human in 1998[10-12]. *In vitro*, these cells are capable of self renewal for a number of passages and are capable of spontaneous or directed differentiation to cells of all the three germ layers.

❖ **Induced Pluripotent Stem Cell:**

Induced pluripotent stem cells, commonly abbreviated as iPS cells or iPSCs, are pluripotent stem cells artificially derived from a non-pluripotent cells, typically adult somatic cells, by 'forced expression' of certain genes. Induced pluripotent stem cells are believed to be virtually identical to ES cells in terms of basic characteristics like colony morphology, gene expression pattern, potency, doubling time, etc. They were first generated in 2006 by Yamanaka et al. from mouse somatic cells, followed by human cells in 2007[13, 14]. The four 'factors' used by Yamanaka were Oct, Sox, c- Myc and Klf4. The ethical issues associated with derivation and use of embryonic stem cells make iPS cells of immense interest and importance.

1.3.4 Stem Cell Niche:

The niche concept was introduced in 1978[15]. Stem cell niche is a phrase loosely used to describe the microenvironment in which stem cells are found, and which interacts with stem cells to regulate stem cell fate. During embryonic development, various factors act on embryonic stem cells within the niche to alter gene expression, and induce their proliferation or differentiation for the development of the fetus. Within the human body, stem cell niches maintain adult stem cells in a quiescent state. When needed, the surrounding microenvironment actively signals to stem cells to either promote self renewal or differentiation to form new tissues.

A Nature Insight review defines niche as follows:

"Stem-cell populations are established in 'niches' — specific anatomic locations that regulate how they participate in tissue generation, maintenance and repair. The niche saves stem cells from depletion, while protecting the host from over-exuberant stem-cell proliferation. It constitutes a basic unit of tissue physiology, integrating signals that mediate the balanced response of stem cells to the needs of organisms. Yet the niche may also induce pathologies by imposing aberrant function on stem cells or other targets. The interplay between stem cells and their niche creates the dynamic system necessary for sustaining tissues, and for the ultimate

design of stem-cell therapeutics...The simple location of stem cells is not sufficient to define a niche. The niche must have both anatomic and functional dimensions" [16].

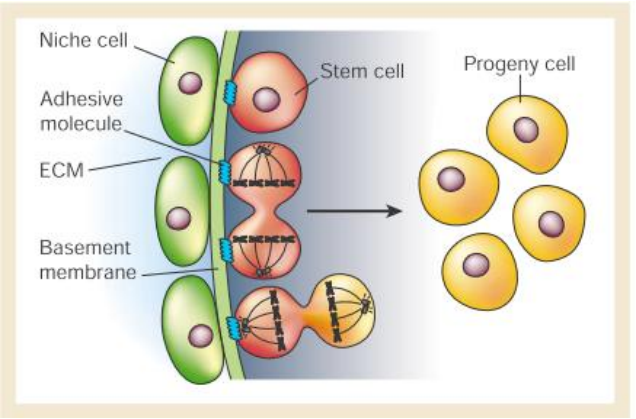


Figure 1.4 Schematic diagram of stem cell niche[17]

The implementation of stem cell technology requires a ready source of these cells along with their differentiated derivatives *in vitro* or, in other words, mimicking the stem cell *in vivo* 'niche'. This has proved to be a challenge for the scientific community, since deciphering the intricacies of the *in vivo* system is still incomplete. The stem cell niche is a complex interplay between a number of intrinsic and extrinsic parameters like cellular interaction, interaction between cells and adhesion molecules, extracellular matrix components, growth factors, cytokines, inhibitors, oxygen tension, fluid flow rate, mass transfer and various physicochemical nature of the environment like the pH.

1.3.5 Need for Optimized Large Scale Culture:

The exponentially growing field of stem cell research requires the implementation of efficient technologies from the field of biotechnology in order to test and optimize the different parameters for efficient and maximum expansion of pluripotent stem cells. The bench top culture methods for stem cells are not enough to suffice its large scale requirement for the purpose of clinical therapy, regenerative medicine, etc. For example, it is estimated that 10^{16} human pluripotent stem cells (hPSCs) – equating to approximately 286 million T175 tissue culture flasks would be needed to treat 250,000 patients with cardiomyocyte replacement[18], highlighting the need for large scale expansion of pluripotent cells and/or their derivatives outside the body.

The level of complexity involved in such a culture is much higher in comparison to the regular laboratory methods, giving rise to the need of a robust bioprocess system. The ideal strategy for such goals is to identify the key factors involved, followed by engineering culture approaches that allow for 3D expansion in a bioreactor based system with finely controlled intrinsic and extrinsic (environmental) conditions. Stem cell fate is highly dependent on the cues of its extracellular environment, which operate at different temporal and spatial scales, drive specific cellular fates and decide on self renewal, differentiation or apoptosis of the cells (Figure 1.5).

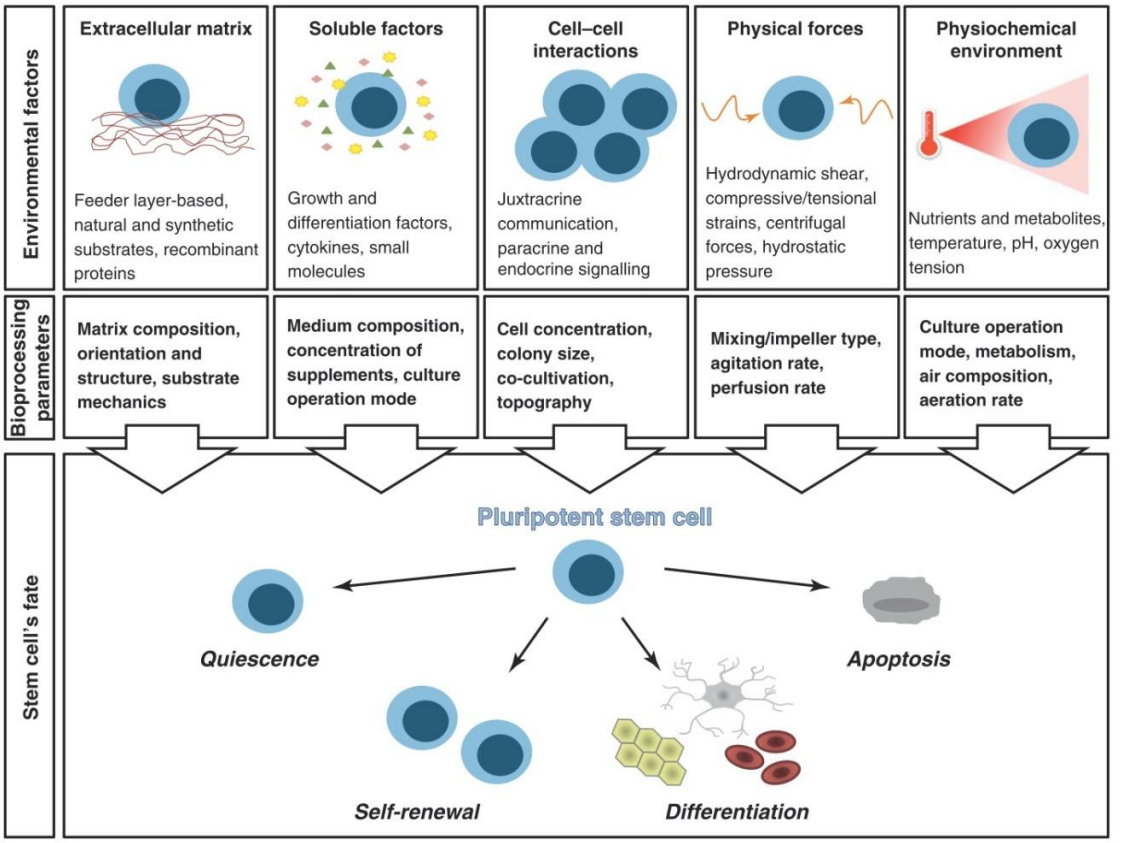


Figure 1.5 Environmental factors and bioprocessing parameters involved in pluripotent stem cell culture and fate decision[19]

Efforts have been made to identify, study and optimize the various cues such as - (i) the extracellular matrix or adherence surfaces (biomaterials), (ii) soluble factors like cytokines and growth factors, (iii) physical forces like hydrodynamic shear, (iv) physicochemical factors like pH, metabolites and (v) cell- cell interactions [19].

The following paragraphs look at the role of some of the above mentioned parameters in expansion and pluripotency maintenance of pluripotent stem cells *in vitro*.

1.3.6 Biomaterials and ECM:

Pluripotent stem cells are adherent in nature; their surrounding matrix along with the associated proteins, cytokines, signaling molecules, etc, are an integral part of the microenvironment of the cells *in vivo*. Traditionally *in vitro*, they are grown as a co-culture on 'feeder cells'. The feeder cells, also known as feeder layer, have a two fold utility; they serve as an extracellular attachment matrix and also supply soluble growth factors necessary for maintaining cell pluripotency. Inactivated mouse embryonic fibroblasts are the oldest and most widely used feeder cells. Recently, various other fibroblast lines have also been tested and used for this purpose, e.g., human fibroblasts have been used for the expansion of human pluripotent stem cells[20]. However, the use of feeder layer is a hindrance to clinical study and use of pluripotent cells. Concerns include cross species contamination and lack of purity in cell population. Also, the use of feeder cells is a time consuming process since it requires the use of fresh feeder cells during every passaging.

Ito et al., in 2007, used formaldedehyde and gluteraldehyde to generate chemically fixed feeder cells to culture mouse embryonic stem cells, thus helping in getting a relatively pure stem cell population[21]. Physical separation of feeder layer and pluripotent cells has also been tested. Human pluripotent stem cells and adipose cells used as feeder cells were grown on the inside and outside of inserts containing 1µm porous membrane, respectively. This system maintained a physical contact between the cells without any inter mixing between the two cell types, and was shown to maintain both viability and pluripotency of the cells. Interestingly, when the two cell types were kept physically apart but in the same culture system, it failed to support the pluripotent cells, highlighting the necessity of direct interaction between the feeder cells and the pluripotent cells[22].

Keeping in mind the importance of feeder cells in terms of secretory factors, decellularized ECM matrix from MEFs was used to culture human ES and iPS cells. The cells grown on such substrates showed colony morphology, pluripotency marker expression, multilienage differentiation capabilities and gene expression patterns similar to cells grown directly on feeder

layer[23, 24]. Currently, efforts are being made to completely replace the feeder layer with suitable biomaterials that resemble the natural ECM, in conjunction with fibroblast conditioned media, specialized commercial media and/or cell specific growth factors or cytokines[25-29]. Advances in the field of biomaterial synthesis and processing have led to the discovery and use of a range of biomaterials for pluripotency maintenance and cell/tissue specific differentiation. Biomaterials can be used as coating for 2D cultures or as scaffolds, hydrogels, microcarriers, hollow fibres, microcapsules, etc, for 3D cultures.

A 3D culture system not only provides an attachment surface for the cells but also helps in mimicking the *in vivo* "niche". The larger surface area: volume ratio offered by a 3D system is also of importance in case of large scale expansion of the cells. 3D cultures like scaffolds, hollow fibres and macroporous carriers in which the cells grown within the system also offer protection to the cells from fluid stress in case of dynamic culture systems. Designing a 3D culture system requires the consideration of a large number of variables: material, porosity, biocompatibility, mechanical properties, and degradability. The production of a 3D culture system is a time consuming one, so in many cases novel biomaterials have been used as 2D coatings on tissue culture systems to test their suitability for pluripotent stem cell culture and/or differentiation prior to their use as 3D systems.

Gelatin and Matrigel coating are the most widely used feeder cell replacement for mouse pluripotent stem cell and have been shown to be equally good in supporting viability and pluripotency maintenance of these cells[30]. Matrigel has been shown to support the growth of human embryonic stem cells in conjunction with MEF conditioned media[28]. Matrigel was also shown to be a better attachment surface than purified human ECM proteins, fibronectin coating and serum matrices for human ES cell culture[31]. 2D systems have also been explored for high throughput and large scale analysis of interaction between pluripotent stem cells and various biomaterials. Harrison et al., in 2004, tested a range of poly (α hydroxy esters) for their suitability to support mouse ES cells. PLGA was found to be the most suitable one in this study. They also showed that polymer surface treatment with potassium hydroxide was beneficial[32]. Li et al., in 2006, used the first completely synthetic ECM composed of a semi-interpenetrating polymer network (sIPN) consisting of poly(N-isopropylacrylamide-co-acrylic acid) [p(NIPAAm-co-AAc)] that was loosely crosslinked with an acrylated peptide Gln-Pro-Gln-Gly-Leu-Ala-Lys-

NH_2(QPQGLAK-NH_2) and polyacrylic acid-graft-Ac-CGGNGEPRGD-TYRAY-NH_2 [p(AAc)-g-RGD] for successful expansion of human ES cells[33]. Coatings of synthetic polymers PNIPAAm and PNIPAAm-PHB-PNIPAAm were also compared with gelatin coating for mouse ES cell expansion. The thermoresponsive polymers were chosen for easier and non enzymatic passaging of cells. The triblock coating was found to be superior to the PNIPAAm homopolymer. Adding gelatin to the synthetic polymer was shown to increase the efficiency of the polymers[34].

Mechanical properties of the biomaterial used are an important factor for its selection. In 2010, Chowdhury et al. showed that a softer surface of polyacrylamide gel with a stiffness of 0.6kPa was able to maintain viability, colony morphology and pluripotency of mouse ES cells even in the absence of LIF, which is known to be an irreplaceable growth factor for mouse pluripotent stem cells[35]. Choice of biomaterial was also shown to affect the differentiation preference of the cells. A comparison of deprotenized bovine bone, β tricalcium phosphate (Cerasorb), PLA/PGA copolymer, insoluble collagenous bone matrix (ICBM) showed different results in terms of cell viability, pluripotency and differentiation capability. ICBM was shown to support ES cell proliferation better than the other materials followed by Cerasorb and PLA/PGA. In terms of genes expression levels, PLA/PGA showed an increase in CD 34 marker after 7 days while ICBM showed a decreased CD 34 and alkaline phosphatase activity. Cerasorb showed decreased CD34 and osteopontin expression level[36].

One of the earliest mentioned scaffolds used for pluripotent stem cell culture was made of biodegradable PLLA and PLGA. PLLA and PLGA scaffolds were shown to support the growth of human ES cells and were able to maintain their pluripotency too. The scaffolds also supported directed differentiation of the ES cells in supplying necessary growth and transforming factors[37]. PEG coated surfaces are well established for culturing of adherent mammalian cells. PEGylated and non PEGylated fibrin scaffolds have been used for the expansion of mouse ES cells. Both these types of scaffolds showed good adhesion and expansion of ES cells. Viability of cells in 3D was also shown to be higher than 2D systems. PEG- fibrin scaffolds, however, showed less differentiation and better pluripotency maintenance of cells in comparison to fibrin on removal of LIF from the culture[38]. Chitosin is another biomaterial that has been used for pluripotent stem

cell expansion in 3D. Porous chitosin scaffold cross linked with sodium triphosphate penta basic was used for mouse ES cell culture.

Surface treatment with collagen also increased the ES cell growth rate[39]. Chitosin-alginate biodegradable scaffolds and sodium alginate- chitin scaffolds have been used for human ES cells and human iPS cells[40, 41]. Some polymers like PET and hyaluronic acid as 3D systems in conjunction with MEF conditioned media have been tested for expansion mouse ES cells and human ES cells, respectively, in the absence of other essential growth factors[42, 43]. Calcium alginate based microcapsules have also been used for human ES cells, along with their differentiation into endoderm lineage[44]. Yang et al., in 2012, used neutral hydrogel poly (N,N - dimethylacrylamide) (PDMAAm) and negatively charged hydrogel poly(2-acrylamido-2-methyl-propane sulfonic acid sodium salt) (PNaAMPS) with gelatin coated polystyrene scaffolds for expansion and pluripotency maintenance of mouse iPS cells. The neutral and negatively charged scaffolds were deemed to be better than the polystyrene ones [45].

Urethane based scaffolds have also been used for pluripotent stem cells expansion. Hollow fibre of polyamino urethane (PAU) coated PFTE was shown to support mouse ES cell culture and its differentiation in to hepatocytes[46]. Electrospun polyurethane scaffolds have been recently used for human ES cells culture, along with its neuronal differentiation[47].

Apart from the various novel materials used, commercially available micro/macro carriers have also been used for the culturing of pluripotent stem cells (both mouse and human). Some of the most popular ones include Cultisphere S, Cytodex 3, Solohill and Hillex II[48-53]. The earliest work on expansion of pluripotent stem cells on microcarriers was carried out in 2005, wherein Fok & Zandstra expanded mouse ES cells on Cytodex 3 carriers and glass carriers. Their experiments suggested that the mouse ES cells were able to expand as well as maintain their pluripotency. However, they also noticed that the survival rate of the cells on microcarriers was different for different cell lines[49]. This cell line dependent efficiency of microcarrier was observed in most of the studies. Several studies using Cytodex 3 carriers were carried out for other mouse ES cell lines as well as for human ES cells[54-57]. MEF coated carriers have also been used for the expansion of mouse ES cells[58]. The macrocarrier, Cultisphere S, has also been used in some cases for the expansion of ES cells[52, 53]. A comparative study between Cytodex 3 and Cultisphere S has also been carried out, which showed Cultisphere S to be more efficient than Cytodex 3[48, 55].

Hillex II is another microcarrier that has been used by more than one group for the expansion of ES cells, both mouse as well as human[50, 51]. Solohill microcarriers (Collagen, FACT III) have also been tested by some groups in comparative studies, but were not used for further experiments since other carriers fared better in comparison[50, 52, 55].

Apart from these widely used carriers, some other commercially based carriers have also been reported for the expansion of ES cells. Lock and Tzanakakis successfully used a collagen coated microcarrier for the expansion of human ES cells[59]. DE 53, a cellulose based microcarrier from Whatman, has been used by several groups for the expansion of human ES cells as well as iPS cells[60-63]. Even with the extensive data and literature available, the selection of an ideal material and 3D system for expansion of pluripotent stem cells is far from decided, leaving us the scope for future studies in this area.

1.3.7 Soluble Factors:

It is hypothesized that apart from giving an attachment matrix, the feeder layer secretes a plethora of growth factors and cytokines necessary for the expansion and pluripotency maintenance of pluripotent stem cells. In an attempt to remove the feeder cells, different growth factors and cytokines have been tested for feeder free expansion of pluripotent stem cells, along with serum containing media[10-12]. For example mouse pluripotent stem cells require LIF for their expansion in absence of feeder; this cytokine is however not suitable for human pluripotent stem cells, which require other growth factors like bFGF, ROCK inhibitor[26, 27, 64-66]. Various commercially available specialized media have been used to replace the serum as well as the feeder layer[67].

However, the use of these growth factors and/or commercially available media is a costly procedure and is a hindrance to large scale expansion of pluripotent cells. As a cost effective solution to this problem, MEF conditioned media have been used to grow pluripotent stem cells, and the expanded cells have been shown to maintain their pluripotency as well as differentiation capability[25, 28, 68]. Analysis of MEF conditioned and unconditioned media demonstrated that ligands like Activin A, IGF1 that activate pluripotency pathways are enriched in the conditioned media from different feeder cell types[69]. Apart from the various growth factors, lipid molecules

have also been shown to effect cell pluripotency and differentiation. Sphingosine - 1- Phosphate (SIP) suppresses spontaneous differentiation of human ES cells when co cultured on MEF feeder cells[70]. Sphingolipid metabolites like SIP were shown to enhance human ES cell self renewal and may also select for undifferentiated cells by promoting apoptosis of certain differentiation cells[71, 72]. The conditioned media are highly variable and efforts have been made to identify and classify the different factors secreted by the fibroblast cells, with the ultimate goal of using a completely defined and optimized conditioned media or manufacture a robust synthetic media for expansion of pluripotent stem cells. Prowse et al. [73] demonstrated that conditioned media derived from mouse and human fibroblasts contain a complex mixture of growth factors, extra cellular matrix proteins and various differentiation factors involved in the growth of human embryonic stem cells. They also showed that the different fibroblast lines had only slight differences in the number and type of proteins secreted. Although a large number of the feeder cells' secreted proteins are identified now, many remain to be identified, classified and purified. Hence, the use of conditioned media is still preferred in many laboratories to date.

However, the use of conditioned media comes with its own set of problems, particularly the change in media pH, osmotic pressure as well as the accumulation of lactate ions. Also, it is to be kept in mind that a defined extra cellular matrix or biomaterial is essential to complement the humanized medium for proper expansion and self renewal of pluripotent stem cells[74].

1.3.8 Physicochemical Factors:

Physicochemical factors like oxygen concentration, pH and metabolites are also of immense importance. The current thesis looks at the specific effects of pH change and accumulation of lactate on pluripotent stem cells and the literature review focuses on the same parameters.

1.3.8.1 Lactate Accumulation:

Lactic acid, a byproduct of glucose metabolism has been reported to be one of the potential growth inhibitors as early as 1958[75]. It has been shown to have significant effect on growth, proliferation, metabolism, antibody production and even differentiation of various mammalian cell lines. It is well established that the influence of lactic acid on different cell lines is diverse and is dependent on their tolerance level, as well as their response to the resultant change in

culture conditions. Addition of sodium lactate externally to the culture system with various cell lines showed that BHK cells have a higher lactate tolerance when compared to Hybridoma cell lines[76]. Studies have also demonstrated that apart from inhibiting cell proliferation addition of sodium lactate or lactic acid also inhibited glucose metabolism but increased the production of by products like antibody, Erythropoietin and recombinant proteins in different cell lines[77-79].

In recent years, stem cells have also been subjected to similar studies. Proliferation of hematopoietic stem cells ceased at a lactic acid concentration higher than 20mM[80], while mesenchymal stem cells could tolerate a lactate concentration of 10mM only[81]. Similar to this, it was also reported that addition of exogenous lactic acid induced chondrogenic differentiation of dermal fibroblasts[82].

Studies have also been carried out to see the effect of lactate on mouse embryonic stem cells although there are deviations in the available data. As a part of their study of embryonic stem cell expansion in 3D matrix, Ouyang et al. showed that mouse embryonic stem cells are extremely sensitive to the presence of lactic acid in media. They inferred that the growth of ES cells is inhibited above a lactate concentration of 16mM and that high lactic acid concentration also affected the pluripotency of the cells[43]. The data published by Chaudhry et al., however, contradict this. This group showed that neither growth rate nor embryoid body formation potential of mouse embryonic stem cells is affected by increasing the initial media lactate concentration up to a value of 40mM[83]. In another brief work, Martinez-Outschroon et al. showed that 10mM sodium salt of L- Lactate boosted feeder dependent ES cell growth by increasing the cell colony size as well as colony numbers[84].

However, detailed study of lactate's effect on growth and pluripotency of pluripotent stem cells (both ES and iPS) has not been carried out to date.

1.3.8.2 pH:

In contrast to the effects of lactate accumulation on cells, studies regarding the effect of pH on mammalian cells mainly highlight two things. In most cases, a change in pH has been shown to be detrimental to cell growth. Patel et al. also showed that hematopoietic stem cells are more sensitive to changes in pH rather than a change in the accumulated lactate concentration[80]. However, contradicting these data, Chen et al. showed that mesenchymal stem cell expansion is

affected by both pH change and lactate accumulation[81]. They also showed that increasing pH or lactate concentration significantly depressed osteogenic differentiation but promoted adipogenic differentiation of mesenchymal stem cells, suggesting a role of pH in cell differentiation. Increasing or decreasing the culture pH has also been shown to modulate the differentiation of various cells[85, 86]. It has been reported that pH change was detrimental to cell growth and embryoid body formation capability of mouse ES cells[83]. It has also been observed that extended exposure of human ES (hES) cell cultures for 1–3hrs to ambient conditions resulted in a rapid increase in pH inhibited cell proliferation and reduced Oct-4 expression levels[87]. Similar to the effect of lactate, in depth study of effect of pH change on pluripotent stem cells is yet to be done.

1.3.9 Shear Stress and Dynamic Culture Systems:

Traditionally, mammalian cells are cultured in static culture systems like T- Flasks, Blood bags, well plates, etc. Although relatively simple and low cost, a static culture comes with certain disadvantages, such as the metabolite and oxygen gradient due to lack of mixing, lack of options for regular monitoring, and restricted surface area for cell growth. To tide over these limitations, various forms of dynamic culture or bioreactors are used for long term expansion of mammalian cells. "A bioreactor is the general term applied to a closed culture environment, that is usually mixed, that enables control of one or more environmental or operating variables that affect biological processes"[88] . Stirring and perfusion flow are the most common methods of dynamic culture. Shear stress in a dynamic culture is one of the most important parameters that need extensive study. *In vivo* mammalian cells are under constant shear stress due to the circulation of blood and other body fluids. The stress in not constant and depends on the cell type under consideration. For example, optimal shear stress for mammary epithelial stem cell aggregate culture was 0.21 Pa[89]. In contrast, it was seen that the optimal shear for mouse ES cell aggregates in stirred culture was 0.61Pa, and they showed excessive clumping at a shear of 0.45 Pa[90]. It has been shown that shear stress affects both cells and their markers; it affects cell shape, physiology, cytoskeleton, etc[91]. A low shear has been shown to result in cell clumping while a high shear is deleterious[92].

To date, different kinds of bioreactors have been designed and used for pluripotent stem cells. The following paragraphs looks at the two major types of bioreactors used for the expansion of

pluripotent stem cells. Table 1.1 highlights the major advantages and disadvantages of the two systems.

Table 1.1 Advantages and Disadvantages of spinner and perfusion reactor systems

Reactor Type	Advantages	Disadvantages
Spinner flask reactor	Scalable well mixed environment. Easy sampling. Easy scale up.	Presence of high shear. Presence of cell agglomeration. Heterogenous culture environment
Perfusion flow reactor	Homogenous culture environment. Low shear environment possible. Inhibition of waste accumulation.	Currently used in a small scale. Continuous removal of secretory materials.

1.3.9.1 Stirred Culture System:

A spinner flask or a stirred culture reactor is one of the oldest dynamic culture systems used until now. They are well characterized for culturing both microbial and animal cells and have been used to overcome the limitations of static culture largely. Stirred systems provide a homogenous mixing environment and are superior to static systems in terms of sampling, scaling up, data collection and controlling of medium, as well as culture conditions like pH and dissolved oxygen. Such a system also provides a scalable controlled environment to study the effects of various factors on cell growth and differentiation. In most cases, it is a closed batch system and hence easily reproducible**.** Despite the presence of high shear stress, it has been used extensively for the expansion of various stem cell lines like hematopoietic stem cells and neural stem cells[93, 94].

Due to its relative simplicity, a spinner flask was one of the first used dynamic systems for embryonic stem cell culture. Spin rates within a range of 25 -120 RPM have been tested for the proliferation of pluripotent stem cells, depending on the cell type, cell line, expansion system (aggregate or adherent) and the type of adherence system when involved. Fok and Zandstra in 2005 reported for the first time the long term culture of mouse ES cells[49]. The group successfully cultured mouse ES cells as aggregates as well as on microcarriers for 15 days, followed by differentiation in the same system. They tested 60 and 100 RPM spin rates and compared the effect of the same in terms of aggregate formation and cell viability. A higher spin rate gave rise

to smaller but uniform cell aggregate, facilitating better oxygen and nutrient transport. They also showed the importance of E cadherin in cellular attachment and expansion for ES cells. The success of this study led to further experimentations by various other groups for mouse and human ES cells using microcarriers or as cell aggregates.

In 2006, Cormier et al. expanded mouse ES cells as aggregates in a spinner reactor, followed by differentiation[90]. They tested a number of spin rates for their system and controlled cell aggregation and differentiation by adjusting the spin rate. A 31 fold expansion was obtained in 5 days in comparison to the 20±11 fold expansion in 15 days obtained in the previous work. In 2007, J.M.S Cabral's group published two different papers on large scale expansion of mouse ES cells in a stirred reactor system using Cytodex 3 and Cultisphere S carriers[48, 54]. The two papers looked at a number of parameters involved in a spinner flask system like cell seeding density, microcarrier concentration and absence of serum in medium. They observed that a low seeding density, however, gave a high fold increase in cell density but the time taken to reach maximum cell density as well as the variability in the culture were also the highest. On the other hand, a high seeding density resulted in very low fold increase in cell number. A serum free medium in conjunction with Cultisphere S was also successfully used for culturing mouse ES cells. It is known that removal of serum from media results in the cells becoming more susceptible to shear stress. Hence Cultisphere S, a macrocarrier, was used in this case to give the cells protection from shear. Mouse ES cell expansion on Cytodex 3 has also been carried out with ES cell medium supplemented with MEF and CHO conditioned media[56]. Apart from maintaining cell viability and pluripotency, this study showed that normal chromosome number was also maintained during long term dynamic culture. Alfred et al. carried out culture of mouse ES cells using serum free medium in 2011[55]. This group also showed that porosity of microcarriers affect cell expansion. A macroporous carrier was found demonstrated to be better than microcarriers and carriers with no pores.

Based on the success of mouse ES cells, spinner flasks or stirred reactor systems were also used for human ES cells. One of the first successful spinner flask expansions of human ES cells was published in 2008 where Hillex II microcarrier was used for successful culture of human ES cells[51]. This study was, however, unable to sustain passaging and maintenance of the cells beyond 6 weeks. Different groups published a number of studies on the expansion of human ES

cells in spinner flask in the year 2009. Oh et al. solved this problem when they were able to expand human ES cells in a spinner flask using microcarriers and serum free defined media with long term passaging over a period of 6 months[63]. The same group also showed that the effect of agitation on growth is cell line specific[62]. They reported that agitation did not affect the growth and pluripotency of the human ES cell line HES- 2 but led to increased cell differentiation for ES cell line HES -3 and human iPS cell line IMR90. It has also been shown that Matrigel and MEF coated microcarriers were able to support a dynamic culture of human ES cells[58]. Additionally, this group showed that cryopreservation of the cells while being attached to microcarriers yielded a higher recovery rate in comparison to cryopreservation as suspension. Successful expansion of human ES cells on microcarriers without any prior coating of Matrigel or feeders was also published during the same time[95]. Lock and Tzanakakis carried this work a step forward and carried out long term human ES cell expansion, as well as definitive differentiation of the cells within the same system[59].

Krawetz et al. expanded human ES cell as aggregates using ROCK inhibitor and rapamycin in their culture[96]. This study demonstrated the need for ROCK inhibitor and rapamycin in the formation and survival of human ES cell aggregates in the dynamic system. Storm et al., in 2010, carried out similar experiments on both mouse and human ES cells successfully, wherein they looked at the effect of initial seeding density on cell aggregation as well as attachment on microcarriers[52]. They also showed that parameters optimized using mouse ES cells could be directly used for spinner flask culture of human ES cells without any prior weaning of the same. This highlighted the importance of mouse pluripotent stem cells as a valuable model for process development and optimization in this field for human cells. Serra et al. incorporated certain parameter control in the traditional stirred reactor for human ES cell culture[57]. They showed that the human ES cell growth and cell metabolism was higher at an oxygen saturation of 30% in comparison to a 5% oxygen saturation. Expansion of human ES cells in spinner flask has also been shown as a proof of concept within a study of microcarrier comparison[97]. Xeno free production of human ES cells in a spinner flask using microcarriers and a novel medium BRASTEM has also been published recently[50].

Figure 1.6 Representative image of a stirred reactor system[88]

The success of both mouse and human ES cell culture in spinner flask has led to its use for dynamic culture of iPS cells in recent times. As a part of their review article, Kehoe et al. presented some preliminary data for expansion of human iPS cells in a spinner flask using microcarriers[61]. Human pluripotent stem cells (both ES and iPS) were cultured under optimized conditions for various parameters, such as agitation rate, use of shear protectant, dissociation process and oxygen concentration[98]. Shafa et al. also achieved successful spinner reactor culture of mouse iPS cells as aggregates in 2012[99]. In a landmark paper, Fluri et al. carried out iPS derivation from terminally differentiated fibroblasts, followed by its expansion and three germ layer differentiation within the same spinner flask system[100]. Recently, microcarrier expansion of human iPS cells in a spinner flask and its directed differentiation towards neural progenitor has also been undertaken[60]. Thus, the continuous success of stirred/ spinner systems for the expansion of pluripotent stem cells makes this system one of the most preferred methods of large scale dynamic culture.

1.3.9.2 Perfusion Bioreactor System:

One of the disadvantages of a batch type spinner flask system is the accumulation of waste and the gradual depletion of essential metabolites, along with the presence of a high shear stress within the system. A perfusion flow system is considered to be the ideal way to solve this

problem and can be incorporated in any kind of reactor with the help of a pump. Designs like parallel plate chamber, packed bed reactors and hollow fibre reactors are exclusively based on a perfusion system of dynamic culture, and allow for a low shear environment helpful for pluripotent stem cell expansion without cell differentiation and death. The use of a perfusion system for stem cell culture was reported as early as 1955 for studying chick heart fibroblasts and snail amoebocytes[101]. The use of perfusion system and its advantages for stem cells was first reported by Koller et al in 1992- 93 wherein a parallel plate perfusion system was used for the expansion of long term hematopoietic stem cells as well as hematopoietic progenitor cells in a controlled environment [102, 103].

One of the earliest uses of a perfusion system with pluripotent stem cells was published in 2005 when such a system was used to generate cardiomyocytes from encapsulated mouse ES cells in a hypoxic environment[104]. Perfusion assisted expansion of human ES cells was carried out in petri dishes and organ culture dishes and showed enhancement of cell number by almost 70%, while maintaining their pluripotency and capability to form teratoma *in vivo* [105]. Similarly, mouse ES cells were also expanded in a perfused petriperm dish with success in comparison to static culture[106]. Serra et al. introduced a perfusion system in a stirred reactor, highlighting the importance of medium perfusion and waste removal[57]. Recently, Baptista et al. used a spinner perfusion reactor for high density continuous generation, expansion and differentiation of iPS cells with success[107]. Ouyang and Yang in their review article mention a two stage perfusion fibrous bed bioreactor that was used to supply MEF conditioned media from one of the reactors to the ES cells in the other reactor[108]. The use of this system resulted in large scale expansion of ES cells without using any growth factors or ECM proteins.

A 3D perfusion based reactor for expansion of mouse ES cells followed by their differentiation was also used by Li et al., in 2003[109]. The ES cells in the 3D perfusion bioreactor maintained their pluripotency and showed a higher cell yield and metabolic activity in 3D when compared to 2D. Perfusion flow was also introduced in a slow turning lateral vessel (STLV) reactor system along with a dialysis chamber for human ES cells and embryoid body culture[110]. The low shear perfusion reactor helped large scale and long term expansion of human ES cells and embryoid bodies along with a more homogenous differentiation of the cells. Gerlach et al., in 2010, used a capillary membrane based perfusion bioreactor system for the expansion of mouse ES cells in a

co- culture system with inactivated MEFs[111]. The system used was a multi compartment one with the capillary membranes being used for medium and gas exchange, while the cells were grown within the inter capillary region allowing proper control of various parameters like oxygen tension, perfusion rate, medium supply pattern, etc. In a very recent article, Fernandes-Platzgummer et al. compared continuous perfusion culture with two different methods of discontinuous medium change[112]. They observed that although the initial cell growth was similar in all three cases, for long term culture after 7 days, the continuous perfusion gave a higher final yield of viable cells, proving its advantage over the discontinuous feeding strategy. Fluctuation in metabolite concentration was also decreased by the use of continuous perfusion system.

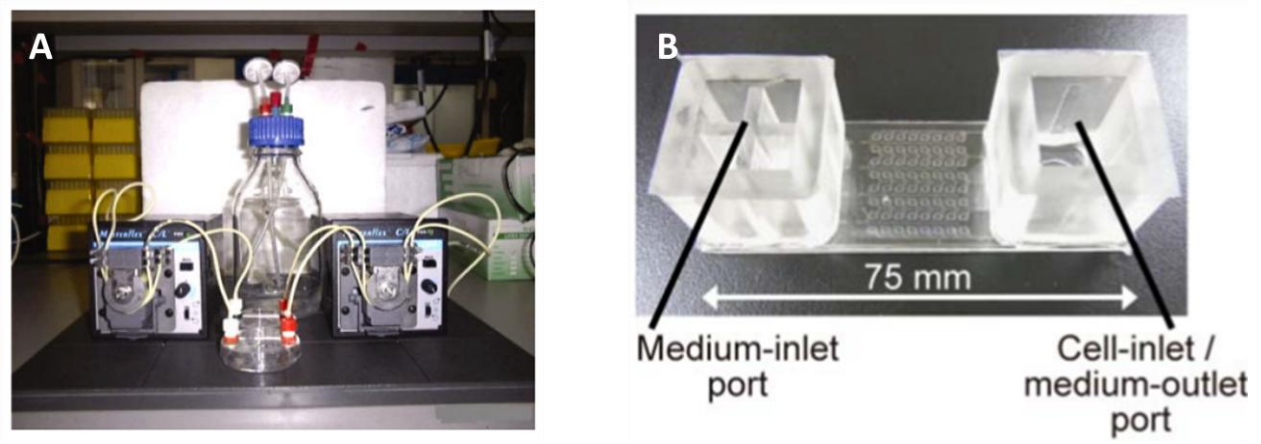


Figure 1.7 Representative images of perfusion bioreactor system (A) Macro scale reactor, (B) Micro scale reactor[106, 113]

Although most potential therapeutic applications of pluripotent stem cells require high cell yield achievable only in a large scale reactor system, the use of a microfluidic system as an efficient system for optimization of culture conditions and for precise controlling of cell microenvironment has also been practised. A perfusion system at a microfluidic level for expansion of mouse ES cells was also used during the same time[114]. The group observed that change in flow rate had significant effect on mouse ES cell colony number and size. For example, a higher flow rate resulted in larger undifferentiated colonies but the number of colonies was lower. In another study, an automated periodic "flow stop" perfusion system at a microbioreactor level was used for culturing of human ES cells on feeder layer[115]. The short pulse flow of this system allowed medium renewal and waste removal without harming the ES

cells, while the longer static incubation period resulted in local accumulation of growth factors from the feeder cells without their getting perfused away instantly.

A perfused microbioreactor has also been used to see the effect of shear stress on human ES cells and for optimizing the same for expansion of the cells[113]. It was seen that at low shear, cell proliferation was negligible while at high shear, there was cell wash out within the reactor system. Intermediate flow rates of 20.8 and 31.3 µl/hr were optimized for human ES cell expansion within this system. Similar work has also been carried out by Giulitti et al. who compared continuous perfusion flow and periodic flow in a microbioreactor for ES cell culture[116]. They observed that continuous perfusion resulted in homogeneity in cell population while a short periodic perfusion resulted in more homogenous colonies with respect to viability, morphology and pluripotency in comparison to a static culture. A recent work describes a perfusion reactor in the form of a fully automated complete multiplexed system for ES cell culture[117]. The system was able to store media at a cooler temperature and can be heated prior to the reactor system. The temperature within the reactor was also controlled and did not require incubation during culture. As a proof of concept, ES cells were grown and imaged within the reactor system, and they were seen to be viable and proliferating after 21hrs of culture. In 2013, Yoshimitsu et al. used a perfused microbioreactor for the expansion of human iPS cells in an undifferentiated state with success[118]. The group also successfully used the same system for cell differentiation and drug testing.

1.4 Conclusion:

Optimized bioprocessing of large scale culture of stem cells and/or their derivatives can translate discoveries from the bench top to a patient's bed side. A number of challenges are associated with such a task and require extensive long term studies. Mimicking and regulating the stem cell 'niche' *in vitro* requires in depth study of various parameters involved like the ECM, growth factors, metabolites, physicochemical culture conditions and hydrodynamic forces. The literature review carried out in this chapter on some of the parameters involved throws light on the advancement achieved to date. However, further studies are still required to recapitulate the *in vivo* stimuli at various levels and require a multidisciplinary collaborative approach involving various fields of biology and engineering.

1.5 Thesis Objectives:

As mentioned earlier, the central theme of this thesis is to study the effect of some of the culture parameters involved on mouse embryonic stem cells. Based on the literature survey, an attempt has been made to answer the following questions in an effort to bridge some of the gaps in the currently available literature.

1. Can novel materials that support ES cells in 2D also support the same in 3D scaffold structure in a static environment?
2. Can iPS cells grow on microcarriers that support the growth of ES cells? Since most preliminary studies are carried out on ES cells prior to their study on iPS cells, this question is of utmost importance.
3. Is it possible to culture iPS cells in a dynamic culture vessel with significant fold expansion in cell number and over a longer time period? A positive answer to this question would be a big step towards the translation of clinical use of pluripotent stem cells.
4. What is the effect of change in pH on pluripotent stem cells?
5. What is the effect of change in lactate concentration in the media on pluripotent stem cells?
6. Are the effects of pH and lactate dependent on the cell type and cell line, i.e., would ES cells and iPS cells show different results on being cultured under variable pH and lactate concentration? In addition, would the effect depend on the presence or absence of feeder cells?

Chapter 2 General Materials & Methods

This chapter reports the common materials and methods used in the entire work.

2.1 Materials:

All chemicals were purchased from Invitrogen unless otherwise mentioned. Plasticwares were purchased from Tarson, India, for the work done at IIT Bombay, Mumbai, and from Corning, SIGMA or BD Biosciences for the work done at Monash University, Melbourne.

Table 2.1 List of Cell Lines Used

Cell Line	Description	Growth Surface
QSMEF fibroblast cells	Mouse embryonic fibroblast cells from Quackenbush Swiss (QS) mice.	Any surface.
SNL fibroblast cells	The SNL cell line is an immortalized subclone of the STO line manipulated to stably express the neomycin resistance and leukaemia inhibitory factor (LIF) genes. STO cell line itself was derived from mouse SIM embryonic fibroblasts.	Any surface.
OCT4B2 ES cells	Embryonic stem cells(129S2/SvPas strain) where the EGFP construct had the regulatory elements of OCT4 promoter driving GFP expression. The cells are puromycin and hygromycin resistant.	0.1% gelatin coated surface.
ESD3 ES cells	129S2/SvPas strain embryonic stem cells.	0.1% gelatin coated surface.
OG2B6 ES cells	Embryonic stem cells (CBA/CaJ X C57BL/6J strain) having GFP expression under the OCT4 promoter region lacking PE region.	Mitomycin c inactivated QSMEF feeder layer.
OG2B6 iPS cells	Induced pluripotent stem cells from fibroblasts of OG2 x B6 strain mice where GFP expression is controlled by the OCT4 promoter.	Mitomycin c inactivated QSMEF feeder layer.

2.2 Methods:

2.2.1 Mouse Embryonic Fibroblast (MEF) Medium:

Dulbecco's modified eagle's medium containing high glucose was supplemented with 10% fetal bovine serum (JRH Biosciences, Sigma, Australia), 1X penicillin- streptomycin, 1X GlutaMAX & 1X MEM non essential amino acid. The medium was filtered through 0.22µm filter (Millipore). MEF medium was always warmed at 37°C in a water bath prior to use.

2.2.2 Mouse embryonic stem (MES) cell medium:

Dulbecco's modified eagle's medium containing high glucose was supplemented with 15% fetal bovine serum, 1X penicillin- streptomycin, 1X GlutaMAX, 1X MEM non essential amino acid, 1000 IU/ml of ESGRO LIF (Millipore) and 2- Mercaptoethanol. The medium was filtered through 0.22µm filter (Millipore). MES medium was always warmed at 37°C in a water bath prior to use.

2.2.3 Cell Freezing Medium:

Cell freezing medium was prepared by mixing 20% DMSO and 80% fetal bovine serum (FBS). The medium was sterile filtered using 0.22µm filters (Millipore).

2.2.4 Gelatin Coating of Culture Vessels:

The MEF and MES cell culture vessels are coated with gelatin for better attachment of cells. 0.1% working solution of gelatin was prepared by diluting 2% stock solution with DPBS (with calcium and magnesium). The solution was filtered using 0.22µm filter (Millipore). In order to coat the culture vessels, appropriate volume of 0.1% gelatin was added. The plates were then kept in the incubator for a few hours. The gelatin solution was aspirated prior to cell plating.

2.2.5 Feeder Layer Preparation:

Feeder cells were prepared by inactivation of mouse embryonic fibroblast cells with mitomycin c. Mitomycin c (SIGMA) is available in powder form of 2mg vials. The working concentration

was made to 8µg/ml by diluting the stock powder in MEF media. The working solution was added to the MEF containing flasks (7ml for T75, 14ml for T175 flask) and incubated for 3-4 hrs. After the stipulated time, the mitomycin c solution was aspirated; the cells were washed with PBS and trypsinised in the regular way. The inactivated cells were either freshly used or frozen down as per need. Frozen feeder cells were thawed and plated on 0.1% gelatin coated dishes at a density of $4x10^6$ cells/cm^2. They were left overnight for proper attachment.

2.2.6 Thawing of Cells:

Cryovials were placed in water bath at 37°C for thawing. The outside of the vials was wiped with 70% ethanol to maintain sterility before being taken inside the hood. The cell suspension was then taken in a conical tube and about 5- 10 ml of medium was added to the cell suspension with gentle shaking. The suspension was then centrifuged at 1600 RPM for 3 minutes. The supernatant was aspirated and cell pellet was resuspended in appropriate volume of medium depending on the cell type. The cells were then plated on to gelatin coated culture vessel or on feeder layer (for feeder dependent pluripotent stem cells). After 24 hours, the medium was replaced to remove dead cells and remnant of DMSO from freezing medium.

2.2.7 Trypsinization and Passaging of Cells:

Cells were regularly passaged for their maintenance. The medium from the culture plates was aspirated and the cells were washed with Dulbecco's phosphate buffered saline (without calcium & magnesium) to remove any remaining serum containing medium. Sufficient amount of Tryple/ trypsin- EDTA was added to the culture dishes in such a way that it just covered the cells. They were then left in the incubator for 2-3 minutes to allow detachment of cells. The cells were periodically checked under the microscope. Once the cells had started to detach, trypsin was inactivated by adding MEF medium. The cells were completely dissociated by gentle tituration to form a single cell suspension. They were then collected in a conical tube and centrifuged at 1600 RPM for 3 minutes. The supernatant was then aspirated and the pellet was resuspended in 1ml of medium. Cell count was done using trypan blue and haemocytometer. The cells were replated at appropriate density on culture vessels precoated with 0.1% gelatin or with feeder layer (for feeder dependent pluripotent stem cells). Both MEF and MES cells were maintained at

37°C, 5% CO_2 in a humidified environment. Cell passaging for regular maintenance was carried out based on the confluency (70- 75 %) and colony morphology. On an average cells were passaged after every two days. For experimental purposes throughout the research, feeder independent ES cells used were between passages 25 – 33 and feeder dependent cells (both ES and iPS) used were between passages 10 -20.

2.2.8 Cryopreservation of Cells:

Suggested volume of Propan- 2-ol was added to 'Mr. Frosty' 1°C cryofreezing container and was equilibrated at 4°C for approximately 1hour prior to use. Cryovials (Nunc/ Tarson) were labeled with necessary information (cell line name, passage number, cell density, date of freezing). Cells were trypsinized following the previously mentioned protocol. Volume of cell suspension was adjusted using either MEF or MES medium. Equal volume of freezing medium was added slowly to the cell suspension with gentle shaking and mixed by pipetting. Cells were frozen as 1ml aliquots and generally contained $0.5x10^6$ – $2x10^6$ cells. The cryovials were then placed in 'Mr. Frosty' and put in -80°C freezer overnight. The vials were transferred to liquid nitrogen (LN_2) for long term storage.

2.2.9 Immunostaining:

Blocking buffer: 2% Bovine serum albumin (BSA) in PBS (with calcium & magnesium). The solution was filtered through 0.22µm filter and stored at 4°C.

Washing solution: 0.1% Tween 20 (dilution in PBS with calcium and magnesium).

Primary antibody dilution: 1:100 (in blocking buffer)
Secondary antibody dilution: 1:500 (in blocking buffer)

Cells for staining were fixed using 4% paraformaldehyde at room temperature for 20 minutes followed by 3 times wash with PBS (Ca+/Mg+). The cells were then washed with 0.05% Triton X to permeabilise the cells (in case of nuclear staining). Blocking buffer was added and incubated at room temperature for an hour. To visualize the presence of different proteins, necessary primary antibodies were added followed by overnight incubation in the dark at 4°C.

The cells were then washed with washing buffer and secondary antibody was added and the cells were incubated for 1 hour in dark at room temperature. Removal of secondary antibody was followed by washing the cells and addition of Hoechst for nuclear staining. Cells were then viewed under fluorescence/ confocal microscope.

2.2.10 MTT Assay:

MTT [3-(4, 5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide] assay was done as a measurement for viability of cells. The assay works on the principle of tetrazolium salt being converted to purple formazon crystals by mitochondrial reductase enzymes of viable cells. The crystals were dissolved using a solubilizing agent to give a purple solution. The absorbance of this solution was quantified at 570 nm with a plate reader[119].

MTT Stock: A stock solution of MTT (5mg/ml) was prepared by dissolving MTT powder in PBS and filtering it through 0.22micron filter. The stock was kept in 4°C wrapped in aluminium foil to avoid light.

Solubilizing agent: A 0.04N HCl in isopropanol was prepared for solubilizing the formazon crystals.

Cells were washed once to remove the unattached cells. 200 µl of fresh media was added followed by 20 µl of MTT stock solution. The cells were then incubated at 37°C and 5% CO2 under dark conditions for 3 – 4 hrs. Once formazon crystals were formed, 200 µl of solubilizing agent was added. The plate was put on a rocker to solubilize the crystals well. Reading was taken at 570nm using a plate reader. The ratio of media: MTT solution: solubilizing agent was maintained in the ratio of 10:1:10 in all experimental set ups.

2.2.11 Scanning Electron Microscopy:

Scanning electron microscopy was carried out both at IIT Bombay and Monash University. The sample preparation method had small differences for the two experiments.

IIT Bombay: The samples were fixed using 3% gluteraldehyde solution. After that they were washed with graded ethanol (10%, 30%, 50%, 70%, 90% and 100%) for 10 minutes each and

then vacuum dried in a desiccator. The scaffolds were then sputter coated (gold coating) and observed under environmental Scanning Electron Microscope (JEOL, Tokyo, Japan).

Monash University: Samples were fixed with 3% gluteraldehyde, followed by drying using graded ethanol. This was followed by a chemical drying by a transition from 100% ethanol to 100% HMDS through a graded series of ethanol- HMDS mixture, ending at 100% HMDS. The samples were then sputter coated and imaged using Hitachi S570 scanning electron microscope (Hitachi, Tokyo)

2.2.12 Propidium Iodide (PI) staining:

20X SSC Buffer: 3M NaCl and 0.3 M sodium citrate

PI stock solution concentration: 1mg/ml

Propidium Iodide: Excitation – 535 nm, Emission – 617 nm.

Cells were fixed using 4% paraformaldehyde at room temperature for 20 minutes followed by 3 times wash with SSC buffer. 1000x diluted PI solution was added and incubated in the dark form 15- 20 minutes. This was followed by washing 3 times (around 15 minutes each time) with SSC buffer and imaged under confocal (LSM 510, Carl Zeiss Pvt. Ltd) or fluorescence microscope.

2.2.13 GFP Analysis by Flow Cytometry:

Oct4-GFP of live cells was measured using flow cytometry. Media were aspirated from the culture vessel followed by a PBS wash to remove all traces of serum. Cells were then trypsinised using previously mentioned protocol followed by a cell count using haemocytometer. About 20,000 - 10^6 cells were resuspended in 1ml of media and the cell suspension was then transferred to a flow cytometry tube. The tubes were kept in ice prior to analysis. GFP was read on flow cytometry (BD FACS Canto at Monash University and BD FACS Aria at IIT Bombay). A minimum of 10,000 cells were counted for a statistically significant datum.

2.2.14 Cell Viability Assay:

Cell Titre- Glo® luminescent (Promega) assay was used to measure cell viability based on the ATP level measurement. The ATP level has a direct correlation to the number of metabolically

active cells. The assay was performed based on the manufacturer's instructions. Briefly, the cells were seeded on to 96 well opaque walled plates under different experimental conditions for the stipulated time. Wells containing only media and no cells were used as the background control (blank). The plate was equilibrated at room temperature for 10 minutes after being taken out from the incubator. Cell Titre Glo® reagent of volume equivalent to the culture medium present was added to each well. The plate was shaken for 2 minutes to ensure lysis of cells. The lucifer luminescence was subsequently analysed using a Fluostar Optima (BMG Labtech, Australia).

2.2.15 BrdU Cell Proliferation Assay:

1mM stock solution of BrdU was diluted in appropriate medium to make a 10µm working solution. The working solution was added to the experimental set ups and incubated for 18-24 hrs. The BrdU incorporated cells were then stained with anti- BrdU secondary antibody conjugated with FITC or Alexa Fluor 647. Fluorescence intensity was then measured using Array Scan (Thermo Fisher)

2.2.16 Feeder Depletion:

- *Gradual Feeder Depletion Method:*

Feeder dependent pluripotent stem cell culture was trypsinised and replated on culture surfaces coated with gelatin and matrigel. The plated culture contained both feeder cells and the pluripotent cells. The cells were passaged after every 2 days to gradually deplete out the inactive fibroblast cells and adapt the cells to a feeder independent condition.

- *Selective Feeder Depletion Method:*

This method was based on the property of different cells to attach to a surface at different rates. Feeder dependent pluripotent stem cell culture was trypsinised and replated on culture surfaces without any coating. They were incubated at 37°C for 30 minutes. The supernatant was then carefully taken, centrifuged and the pellet was resuspended in ES media. The cells were then plated on culture surfaces coated with gelatin and matrigel. The cells were passaged after every 2 days in the same way to deplete the inactive fibroblast cells and adapt the cells to a feeder independent condition.

Chapter 3 Expansion of Embryonic Stem Cells on Different Biomaterials in 2D and 3D Culture Systems

3.1 Introduction:

As highlighted in the first chapter, a suitable biomaterial is of immense importance in stem cell culture in order to replace the feeder layer. Even with the large number of biomaterials tested for expansion of pluripotent stem cells, the search for better materials is still ongoing. The current chapter deals with the expansion of mouse embryonic stem cells on different biomaterials in 2D and 3D culture systems. Gelatin, being the most widely used polymer for pluripotent stem cell culture, was used as the control material. Tropoelastin, a novel material was compared to Matrigel and gelatin for expansion of mouse ES cells in 2D, while PMVE- MA was tested as a novel material for mouse ES cell expansion in 3D scaffolds. Preliminary studies were also done to test the suitability of the commercially available microcarriers Cytodex 3 and Hillex II for expansion of mouse ES cells for their use in later work.

Hypothesis:

For the current work, it is hypothesized that the new materials tropoelastin and PMVE - MA will support the long term expansion and pluripotency maintenance of mouse ES cells in 2D and 3D culture, respectively. Being a precursor of elastin, tropoelastin is expected to support cell adhesion and expansion and hence is being tested. PMVE- MA has recently been shown to support pluripotent stem cell in 2D. Our experiments study its suitability in a 3D system.

Gelatin: Gelatin is a water soluble heterogeneous mixture of protein and is derived from partial hydrolysis of collagen. It has widespread use in the field of biology ranging from coating of cell culture plates in 2D to its use as biomaterial for 3D scaffolds for tissue engineering[120-122]. It has also been used as delivery vehicle for release of bioactive molecules[123]. Mouse pluripotent stem cells are regularly cultured on gelatin coated cell culture systems on its removal from feeder layer.

Matrigel: Matrigel is the trade name for a protein mixture secreted by Engelbreth-Holm-Swarm (EHS) mouse sarcoma cells and marketed by BD Biosciences as a reconstituted basement membrane for culturing adherent cells. It contains laminin, collagen IV, entactin, heparan

sulphate proteoglycan and various growth factors. The concentration of the stock solution varies from lot to lot. Although BD Biosciences gives a guarantee for lot-to-lot consistency, it is not a defined substrate. Currently, Matrigel remains as one of the most widely used polymers used in 2D culture as well as in coating for microcarriers[61].

Tropoelastin: Tropoelastin is the water soluble precursor of elastin molecule responsible for the stretch properties of skin, arterial walls and ligaments. It forms elastin by cross linking with each other using deaminated lysine residues, the microfibril structures functioning as a scaffold.

The tropoelastin used for experiments as reported here is a synthetic form containing the recombinant human tropoelastin of the form SHELΔ26A (synthetic human elastin without the domain 26A). It is the most common splice variant of tropoelastin. It is >95% pure and lyophilized in acetic acid and re dissolved in PBS. The supplied concentration of tropoelastin was 3mg/ml, which was diluted as needed. Although tropoelastin has not been used for pluripotent stem cells, it has been shown to support the expansion of dermal fibroblasts, HUVEC and HCASMC, when used to form microfibres[124].

Poly Methyl Vinyl Ether- alt- Maleic Anhydride: PMVE- MA is a biodegradable synthetic anhydride FDA approved polymer with low toxicity. It has been widely used in various pharmaceutical purposes like forming micro particles for nasal spray[125], denture adhesive and transdermal patches for wound healing[126]. Recently in 2010, Brafman et al. used a high throughput array system to test synthetic polymers for expansion of human pluripotent stem cells. PMVE-MA was found to support long term culture of both human ES and iPS cells[127].

Cytodex 3: Cytodex 3 are commercially available microcarriers manufactured by GE Healthcare. Cytodex 3 consists of a matrix of cross-linked dextran chemically coupled with a thin layer of denatured collagen. The carriers are microporous in nature and have a diameter in the range of 141-211 µm, a density of 1.04 gm/ml and an approximate surface are of 2700 cm^2/gm. Cytodex 3 was used for mammalian cell culture as early as the 1980s when Lewis and Morrison used Cytodex 3 to culture human kidney cells[128]. Cytodex 3 has been extensively used in the culture of embryonic stem cells both human and mouse[48, 95] but has not been tested for induced pluripotent stem cells.

Hillex II: Hillex II is another commercially available microcarrier marketed by Solohill Engineering Inc. These are modified polystyrene carriers with an amine coating on the surface resulting in a cationic surface charge. The carriers have an approximate diameter in the range of 160- 200 µm. They have a density in the range of 1.09 - 1.15gm/ml and an approximate surface area of 515 cm^2/gm. Hillex II has been also used for the expansion of mouse and human embryonic stem cells[51].

3.2 Materials & Methods:

The following paragraphs report materials and methods used specifically for this chapter.

3.2.1 Materials:

Gelatin & PMVE-MA were procured from Sigma Aldrich (St Louis, MO, USA). Matrigel was bought from BD Biosciences (Franklin Lakes, New Jersey, USA). Elastagen Pty Ltd, Australia kindly supplied the Tropoelastin used for experimental purpose. Cytodex 3 and Hillex II were procured from GE Healthcare Biosciences AB (Uppsala, Sweden) and Solohill Engineering Inc. (Ann Arbor, MI, USA), respectively.

Table 3.1 Composition and/or dilution of biomaterials used in 2D and 3D culture

Biomaterial	Composition & dilution
Gelatin (2D)	0.1% v/v solution diluted in PBS (with Ca^{+2} , Mg^{+2})
Matrigel (2D)	0.33mg/ml diluted in DMEM media
Tropoelastin (2D)	3mg/ml (stock solution from company)
Tropoelastin (2D)	1.5mg/ml diluted in PBS (with Ca^{+2} , Mg^{+2})
Gelatin (3D)	0.4% w/v dissolved in 0.5N acetic acid
PMVE-MA+ Gelatin (3D)	0.5% PMVE- MA & 4% gelatin (w/v) dissolved in 0.5N acetic acid

3.2.2 Cell expansion on 2D surface coated with Gelatin, Matrigel and Tropoelastin:

These experiments combined the effect of the three biomaterials, along with three different media - a) regular ES cell media, b) conditioned media from untreated MEFs, and c) Conditioned media from MEFS treated with mitomycin c. Wells of 24 well plates were coated in duplicate with gelatin, Matrigel, tropoelastin and kept overnight at 4°C. Each well was seeded with 50,000 Oct4B2 ES cells and observed over a period of 5 days. Cell count was done using a haemocytometer, while pluripotency was measured via GFP analysis using flow cytometry on day 3 and day 5. pH of the media over the different biomaterials was estimated using pH strips.

3.2.3 Conditioned Media preparation:

2.5×10^6 QSMEF and mitomycin c treated MEF cells were seeded in a T-75 tissue culture flask with MEF media. After 24 hours, cells were changed over to ES media. The media from both the sets were collected and changed after every 24hrs for 7 days. They were filtered through a 0.22μm filter and frozen in aliquots.

3.2.4 Cell seeding in Gelatin and PMVE-A+ gelatin scaffolds:

The scaffolds were dipped in ethanol overnight for sterilization purposes. They were then washed with PBS three times and dried inside the laminar hood, followed by UV sterilization for 20 minutes. Required amount of Oct4B2 ES cells (10,000 cells/ cm^2) was suspended in media just enough to wet the scaffolds (approximately 100μl for a $2cm^2$ area scaffold) and added drop wise to the scaffolds in a uniform manner. The scaffolds were then incubated in a humidified environment at 37°C and 5% CO_2 for 2-3 hrs for attachment of the cells in the scaffold. Fresh medium was then added to the scaffolds and incubated again for a total time period of 7 days. Characterization of cell growth, proliferation and pluripotency was done by MTT assay, propidium iodide staining, scanning election microscopy, OCT4 - GFP visualization and immunofluorescence assay for SSEA-1.

3.2.5 Cell seeding on Cytodex 3 and Hillex II microcarriers in static culture:

Cytodex 3 carriers require swelling prior to its use. To maintain the same conditions for both the carriers, appropriate amounts of Cytodex 3 and Hillex II were soaked in PBS (with Ca^{++} and

Mg^{++}) overnight. The PBS was then changed and the carriers were then autoclaved in PBS for sterilization. The culture was carried out in low adherence 6 well plates. The microcarriers were incubated in appropriate media for 1 hour prior to their use. Carrier volumes equivalent to 20 cm^2 surface area were put in each well of the plate. The area was kept in excess to give maximum carrier surface for the cells to attach.

Feeder independent mouse embryonic stem cells Oct4B2 and feeder dependent mouse induced pluripotent stem cells OG2 iPS were plated on to the carriers at three different seeding densities - 5,000 cells/cm^2,10,000 cells/cm^2 and 15,000 cells/cm^2 for optimization and comparison purposes. The cells were then incubated in a humidified environment at 37°C and 5% CO_2 for 3 days. Cell growth was monitored every day by microscopy. At the end of day 3, ES cells were trypsinized, filtered through 100µm filter to remove the carriers, and a cell count was done using a haemocytometer. The iPS cells were trypsinized after 7 days.

3.2.6 Cell viability, attachment and pluriptency analysis:

Cell viability was measured using MTT assay while propidium iodide staining and scanning electron microscopy visualized cell attachment in 3D scaffolds. SSEA 1 staining and Oct4- GFP fluorescence analysis was carried out for pluripotency analysis. The protocols for all the assays are reported in *Chapter 2***.**

3.3 Results and Discussions:

3.3.1 Comparison of Gelatin, Matrigel and Tropoelastin as 2D Surface for Expansion of Mouse ES Cells:

In this study, a novel material tropoelastin was used to test its suitability as an attachment surface for mouse ES cells. The novel material was compared to gelatin and Matrigel, which have been widely used for ES cell culture.

Conditioned media from untreated as well as inactivated MEF were also compared to regular ES media in an effort to replace the use of LIF.

Effect of biomaterial on system pH: The pH for the various biomaterials over regular media was found to be more or less similar, negating any pH effect due to the material itself.

3.3.1.1 Cell Expansion & Pluripotency:

The Oct4B2 ES cells used have a GFP transgene under the control of the promoter for the pluripotent gene Oct4. This gives us an easy method to differentiate between the pluripotent and differentiated cells. Visual inspection and images of cells on gelatin and Matrigel showed heterogeneous populations of flat colonies, pluripotent as well as differentiated. Cells grown on tropoelastin coating, however, showed different morphology with the colonies being much smaller and less flat, giving the appearance of embryoid bodies.

Conditioned media (both from untreated and inactivated MEFs) were used to test their capability to maintain cell pluripotency on the different biomaterials used. It was observed from visual inspection that both the conditioned media showed more cell differentiation than normal ES media for gelatin and Matrigel coating but the cells growing on tropoelastin did not show any discernible difference in terms colony morphology on changing the media (Figures 3.1, 3.2, 3.3).

Efficiency of cell expansion was measured by cell counting using a haemocytometer. Both biomaterial and media type affected the expansion of mouse ES cell on 2D surface. As observed in figure 3.4, Matrigel coating coupled with normal ES cell media was seen to give the highest yield in terms of cell number. However, cell yield on Matrigel decreased when conditioned media from untreated and inactivated MEFs was used. Cell expansion on gelatin coating was almost uniform irrespective of the media used. Cell expansion on gelatin was equivalent to that of Matrigel on conditioned media. Cell number on tropoelastin coating was the lowest in all cases, being not affected by the type of media or the concentration of tropoelastin used.

Flow cytometric analysis of OCT-GFP showed that percentage of GFP positive cells remained greater than 80% in all cases (Figure 3.5), irrespective of the biomaterial and media used.

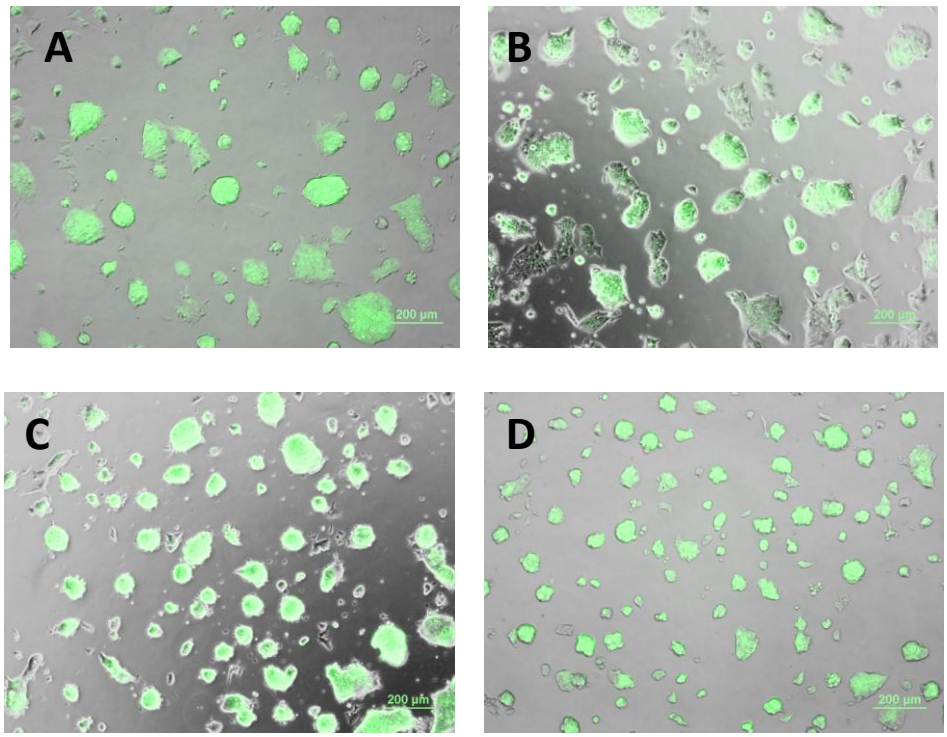


Figure 3.1 Oct4B2 ES cell culture in ES media and various biomaterial coatings on day 2.(A) Gelatin,(B) Matrigel,(C) Tropoelastin (1.5mg/ml),(D) Tropoelastin (3mg/ml). Scale bar= 200µm

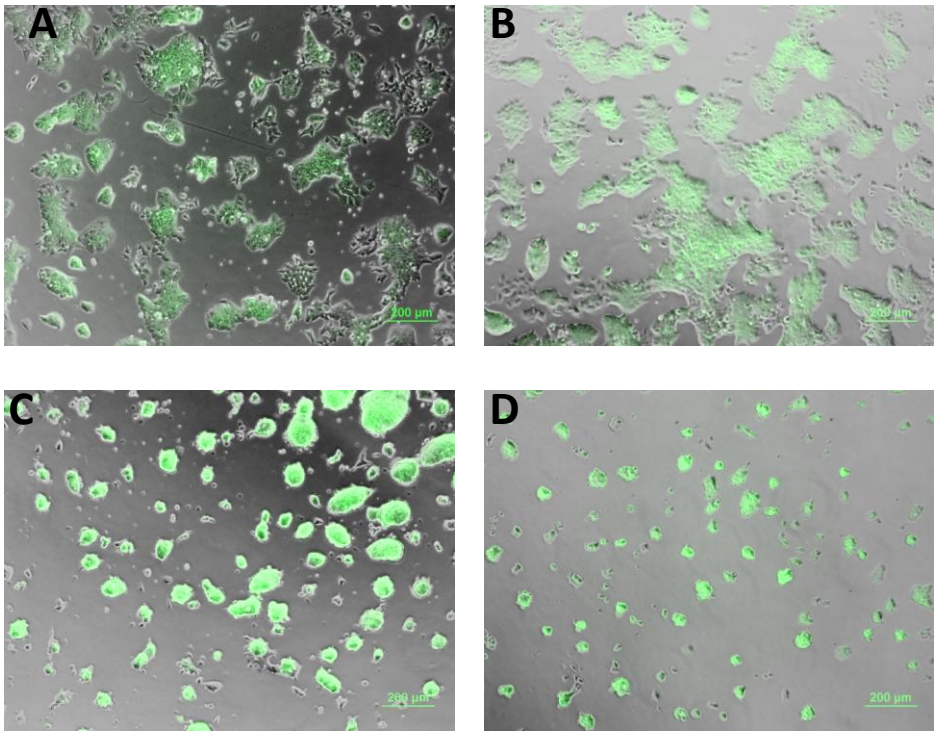


Figure 3.2 Oct4B2 ES cell culture in untreated MEF conditioned media and various biomaterial coatings on day 2.(A) Gelatin, (B) Matrigel, (C) Tropoelastin (1.5mg/ml), (D) Tropoelastin (3mg/ml).. Scale bar = 200µm

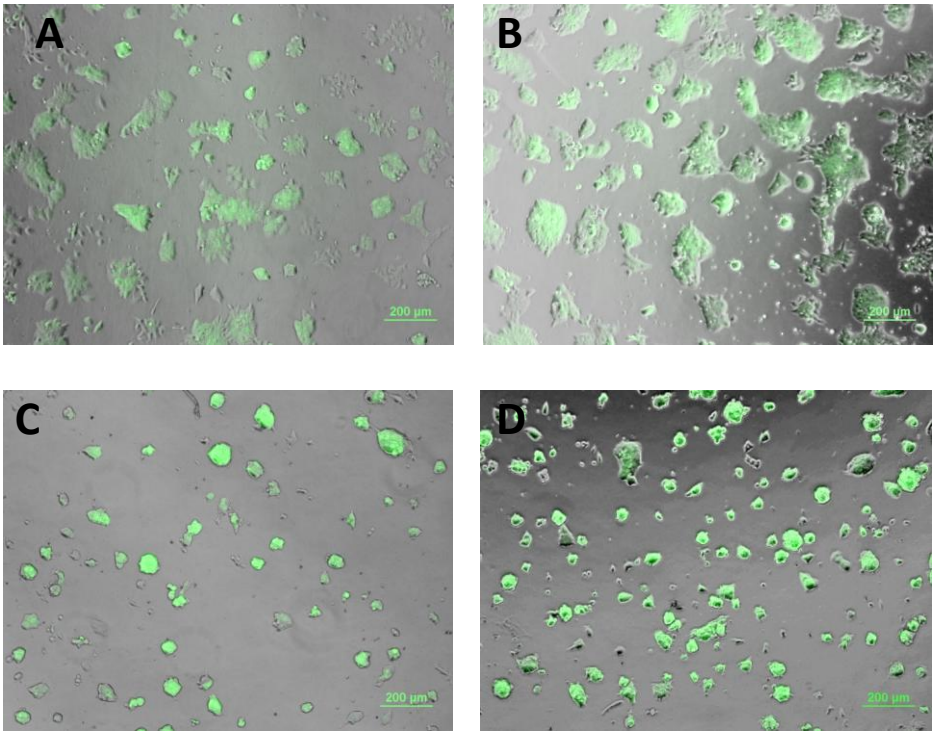


Figure 3.3 Oct4B2 ES cell culture in inactivated MEF conditioned media and various biomaterial coatings on day 2. (A) Gelatin, (B) Matrigel,(C) Tropoelastin (1.5mg/ml),(D) Tropoelastin (3mg/ml). Scale bar = 200µm

It is to be noted that limited amount of Tropoelastin made available as a gift prevented us from carrying out higher number of replicates and more extensive experiments.

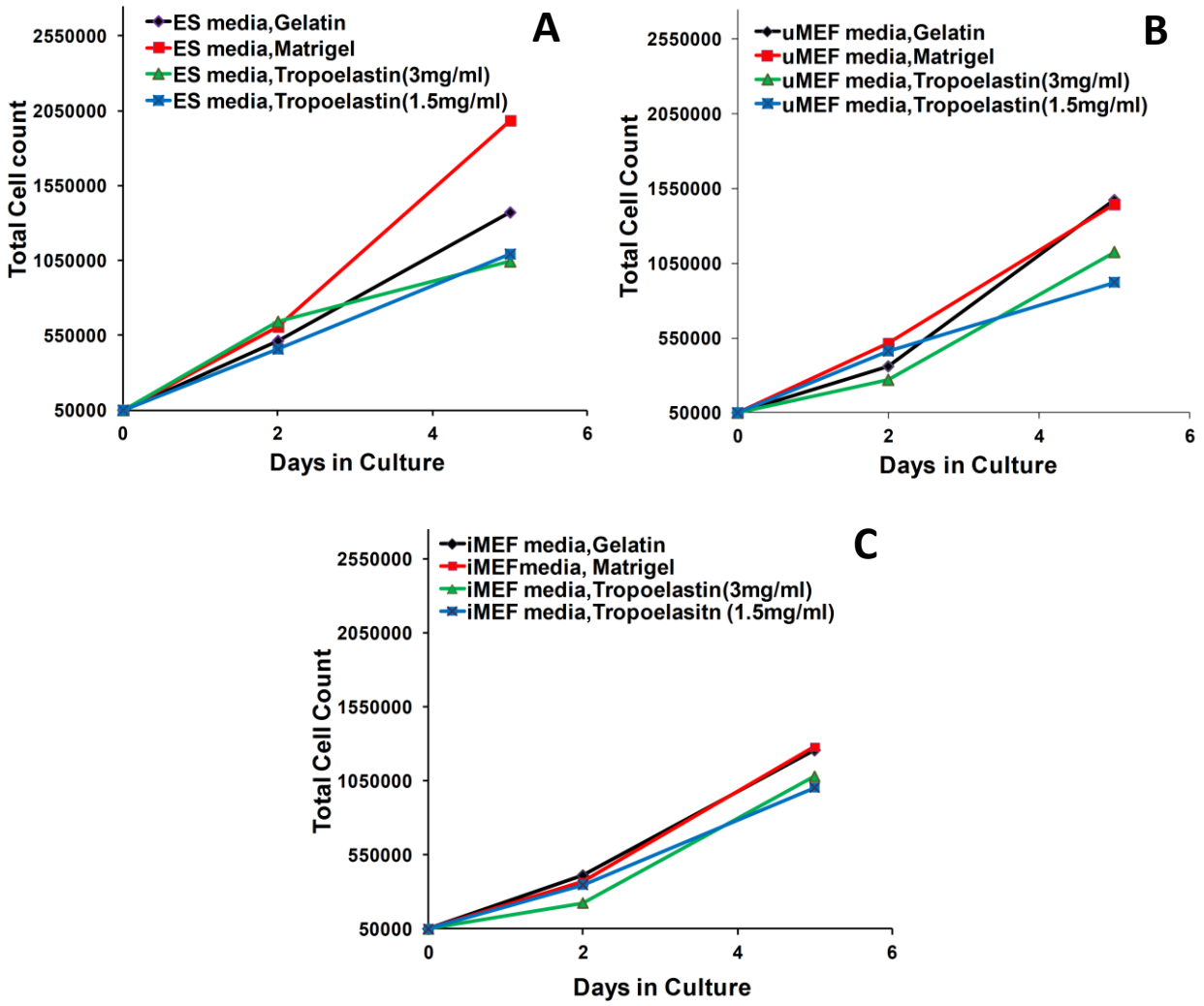


Figure 3.4 Cell count on different matrices and media (A) ES media, (B) untreated MEF conditioned media, (C) inactivated MEF conditioned media (data represent mean of n=2 independent experiments)

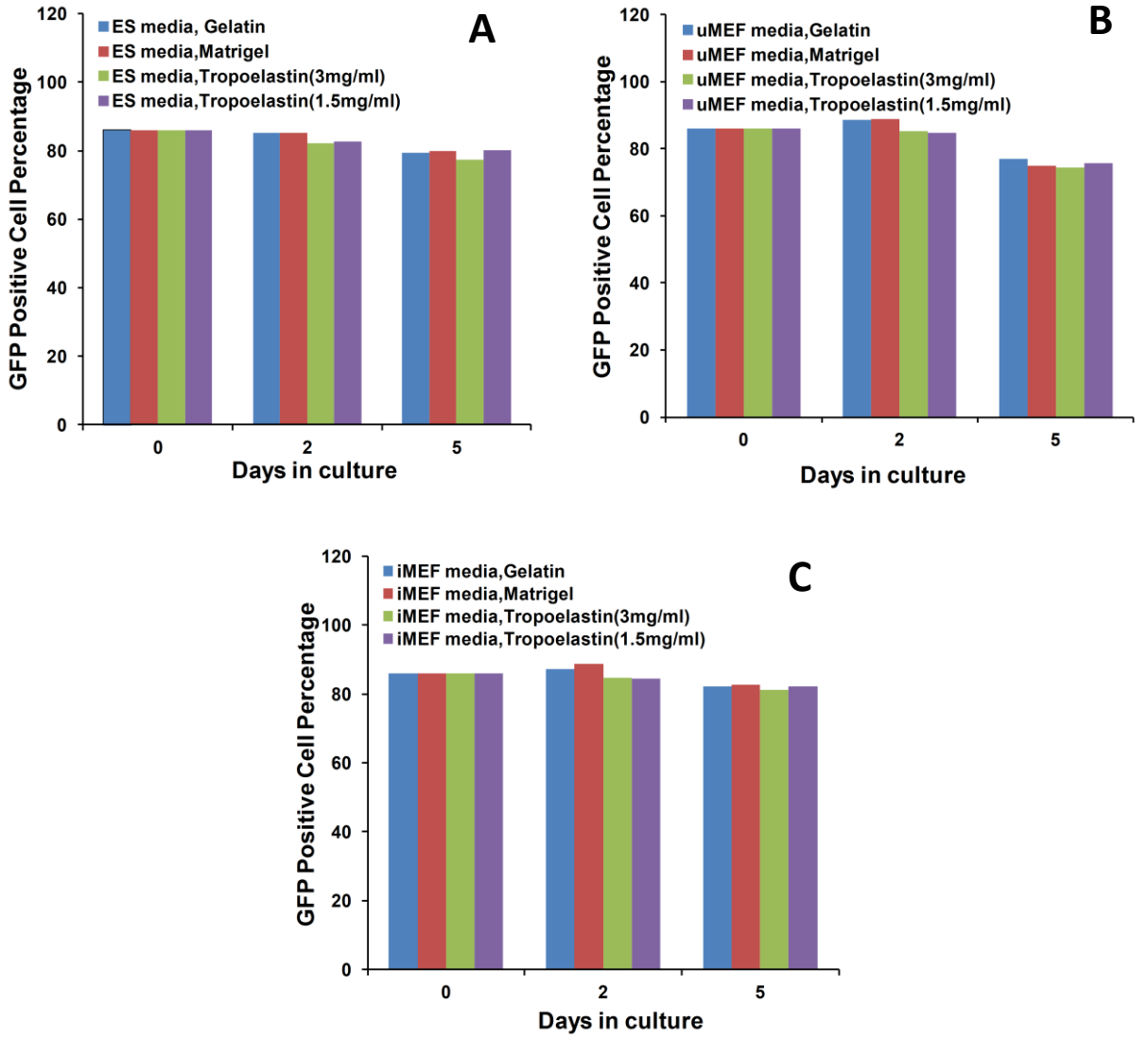


Figure 3.5 Cell count on different matrices and media (A) ES media, (B) untreated MEF conditioned media, (C) inactivated MEF conditioned media (data represent mean of n=2 independent experiments)

3.3.1.2 Conclusion:

Although tropoelastin was able to maintain cell pluripotency, cell expansion on it was lower than gelatin and Matrigel; it also resulted in changed colony morphology. Hence, the hypothesis that Tropoelastin would significantly improve cell expansion could not be proved with significance. Matrigel was able to maintain cell pluripotency and result in a high yield, but its efficiency was dependent on the type of media used. Also, the use of Matrigel for long term or 3D culture is not a very cost effective option. Gelatin, on the other hand, gave a consistent cell yield in all

conditions and also maintained colony morphology and cell pluripotency. Taking in to account all the data, gelatin's suitability for ES cell culture was confirmed and it was taken one step further and used for 3D culture of ES cells. Both commercially available gelatin based microcarriers and novel gelatin based scaffolds, prepared in house, were used for ES cell culture.

Although pluripotency of the cells was maintained in all the three types of media used, the cell number was affected negatively when conditioned media was used. This demonstrated that further study of conditioned media and the effect of media related parameters on pluripotent cells was needed prior to its use.

3.3.2 3D culture of ES cells in gelatin and PMVE-MA+gelatin scaffolds:

Based on the previous results, gelatin was chosen as the base material for the construction of novel scaffolds for 3D expansion of ES cells. This work was carried out in collaboration with Miss Hemlata Chhabra, research scholar, Chemical Engineering Department, IIT Bombay. Miss Hemlata did scaffold preparation, mechanical characterization and initial biocompatibility analysis of the scaffolds. Culturing and characterization of ES cell expansion in the scaffolds only falls within the scope of this thesis.

3.3.2.1 Characteristics of Scaffolds:

Two different types of scaffolds - 4% gelatin, composite of 4% gelatin and 0.5% PMVE-MA were used for expansion of mouse ES cells after initial screening of composite with various concentrations of PMVE-MA. The scaffolds were cross linked with HMDI (10% v/v in isopropanol) and were prepared by simple freeze - drying method. The scaffolds were physically in the size and shape of a well in 24 well plates and had a surface area of approximately 2.0 cm^2.

The gelatin scaffold had a pore size of 155±30 microns while the PMVE-MA + gelatin composite scaffold (in future referred to as only composite scaffold) had a pore size of 190±40 microns. Figure 3.6 shows the honeycomb-like structure of the composite scaffold as seen under scanning electron microscopy (SEM). Similar structure was also seen for the pure gelatin scaffold. Swelling ratio of the composite was found to be higher than pure gelatin scaffolds. The

composite also had a higher tensile strength and compression modulus in comparison to the pure gelatin scaffold. Both the scaffolds were haemocompatible.

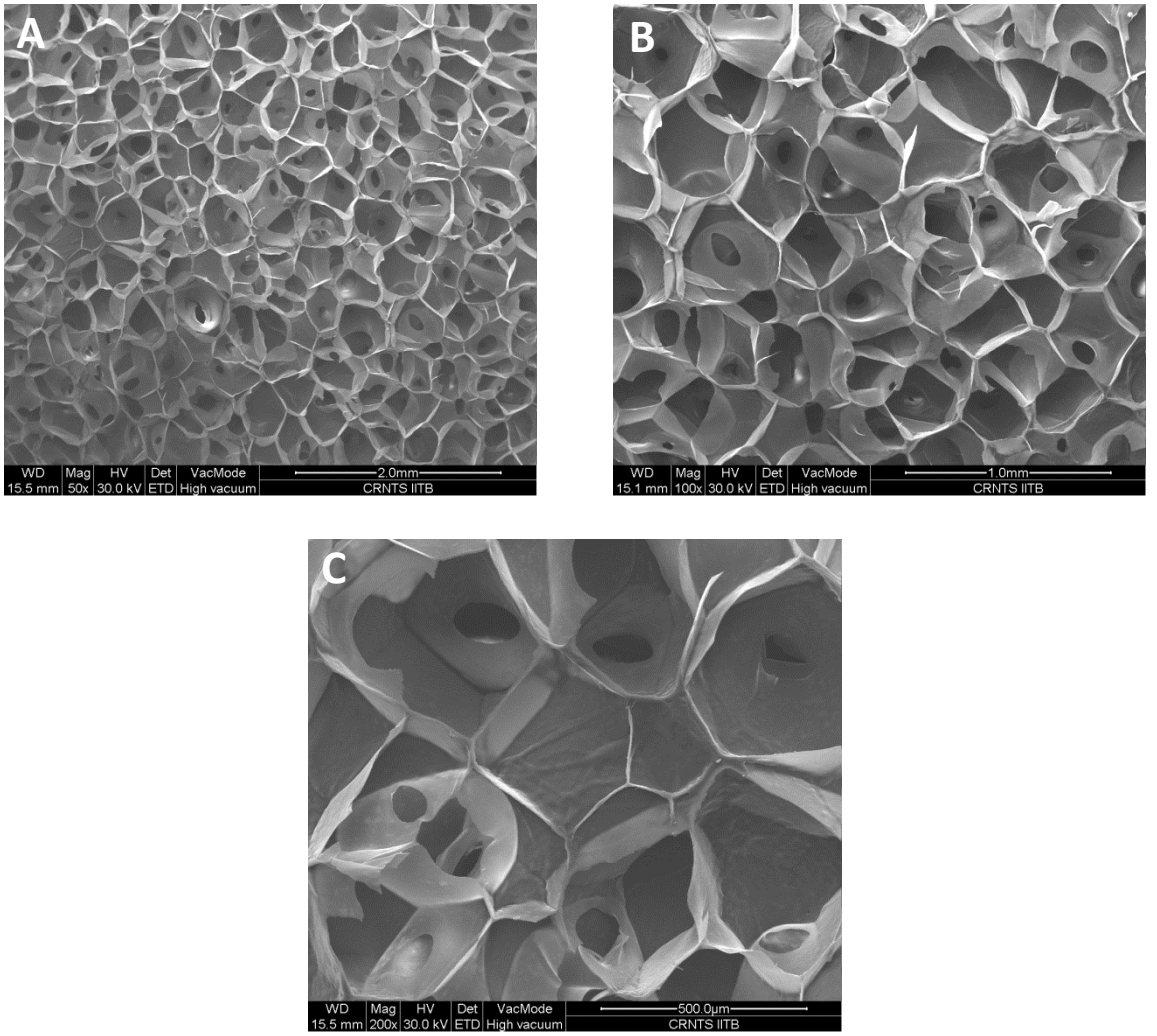


Figure 3.6 SEM images for PMVE- MA+gelatin composite scaffolds. (A) 50X magnification (scale bar=2.0 mm), (B) 100X magnification (scale bar=1.0 mm), (C) 200X magnification (scale bar=500 µm)

3.3.2.2 Mouse ES cell viability:

Oct4B2 mouse ES cells were cultured within the two scaffolds for a time period of 7 days. Cell viability was measured by MTT assay done on days 2, 5 and 7 (Figure 3.7). Cells grown in 24 well plates at the same seeding density were used as control. Data here represent mean±SEM of n=4 independent experiments, with each independent experiment being done in duplicates.

Although not significant, the composite scaffold showed lowest cell viability on day 2. However, it became comparable to the gelatin scaffold on day 5, although both the scaffolds had lower cell density in comparison to the control. However, by day 7, the composite scaffold showed a slightly higher cell number than the gelatin scaffold although it was not statistically significant. Cell number within the scaffold was found to be significantly higher on day 7 in comparison to day 3 and day 5. The results proved that the novel gelatin + PMVE- MA composite scaffold was able to support cell proliferation. Since the Oct4B2 mouse ES cells are feeder independent and were routinely cultured on gelatin coated dishes in 2D, the gelatin scaffolds were almost a regular surface for them; the initial delay in growth within the composite scaffold can be attributed to the time required by the cells to adapt to the new material prior to rapid expansion.

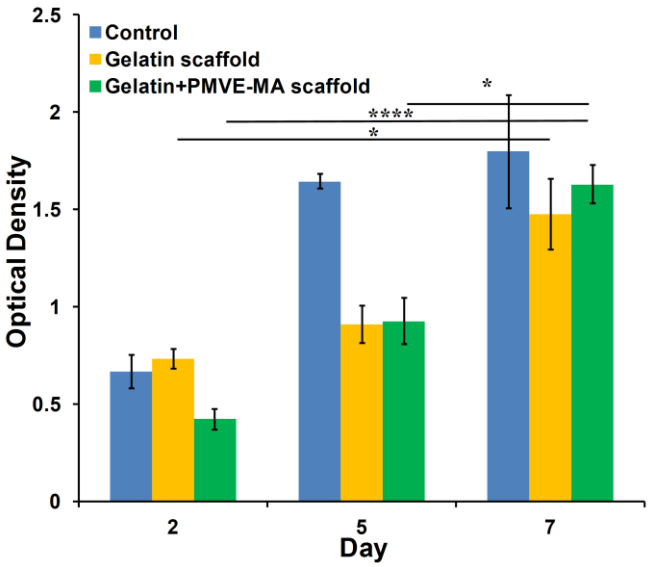


Figure 3.7 Cell viability in different scaffolds measured by MTT assay in comparison to 2D culture system as control (data represent mean±SEM of n=4 independent experiments)

3.3.2.3 Cell attachment, morphology and colony formation:

Cell attachment, their morphology and colony formation over time was observed using scanning electron microscopy on days 2, 5 and 7 and propidium iodide staining on days 2 and 7. SEM images (Fig 3.8-3.10) showed that the cell density on composite scaffold was less than the pure gelatin scaffold on day 2, corroborating the MTT data. By day 7, mouse ES cells had formed a dense layer on both the scaffolds, proving that both the gelatin scaffolds and the novel composite scaffold were able to support the attachment and expansion of mouse ES cells.

Propidium iodide staining on cells on days 2 and 7 also supported the SEM images and clearly showed the expansion of ES cells in both the scaffolds (fig 3.11, 3.12).

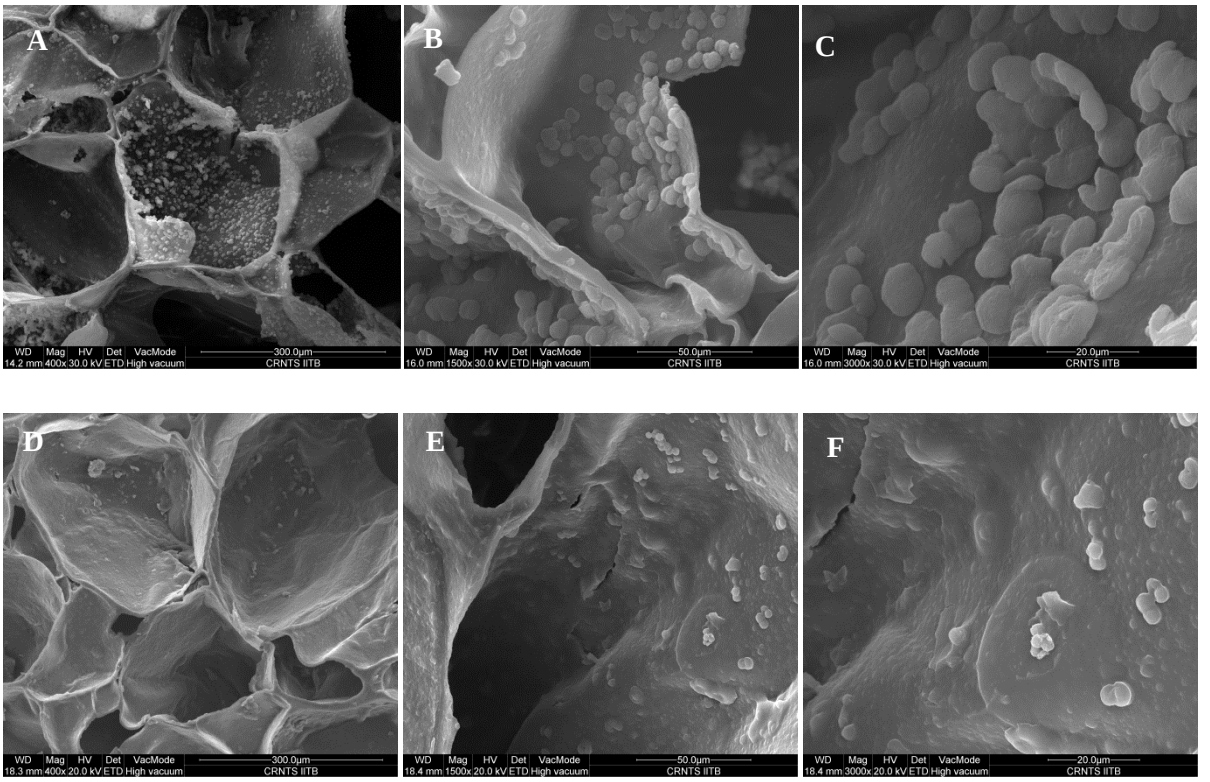
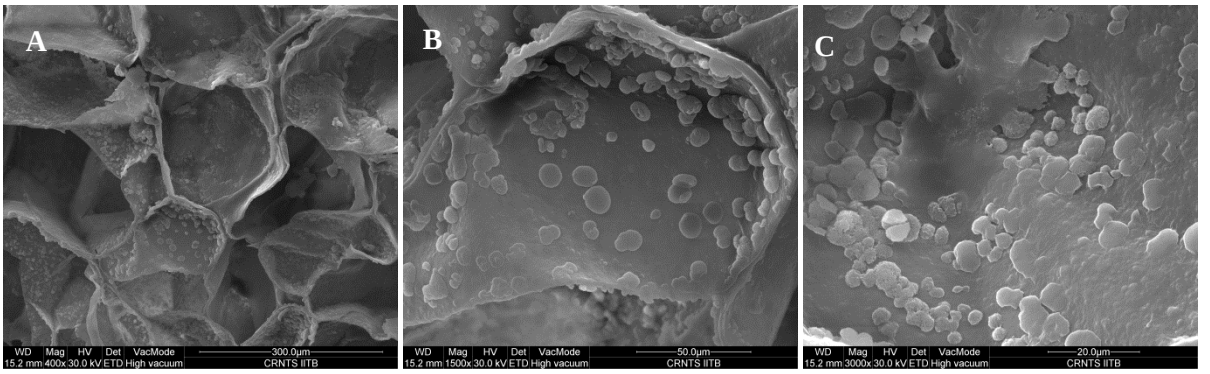


Figure 3.8 SEM images for Oct4B2 cell growth on scaffolds on day 2. (A-C) Gelatin scaffold, (D-F) Composite scaffold. Magnifications: (A, D) = 400X (scale = 300µm), (B, E) = 1500X (scale = 50µm), (C, F) = 3000X (scale = 20µm)



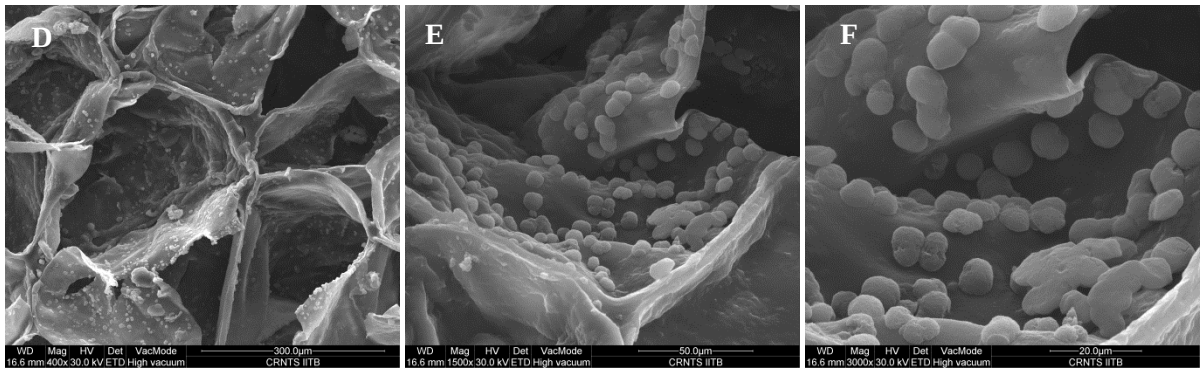


Figure 3.9 SEM images for Oct4B2 cell growth on scaffolds on day 5. (A-C) gelatin scaffold, (D-F) Composite scaffold. Magnifications: (A, D) = 400X (scale = 300µm), (B, E) = 1500X (scale = 50µm), (C, F) = 3000X (scale = 20µm)

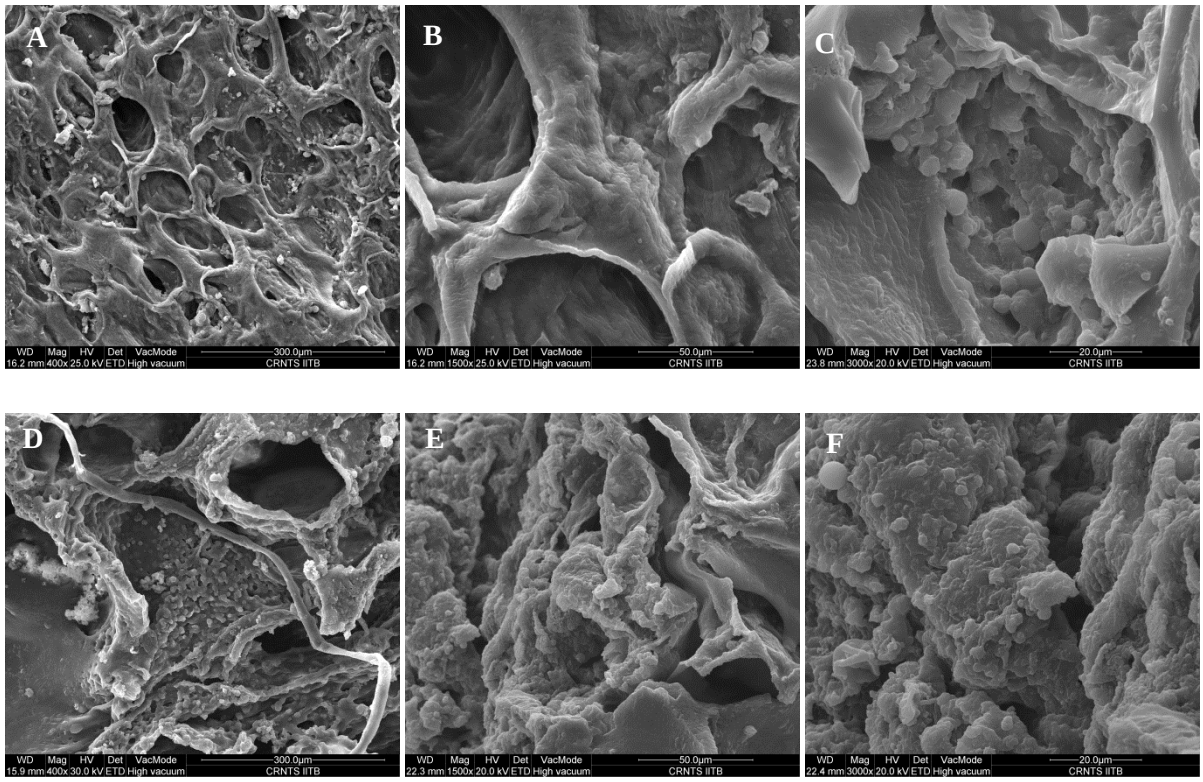


Figure 3.10 SEM images for Oct4B2 cell growth on scaffolds on day 5. (A-C) gelatin scaffold, (D-F) Composite scaffold. Magnifications: (A, D) = 400X (scale = 300µm), (B, E) = 1500X (scale = 50µm), (C, F) = 3000X (scale = 20µm)

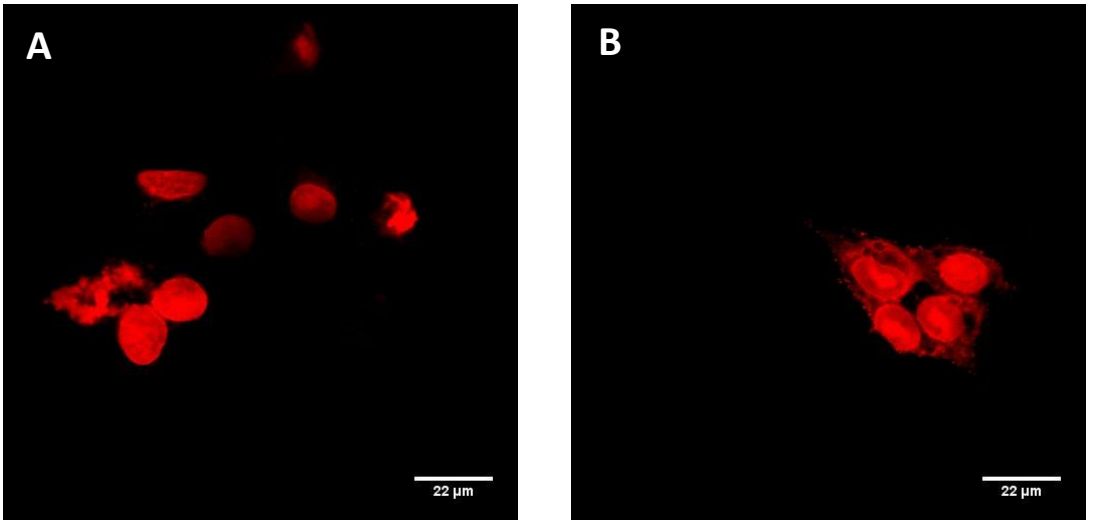


Figure 3.11 Propidium iodide staining of Oct4B2 ES cells in scaffolds on day 2. (A) Gelatin scaffold, (B) Composite scaffold. 63X magnification (scale = 22µm)

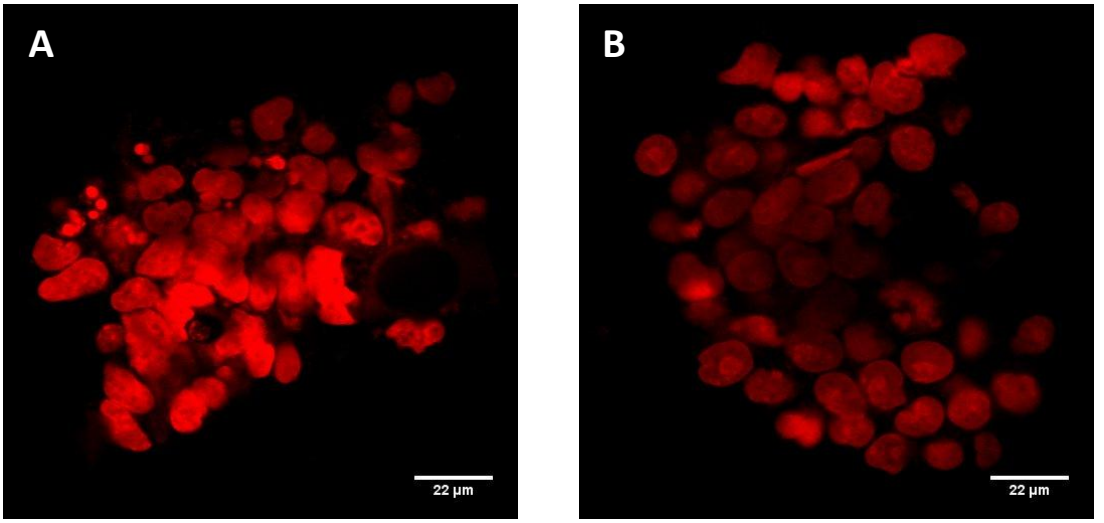


Figure 3.12 Propidium iodide staining of Oct4B2 ES cells in scaffolds on day 7. (A) Gelatin scaffold, (B) Composite scaffold. 63X magnification (scale = 22µm)

3.3.2.4. Cell penetration within scaffolds:

Propidium iodide staining was also used for z - stacking of images to estimate the cell penetration within the scaffolds. The penetration of the cells within the scaffold is of importance for the proper utilization of the available surface area and also for protection of the cells within a dynamic culture system. The cell penetration for both the scaffolds was compared. On day 7, cells were visible up to a depth of about 50µm in pure gelatin scaffold and to a distance of about

30µm in the composite scaffold (fig 3.13, 3.14), suggesting greater cell penetration in the pure gelatin scaffold in comparison to the composite scaffold.

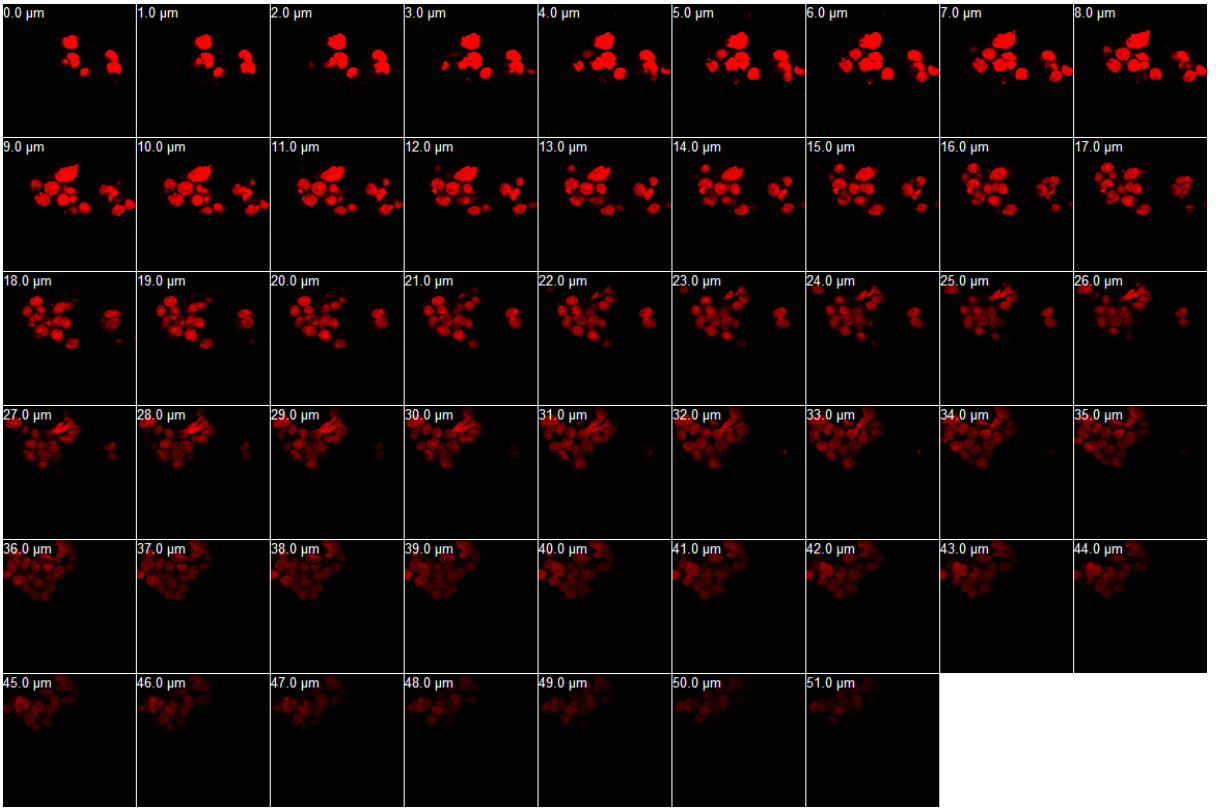


Figure 3.13 Z stacking of Oct4B2 ES cells in pure gelatin scaffold on day 7 (63X magnification, scale bar = 22µm)

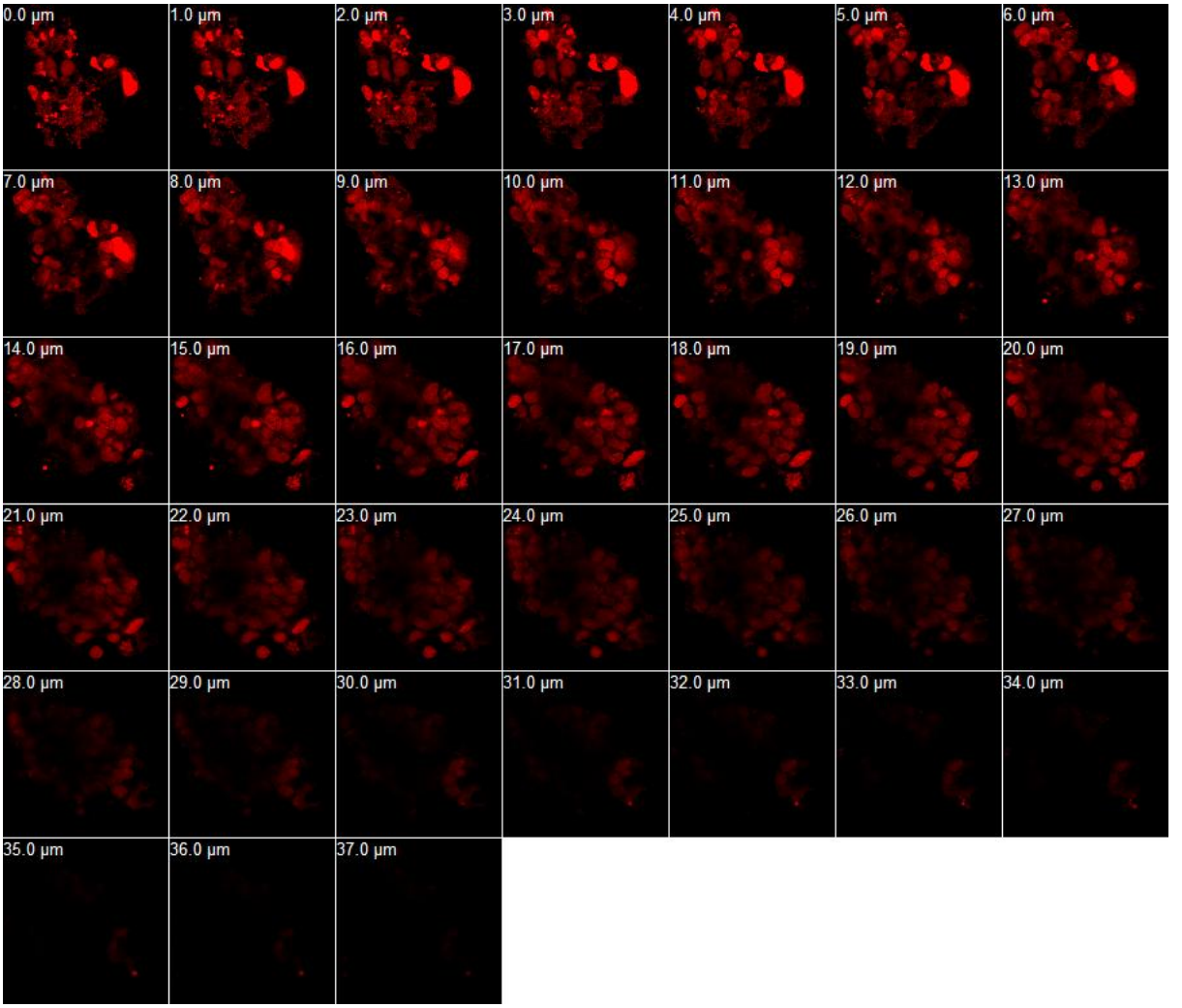


Figure 3.14 Z stacking of Oct4B2 ES cells in composite scaffold on day 7 (63X magnification, scale bar = 22µm)

3.3.2.5 Pluripotency Maintenance:

The ability of the two scaffolds to maintain the pluripotency of mouse ES cells was tested by SSEA-1 immunostaining. SSEA-1 is a pluripotency marker for mouse ES cells. Immunostaining was done on days 2 and 7 and visualised using a confocal laser scanning microscope. The images in figures 3.15 and 3.16 clearly showed that the mouse ES cells on both scaffolds were able to maintain their pluripotency during their long term expansion.

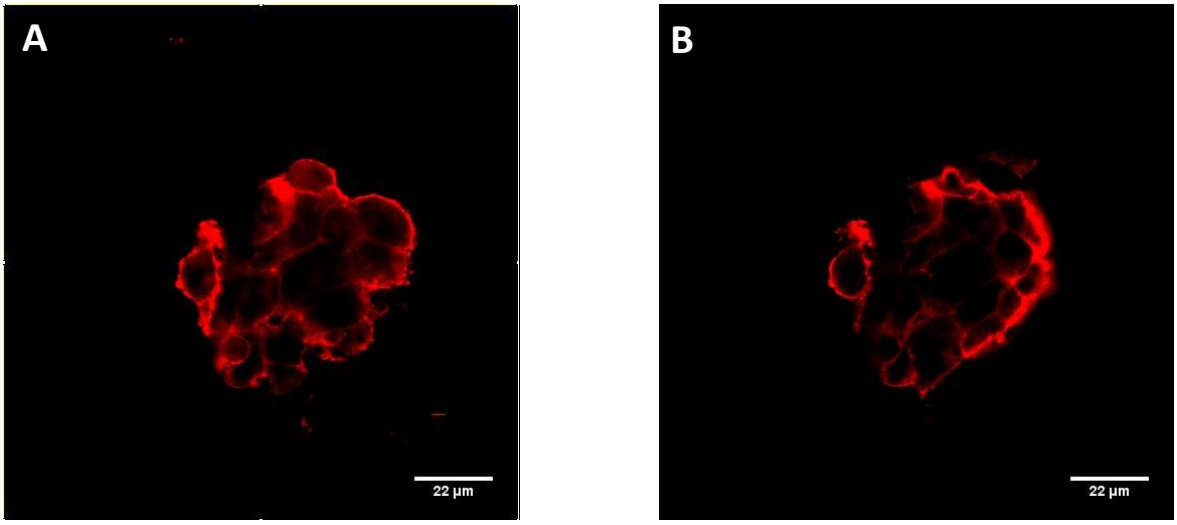


Figure 3.15 SSEA 1 staining of OCT4B2 ES cells on day 2 at 63X magnification (scale bar = 22µm). (A) Gelatin scaffold, (B) Composite scaffold

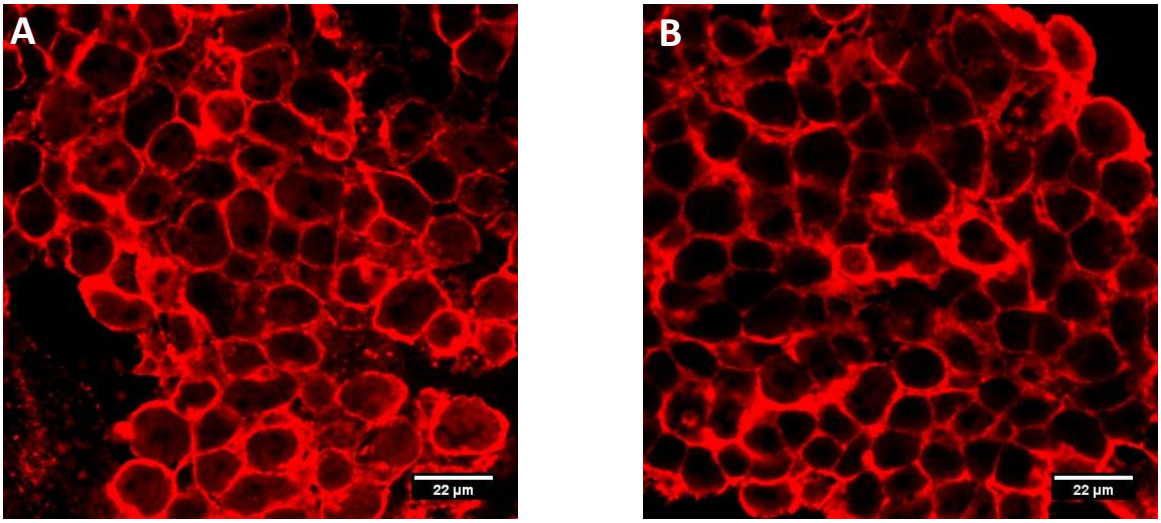


Figure 3.16 SSEA 1 staining of OCT4B2 ES cells on day 7 at 63X magnification (scale bar = 22µm). (A) Gelatin scaffold, (B) Composite scaffold

3.3.2.6 Conclusion:

Both the gelatin scaffold and the composite scaffold were proven to be suitable for long term expansion and pluripotency maintenance of mouse ES cells. Being a new attachment surface for the cells, the initial cell expansion in the composite scaffold was slower than the expansion in the gelatin scaffold. However, by day 7, the cells had expanded well within the composite scaffold. MTT assay data showed the composite scaffold was comparable to the pure gelatin scaffold in

terms of cell number at at the end of day 7. Propidium iodide staining and SEM imaging showed the attachment, colony formation and expansion of ES cells on both scaffold and supported the MTT data. SEM images also showed that by day 7, ES cells have formed layers on the scaffold covering its surface.

Z stacking using confocal microscopy showed that the cells had penetrated within the honeycomb structure of the scaffolds. The cells were visible until a depth of about 50µm in gelatin scaffold and 30µm within the composite scaffold. Taking all the results together, it can be concluded that the novel composite scaffold was able to support cell attachment, penetration, expansion and pluripotency for mouse ES cells. However, the recovery of cells from within the scaffold was not easy and hence limited the scaffold's usage. The composite scaffold was thus demonstrated to be a viable option of culturing of ES cells, depending on the end goal.

3.3.3 Static culture of pluripotent stem cells on Cytodex 3 and Hillex II microcarriers:

The current work looks at preliminary studies to compare two commercially available microcarriers for 3D culture of mouse ES (feeder independent) and iPS (feeder dependent) cells in terms of cell attachment, expansion and pluripotency maintenance.

3.3.3.1 Cell attachment, morphology, expansion and pluripotency:

Cell attachment, culture morphology and pluripotency of the mouse embryonic stem cells were monitored via microscopy. The Oct4 promoter driven GFP fluorescence was used as an indicator for pluripotency. Cell expansion was measured by cell count using haemocytometer on day 3 for the ES cells while on day 7 for the iPS cells.

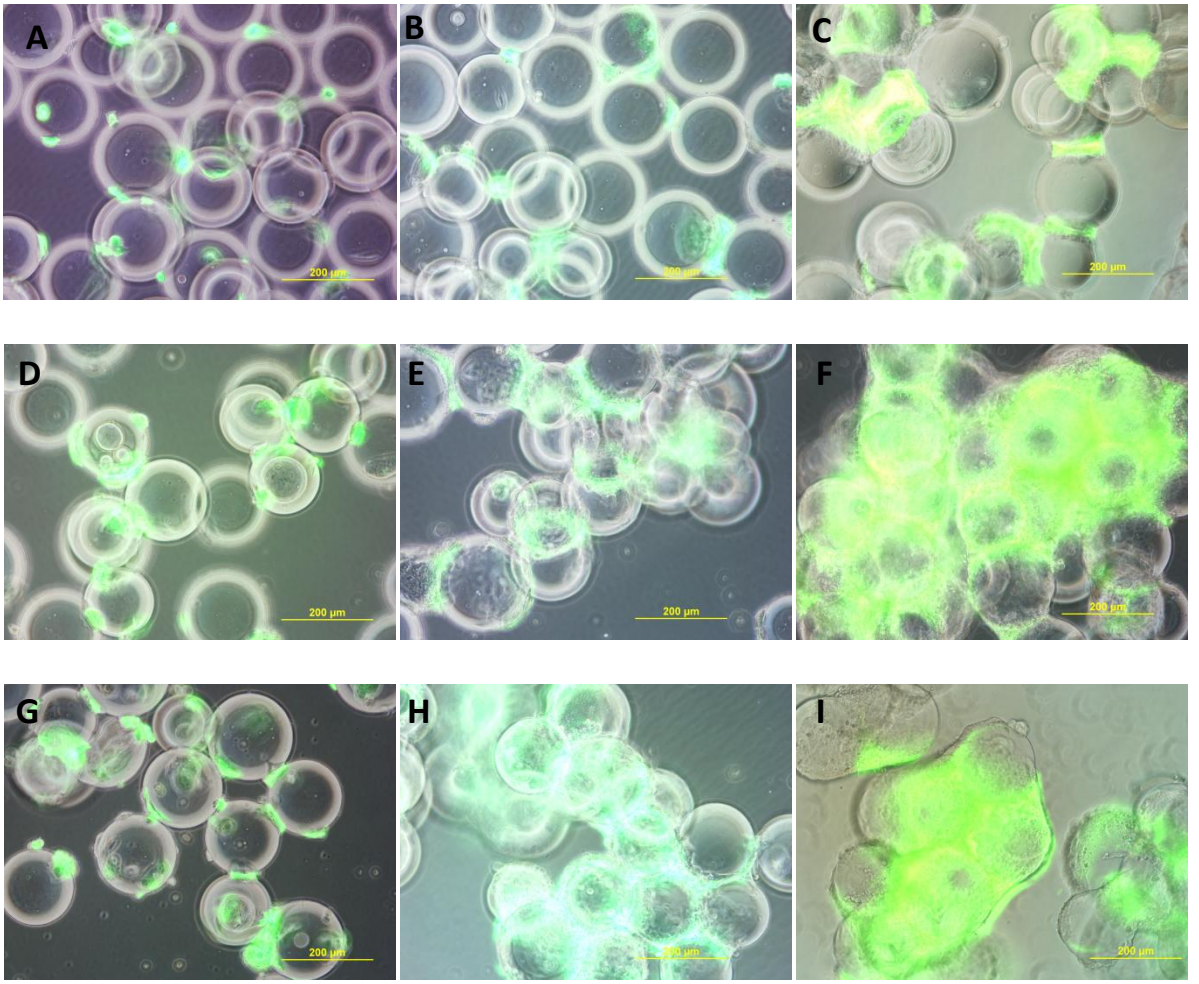


Figure 3.17 Attachment and growth of Oct4B2 mouse ES cells on Cytodex 3 at different seeding densities on days 1, 2 and 3. (A-C): Seeding density: 5000cells/cm^2; (D-F): Seeding density: 10,000cells/cm^2; (G-I): Seeding density: 15,000cells/cm^2. Scale bar = 200µm

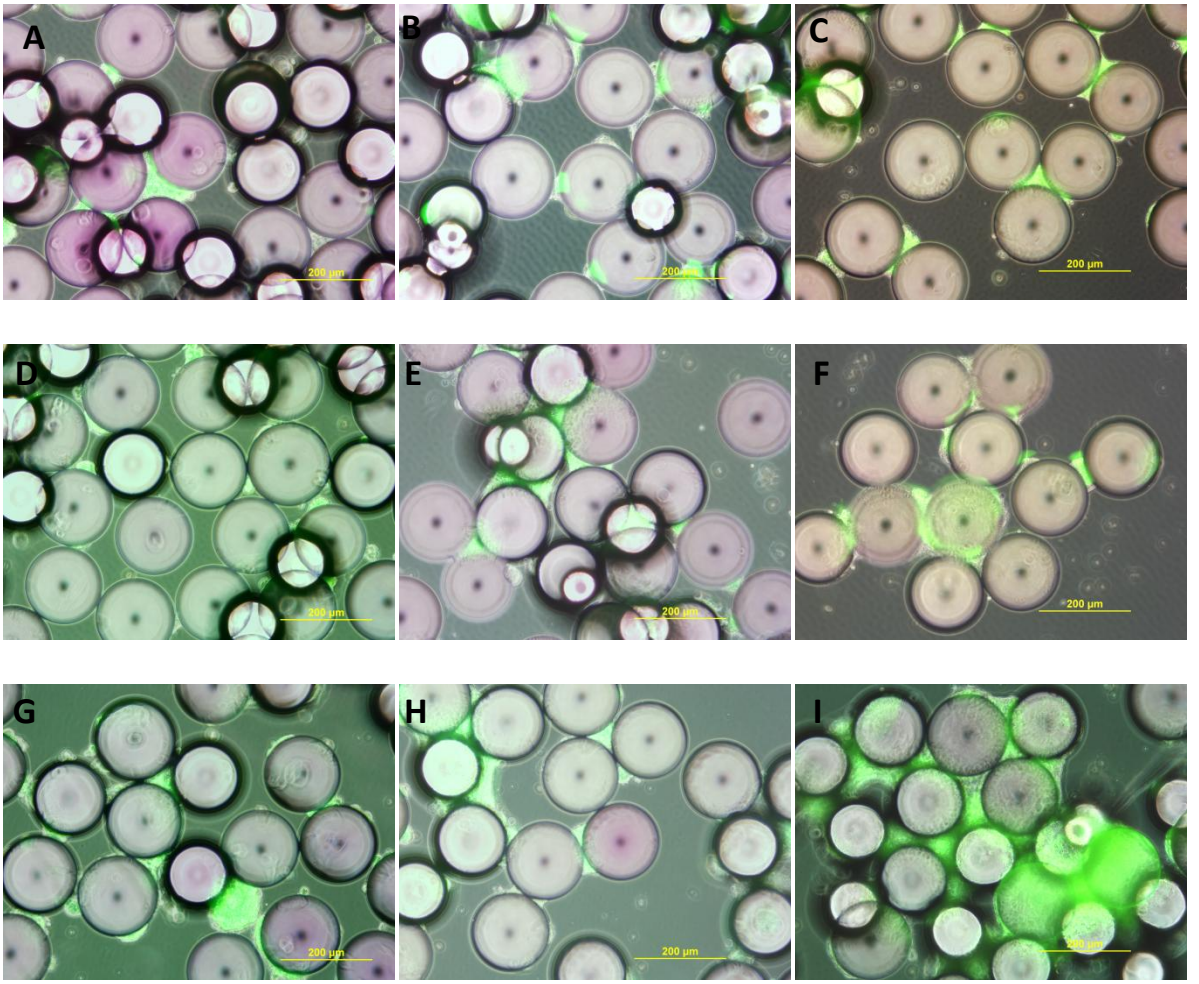


Figure 3.18 Attachment and growth of Oct4B2 mouse ES cells on Hillex II at different seeding densities on days 1, 2 and 3. (A-C): Seeding density: 5000cells/cm^2; (D-F): Seeding density: 10,000cells/cm^2; (G-I): Seeding density: 15,000cells/cm^2. Scale bar = 200µm

As seen from microscopy images of day 1, ES cells were able to attach on both the carriers, although the attachment was better on Cytodex 3 than Hillex II. Due to the static nature of the culture system, cell aggregation and "bead bridging" were observed on days 2 and 3 for both the carriers. Bead bridging was the phenomenon where the microcarriers became attached together due to the aggregation of the expanding cells on the carriers. Higher seeding density also increased cell aggregation. Visual examination showed higher cell expansion on Cytodex 3 than Hillex II. The Hillex II culture also showed higher number of non attached floating cells (figures 3.17, 3.18).

The feeder independent mouse iPS cells showed low cell adhesion in the initial days. However, by day 7 the attached cells had expanded well on Cytodex 3 microcarriers. Cell expansion on Hillex II was found to be very low (Figures 3.19, 3.20)

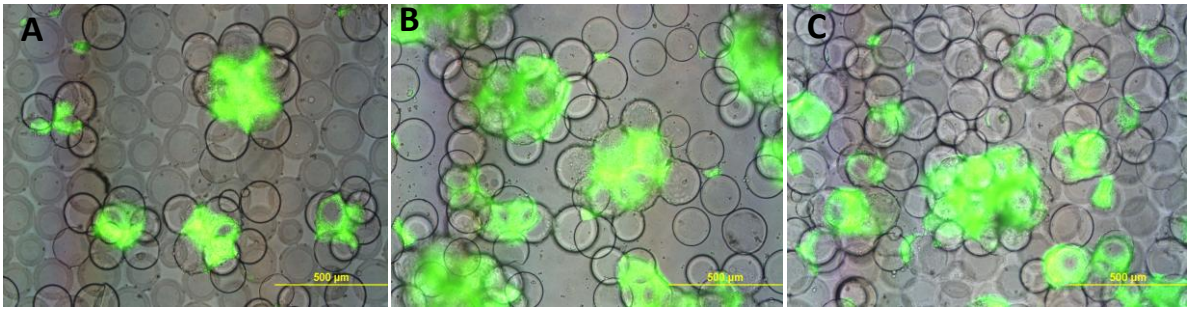


Figure 3.19 Attachment and growth of mouse iPS cells on Cytodex 3 at different seeding densities after days 7. (A) Seeding density: 5000cells/cm^2; (B) Seeding density: 10,000cells/cm^2; (C) Seeding density: 15,000cells/cm^2 . Scale bar = 500µm

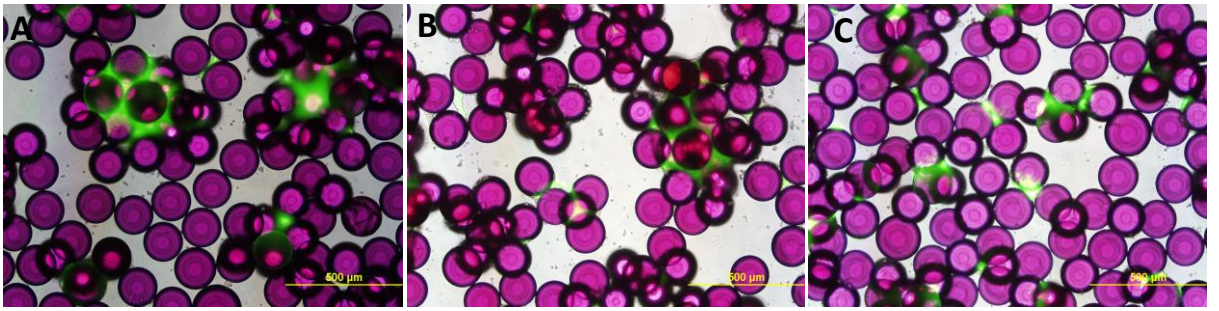


Figure 3.20 Attachment and growth of mouse iPS cells on Hillex II at different seeding densities after day 7. (A) Seeding density: 5000cells/cm2; (B) Seeding density: 10,000cells/cm2; (C) Seeding density: 15,000cells/cm2 . Scale bar = 500µm

Cell count on day 3 (figure 3.21) showed higher cell number in Cytodex 3 culture in comparison to Hillex II for both the cell types. Higher cell number was obtained for a seeding density of 15,000 cells/cm^2 in case of Hillex II. Surprisingly, for Cytodex 3, the 10,000cells/cm^2 seeding density gave the highest fold increase in cell number. This may be due to the fact that the highest seeding density resulted in large cell aggregate formation, giving rise to a decrease in cell expansion. Visualisation of carriers after cell detachment (figure 3.22) showed that the cells had completely come off from the carriers and can be rejected as a reason for lower cell number in case of 15,000 cells/cm^2 seeding density.

As observed by visual inspection of Oct4 driven GFP, pluripotency of the attached cells was maintained by both the microcarriers.

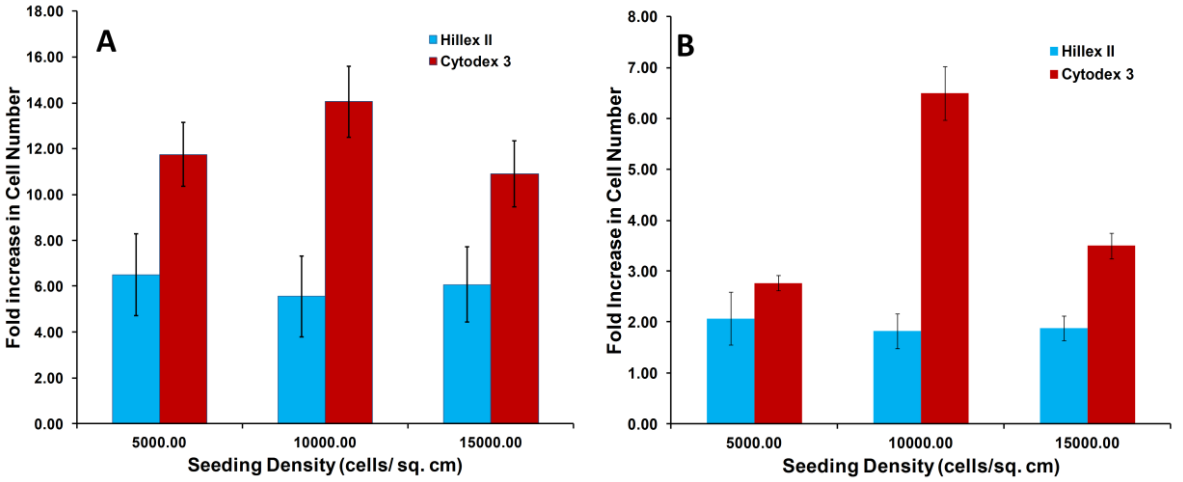


Figure 3.21 Fold increase in cell number on microcarriers (A) Feeder independent embryonic stem cells after day 3, (B) Feeder dependent induced pluripotent stem cells after day 7. Data represents mean ±SEM of n=3 individual experiments

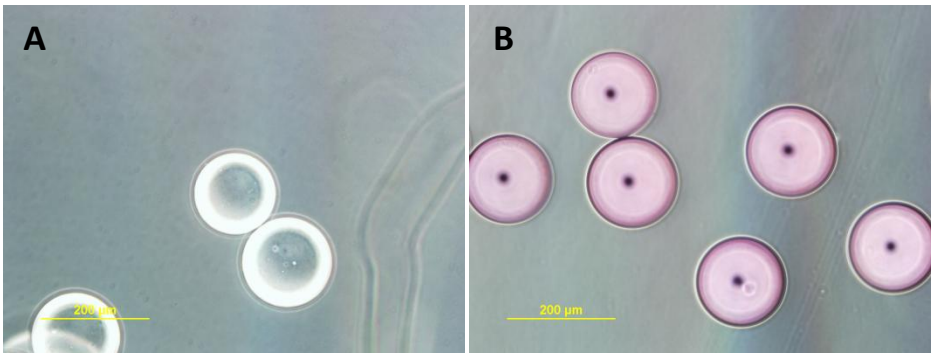


Figure 3.22 Carriers after cell detachment using Tryple. Both carriers show completely detachment of cells. (A) Cytodex 3, (B) Hillex II. Scale bar = 200µm

3.3.3.2 Conclusion:

Both Cytodex 3 and Hillex II have been previously used for expansion of mouse ES cells. However, a review of the literature shows that the efficiency of these carriers is cell line dependent. In the current work, both Cytodex 3 and Hillex II were able to support the expansion of mouse ES cells in static culture. A seeding density of 10,000 cells/cm^2 resulted in the highest

cell density. Cytodex 3 had a higher final cell density than Hillex II. Cytodex 3 was also able to maintain cell attachment and expansion of mouse iPS cells. The feeder dependent iPS cells initially showed a low cell number which can be attributed to the cells' adaptation on the feeder free system. The expansion of cell on the carriers resulted in easier cell collection, thus making it an excellent option for dynamic culture set ups where the direct use of expanded cells would be the main aim.

3.4 Conclusion:

The presence of feeder layer is one of the biggest hindrances in large scale expansion of pluripotent stem cells. Efforts have been made to replace the feeder cells with suitable biomaterials, both natural and artificially in the form of 2D coatings or 3D culture systems.

The results in this chapter demonstrated the suitability of various biomaterials for culturing mouse embryonic stem cells in 2D and 3D systems. Both 2D and 3D systems have their own importance in terms of cell culture. The 2D system is required for initial testing of novel biomaterials and other parameters at a small but high throughput scale. A 3D system is important for large scale expansion of cells and to mimic the *in vivo* conditions.

Gelatin, Matrigel and tropoelastin were compared as 2D coatings for the expansion of mouse embryonic stem cell Oct4B2. The suitability of gelatin and Matrigel as a matrix for ES cells has been well demonstrated. The study here compared the novel material tropoelastin with the other two. All three materials supported the growth of ES cells and maintained their pluripotency but Matrigel and gelatin were found to be better than tropoelastin in terms of final cell density achieved.

Based on the above results, gelatin based 3D culture system in the form of scaffolds was tested for the growth and expansion of mouse embryonic stem cells. A 3D scaffold structure is highly coveted for its high surface area: volume ratio and also for its ability to provide protection to sensitive cells from external damages in a dynamic culture system. Although the novel gelatin +PMVE- MA scaffold was able to support ES cell culture, but the hindrance in cell collection deemed it unfit for usage in situations where the main aim was to use the expanded cells directly.

However, the scaffolds can be alternatively used for various other purposes where the cell secretions and not the cells are more important.

Our initial hypothesis was confirmed to be partially correct for the current work. Although tropoelastin has been previously used for the expansion of other cell lines and was able to support the growth of mouse ES cells for our work, it did not turn out to be a better material in comparison to already established gelatin and Matrigel.

As hypothesized, the PMVE- MA + gelatin scaffold was able to support the 3D culture of mouse ES cells and to maintain their pluripotency too.

Commercially available gelatin based microcarrier Cytodex 3 was compared to a polystyrene microcarrier Hillex II. Both carriers supported the attachment and expansion of mouse pluripotent stem cells; however, the final cell density was higher on the Cytodex 3 carriers. Based on these results, Cytodex 3 was used for the expansion of induced pluripotent stem cells in a dynamic system as described in the next chapter. In conclusion, tropoelastin was not considered to be a good candidate for pluripotent stem cell culture, while Cytodex 3, Hillex II and gelatin + PMVE- MA composite scaffolds were found to be suitable platforms for pluripotent stem cell expansion. The choice of the type of 3D structure used would, however, depend on the experimental procedure and end results expected.

Chapter 4 Expansion of Mouse Induced Pluripotent Stem Cells in a Spinner Flask System: Effect of RPM on Microcarriers and Cells

4.1 Introduction:

The use of the spinner flask is one of the oldest and easiest methods for large scale expansion of mammalian cells. It has also been used for the expansion of embryonic stem cells in the form of cell aggregates or as adherent culture on microcarriers[49,90,129]. Recently, induced pluripotent stem cells have also been expanded in spinner flasks[60, 61, 99, 100]. However, expansion as aggregates has some disadvantages associated with it. The formation of aggregates may lead to concentration gradients of nutrients and oxygen, resulting in their uneven distribution. In case of larger aggregates, this may even result in improper waste removal, along with cell death and necrosis at the centre. Aggregates also induce differentiation, so are a problem for expansion of pluripotent stem cells. The use of microcarriers avoids such problems to a large extent. A successful microcarrier expansion is dependent on several factors; these include the type of carrier used, the cell line being expanded, the spinner flask set up, the position of the impeller and the spin rate (RPM) used. It is well known that the sensitivity of the cells to the spinning is of utmost importance, but it is equally important to know the tolerance level of the microcarriers themselves. As mentioned in the first chapter, to date, only two groups have looked at the expansion of iPS cells using microcarriers. Interestingly, although both groups used the same microcarriers and the same cell type (hiPS), the spin rate used varies widely.

In the current work, the expansion of mouse iPS cells on Cytodex 3 microcarriers was carried out. Cytodex 3 has been widely used for the expansion of embryonic stem cells but its suitability for the expansion of iPS cells has yet to be tested. A wide range of spin rate was tested to see its effect on the microcarriers, and an attempt was made to find an optimized spin rate for the maximum expansion of mouse iPS cells.

Hypothesis:

Preliminary comparative studies in the previous chapter showed that Cytodex 3 microcarriers supported pluripotent stem cell attachment and expansion in static culture. Based on the results, it is hypothesized that expansion of mouse iPS cells on Cytodex 3 is possible in a spinner flask at an optimum spin rate while maintaining their pluripotency and differentiation capabilities.

Objectives: In order to test the hypothesis, the work was divided into the following objectives:

- Test the effect of spin rate on Cytodex 3 and find the tolerance threshold for them in terms of carrier breakage.
- Based on the microcarrier threshold, test the effect of spin rate on mouse iPS cell attachment and expansion in spinner flask.
- Long term expansion of mouse iPS cells in a spinner flask at optimized spin rate followed by characterization assays to verify cell expansion, pluripotency maintenance and differentiation capability of expanded cells.

4.2 Materials and Methods:

4.2.1 Cytodex 3 Carrier Preparation:

Cytodex 3 microcarriers were purchased from G. E Health Care (Little Chalfont, UK) and prepared as per manufacturer's instructions. Required amount of dry Cytodex 3 powder was suspended in PBS. A small amount of Tween 80 was added to facilitate settling of the carriers. The microcarriers were hydrated overnight followed by autoclaving. The carriers were usually prepared fresh but they can be stored for a few weeks if kept at 4°C.

4.2.2 Spinner Flask Culture:

100 ml spinner flasks from Bellco Biotechnology (Bellco Glass, Inc., Vineland, NJ) were used with a final volume of 50 ml. The spinner flasks were coated with SigmaCote (Sigma Aldrich, St. Louis, MO) prior to usage in order to avoid the sticking of carriers to the glass walls. Cytodex

3 carriers equivalent to a surface area of 200 cm^2 / flask were used. The carriers were equilibrated prior to use by incubating them in mES culture medium for around 1 hr. A seeding density of 2 $x10^5$ cells/ ml was used for each flask. The seeding density was kept on the higher side since the iPS cells used were feeder dependent under normal circumstances and cell death and non adherence was expected when transferred to a feeder free culture. The cells were inoculated with the carriers in a low adherence 10 cm dish for about 24 hrs in order to facilitate cell attachment. After 24 hrs, the carriers with cells were transferred to the spinner flasks and the culture volume was adjusted to 50 ml. The impeller was adjusted after initial trial and error experiments in such a way that the stirrer was half immersed, in order to minimise mechanical damage to the carriers as well as the cells as far as possible. We observed more carrier breakage if the impeller was submerged too much and if the motor rotation was not smooth. The spinner flask's impeller was directly connected to a stepper motor using a custom-made coupling shaft (figure 4.1). The speed of the motor was controlled through a motion controller (National Instrument Australia), allowing precisely controlled variation of speed. The iPS cells were expanded for 7 days in the spinner flask with 50% medium replacement every day.

Control:

As control, the iPS cells were grown on 0.1% gelatin coated 24 well plate in the same seeding density. The medium was changed at the same frequency as the spinner flasks.

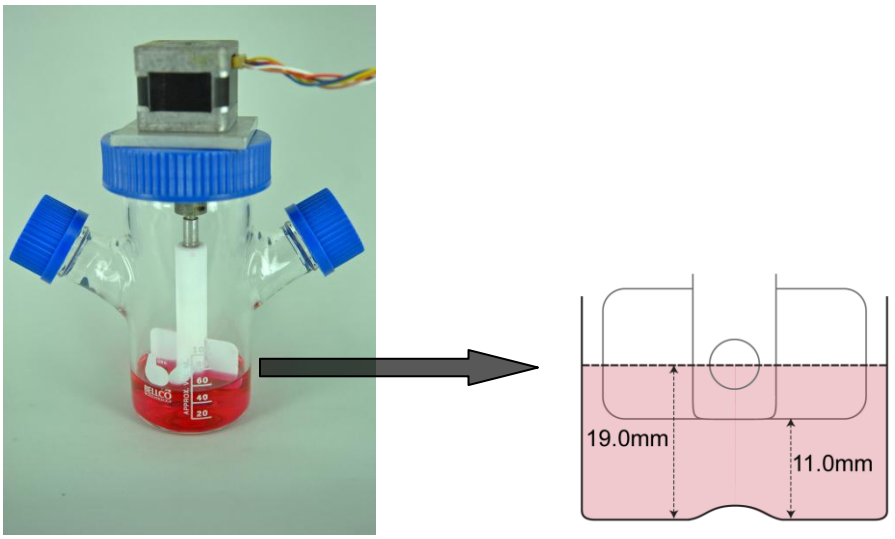


Figure 4.1 Spinner flask set up with the impeller directly connected to a stepper motor

4.2.3 Effect of RPM on Microcarriers:

In order to study the effect of different RPMs on Cytodex 3 microcarriers, the carriers were suspended in PBS in spinner flasks for 2 days at various RPMs. After 2 days, 1 ml of the suspension was taken out and studied under microscope.

4.2.4 Cell Count and Microscopy:

Samples of microcarriers (1ml) were taken on days 3, 5 and 7 and viewed under a microscope. The cells were then washed with PBS and trypsinized for cell detachment. The suspension was then passed through a 100µm strainer to remove the microcarriers. Cell counting was done using trypan blue and haemocytometer. The sampling and counting each time were done in duplicates.

4.2.5 Spontaneous Differentiation:

The differentiation capability of the cells was measured by *in vitro* EB formation in suspension culture. After 7 days of culture, iPS cells from control set-up as well as the spinner flask culture were seeded on to low adherence 6 well plates with MEF (mouse embryonic fibroblast) media at a seeding density of 50,000 cells/ well. Media was changed on alternate days. At the end of 10 days culture, the EBs were collected for RT- PCR and immunostaining in order to analyse for the presence of three germ layer markers.

4.2.6 RT PCR Analysis:

Cells for RT PCR analysis were snap frozen on dry ice and stored at - 80°C until analysis. RNA was isolated from the cells using RNeasy Mini kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Isolated RNA was cleaned using Ambion RNA Turbo DNAfree kit (Invitrogen) to remove any contaminating genomic DNA. Cleaned RNA was quantified using NanoDrop ND- 1000 (NanoDrop Technologies, Australia). cDNA synthesis was done using Superscript III kit (Invitrgen) following the manufacturer's instructions. β actin PCR was done after RNA clean up and cDNA synthesis to verify the procedures. The PCR amplification included a total 35 cycles of denaturation at 95°C for 30 seconds followed by annealing at appropriate temperature for 30 seconds and extension at 72°C for 1 minute with an initial

denaturation step at 95°C for 4 minutes and a final extension step at 72°C for 10 minutes. The PCR products were run on a 1% agarose gel at 80V. Gels were visualised using the Bio Rad Universal Hood II GelDoc system (Bio Rad Laboratories Inc.) and images were taken using Quantity One (version 4.6.3) software. The Primer sequences along with their respective annealing temperatures are mentioned in Table 4.1.

Table 4.1 Primer Sequences for RT PCR Analysis

Gene	Annealing Temperature	Primer Sequence
β - actin	58°C	F: GGA ATC CTG TGG CAT CCA TGA AAC R: AAA ACG CAG CTC AGT AAC AGT CCG
Oct 3/4	58°C	F: TCT TTC CAC CAG GCC CCC GGC TC R: TGC GGG CGG ACA TGG GGA GAT CC
Nanog	58°C	F: TCA AGG ACA GGT TTC AGA AGC A R: GCT GGG ATA CTC CAC TGG TG
c- myc	58°C	F: AAG TTT GAG GCA GCA GTT AAA ATT ATG GCT GAA R: TGA CCT AAC TCG AGG AGG AGC TGG AAT C
Nestin	58°C	F: TCT GGA AGT CAA CAG AGG TGG R: ACG GAG TCT TGT TCA CCT GC
Brachyury	55°C	F: CAT GTA CTC TTT CTT GCT GG R: GGT CTC GGG AAA GCA GTG GC
Fox A2	55°C	F: TGG TCA CTG GGG ACA AGG GAA R: GCA ACA ACA GCA ATA GAG AAC

4.2.7 Immunostaining:

Immunostaining for pluripotency markers was carried out for cells fixed on microcarriers following the protocols described in chapter 2.

Cells were also trypsinized from the microcarriers after 7 days of culture, seeded on to 24 well plates and stained after 3 days for SSEA 1 and Nanog markers following the same protocol.

EBs were stained for Brachyury, Nestin and FoxA2, as markers for the three germs layers. Alexa Fluor 594 was used as the secondary antibody conjugate for all three markers.

The primary antibodies and their respective secondary antibodies are listed in table 4.2

Table 4.2 Antibodies Used

Marker	Primary Antibody	Secondary Antibody
SSEA -1	Anti SSEA 1, mouse IgM	Rhodamine
Nanog	Anti Nanog, rabbit IgG	Alexa Fluor 594
Nestin	Anti Nestin, mouse IgG	Alexa Fluor 594
Brachyury	Anti Brachyury, rabbit IgG	Alexa Fluor 594
FoxA2	Anti FoxA2, goat IgG	Alexa Fluor 594

4.3 Results and Discussions:

4.3.1 Effect of Spinner Flask Speed on Microcarrier Breakage:

Expansion of iPS cells in spinner flasks is dependent on the survival of the microcarriers themselves. Both fluid shear stress as well as mechanical damage from the impeller and the flask wall can result in the breakage of the microcarriers. Experiments were carried out to see the effect of different spin rates on the Cytodex 3 microcarriers. The aim was to find out the maximum speed that the carriers can tolerate within the spinner flask without any breakage. 100 RPM was taken as the upper limit based on literature survey along with set-ups at 60, 55 47, 45, 42 and 40 RPM. As seen in figure 4.2, RPMs between 42- 100 resulted in the breakage of the Cytodex 3 carriers after 2 days in the spinner flask. However, at 40 RPM, no carrier breakage was observed. 40 RPM was thus optimal as far as being the highest RPM that the Cytodex 3 carriers could tolerate.

The proper suspension of microcarriers in the spinner flask was also considered to be an important aspect for long term culture of cells in a dynamic environment. For this purpose, suspension of Cytodex 3 carriers at lower RPMs was also studied. It was observed that the suspension of the carriers decreased considerably after 25 RPM and was negligible at 20 RPM and below.

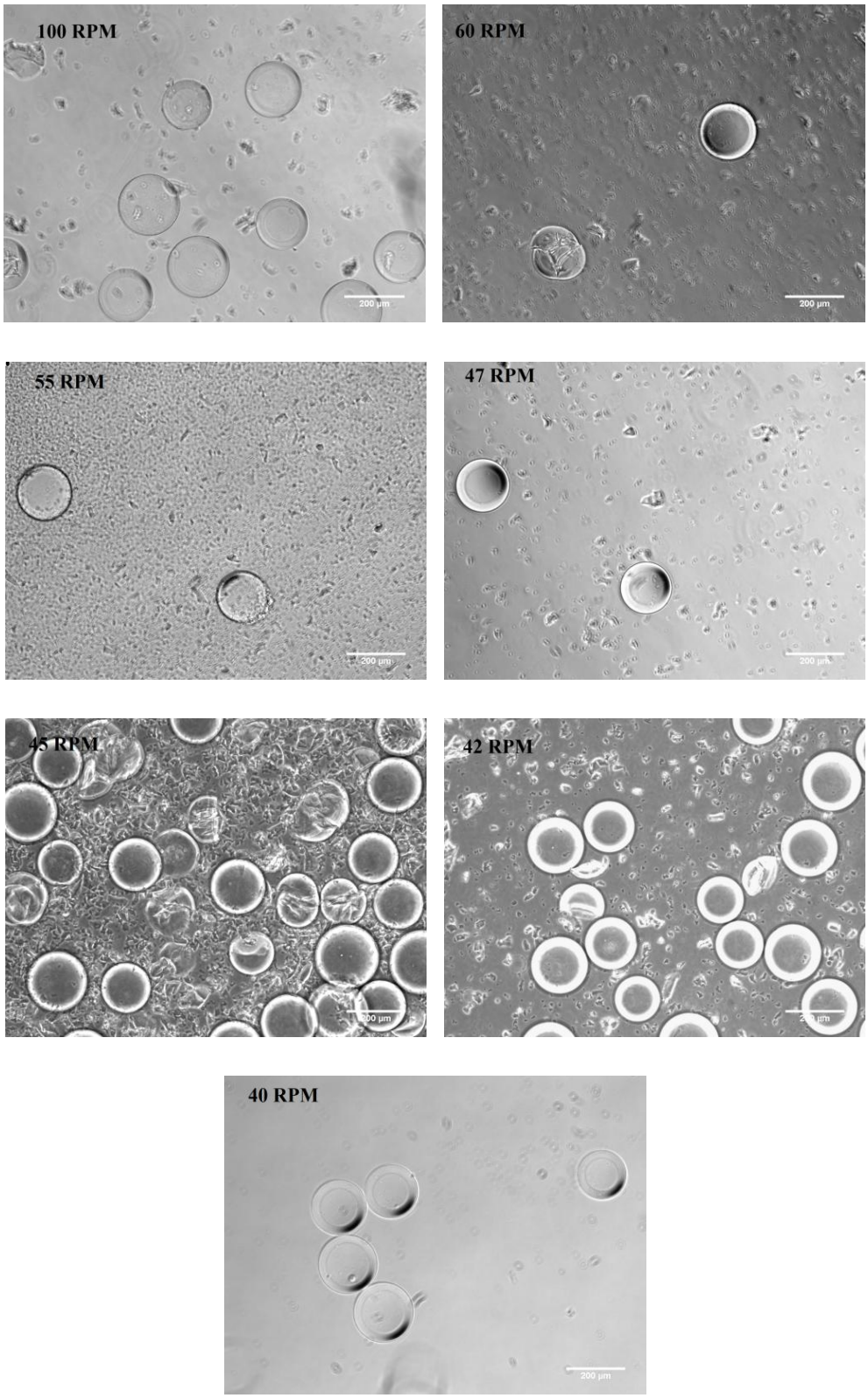


Figure 4.2 Effect of different spin rates on Cytodex 3 microcarriers. Carriers were put in spinner flask and kept in a dynamic motion for 2 days. Samples were then taken out and observed under microscope. Scale bar = 200µm

Based on these results, RPMs of 40 and below were tested for attachment and expansion of mouse iPS cells in spinner flask using Cytodex 3 microcarriers. The aim was to find the RPM at which the carriers were properly suspended and, at the same time, best supported long term cell expansion.

4.3.2 Attachment and Expansion of iPS Cells in Spinner Flask at Different RPM:

Similar to any mammalian cell line, the mouse iPS cells have a threshold RPM in terms of cell attachment, expansion and pluripotency maintenance. Based on the above results of RPM threshold for Cytodex 3, attachment and expansion of mouse iPS cells on Cytodex 3 carriers were carried out at 40, 30, 28 and 25 RPM in order find out the optimum RPM for their long term expansion

Cells and microcarriers were put in spinner flasks after 18- 24 hrs of static attachment and subjected to 7 days spinning. Sampling and imaging were done every day to check cell attachment and growth. Cell count using haemocytometer was performed on days 3, 5 and 7.

It was observed that at 40 RPM, the cells detached within 24 hrs of spinner flask culture and thus it was deemed unfit for further studies. Microscopic images showed cell attachment at 28 and 30 RPM in the initial culture days, but they were not able to support long term cell culture (figure 4.3).

The 25 RPM case, on the other hand, showed cell expansion until day 7. Cells were able to grow on the surface of microcarriers but they also showed some amount of 'bead bridging', resulting from the tendency of iPS cells to form clustered colonies. The low RPM used also acted as an added factor towards this. The presence of Oct4 - GFP fluorescence suggested that the cells were able to maintain their pluripotency (figure 4.3). Scanning electron microscopy images of cells on Cytodex 3 carriers at the end of 7 days culture shows cell attachment, spreading and expansion on the microcarriers (figure 4.4)

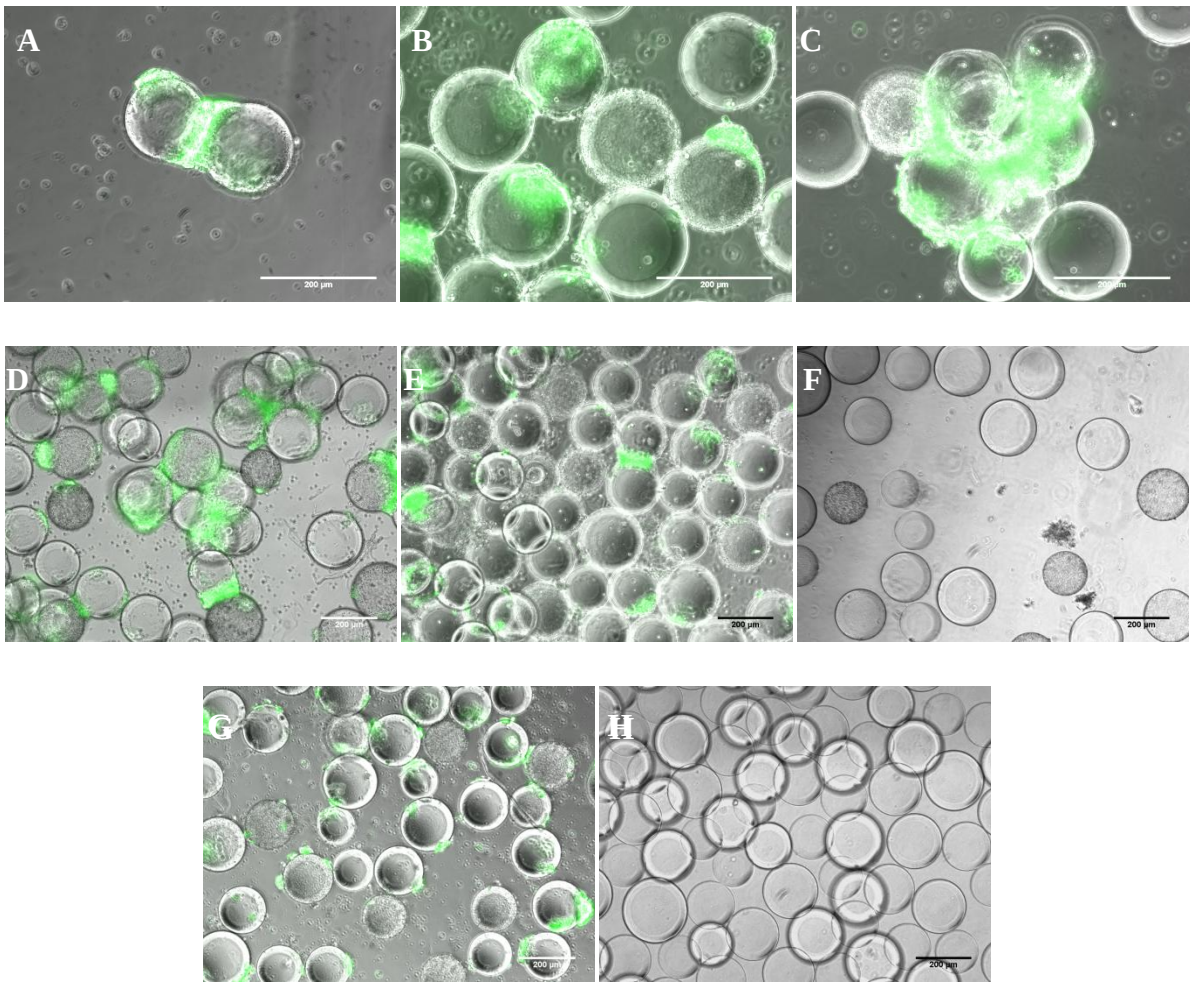


Figure 4.3 Expansion of mouse iPS cells on Cytodex 3 carriers at different RPM over days 3,5 and 7. (A- C) - 25 RPM, (D-F) 28 RPM, (G-H) 30 RPM. (A,D, G) - day 3, (B,E, H) - day 5, (C, F) - day 7. Scale bar = 200 µm

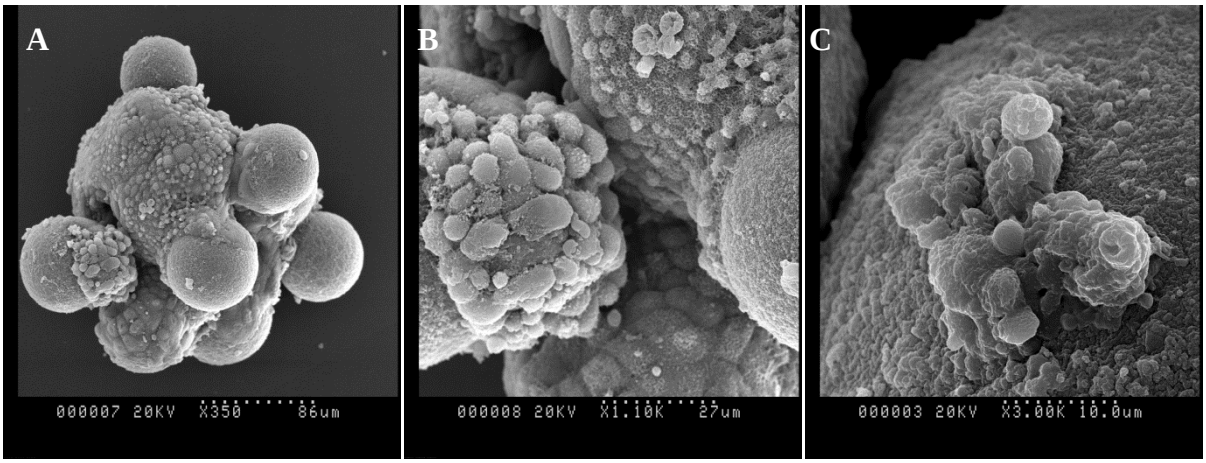


Figure 4.4 Scanning Electron Microscopy (SEM) images of cells growing on Cytodex 3 microcarriers at the end of 7 days culture in spinner flask at 25 RPM

Cell count using a haemocytometer was also undertaken for estimating cell growth and expansion on the microcarriers. These data also supported the microscopy observation (figure 4.5). At 25 RPM, cell density increased from $2x10^5$ cells/ml to around $8x10^5$ cells/ml at the end of 7 days giving rise to a total cell number of $40x10^6$ while the total cell number in the control set up was $6x10^6$. Since the cells were put in the spinner flask directly from 2D culture on feeder layer, it is possible that the cells initially took some time to adjust to the new culture environment.

However, at 28 and 30 RPM, the cell number decreased over time. Cell count became negligible between days 4 to 6 for both the RPMs. The ability of the miPS cells to expand in the spinner flasks also highlighted the cells' capability of adapting to changed culture conditions.

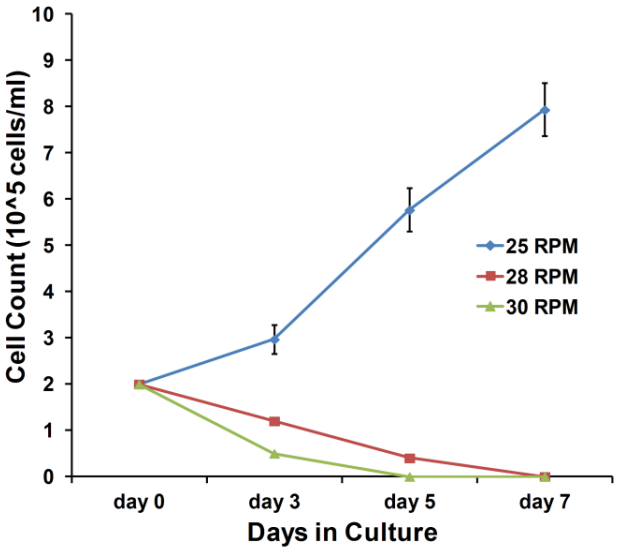


Figure 4.5 Density of miPS cells on days 3, 5 and 7 at RPMs 25 ( ), 28 ( ) and 30 ( ). Based on the preliminary data from microscopy and cell count, 25 RPM (n= 3 independent experiments)

4.3.3 Cell Pluripotency:

Along with cell attachment and expansion, the maintenance of pluripotency of the miPS cells was also considered to be an important aspect for dynamic culture optimization. Immunofluorescence and PCR were done to study cell pluripotency after dynamic culture. RT - PCR analysis for cells collected on days 3, 5 and 7 for 25, 28, and 30 RPM was carried out for pluripotency analysis. As shown in figure 4.6, it was found that Oct 4, Nanog and c- myc

expression was observed in all cases although Nanog and c- myc expression was very low for cells expanded at 28 RPM on day 5. This suggested that although cells were present on day 5 at 28 RPM (figure 4.5), their pluripotency was negatively affected.

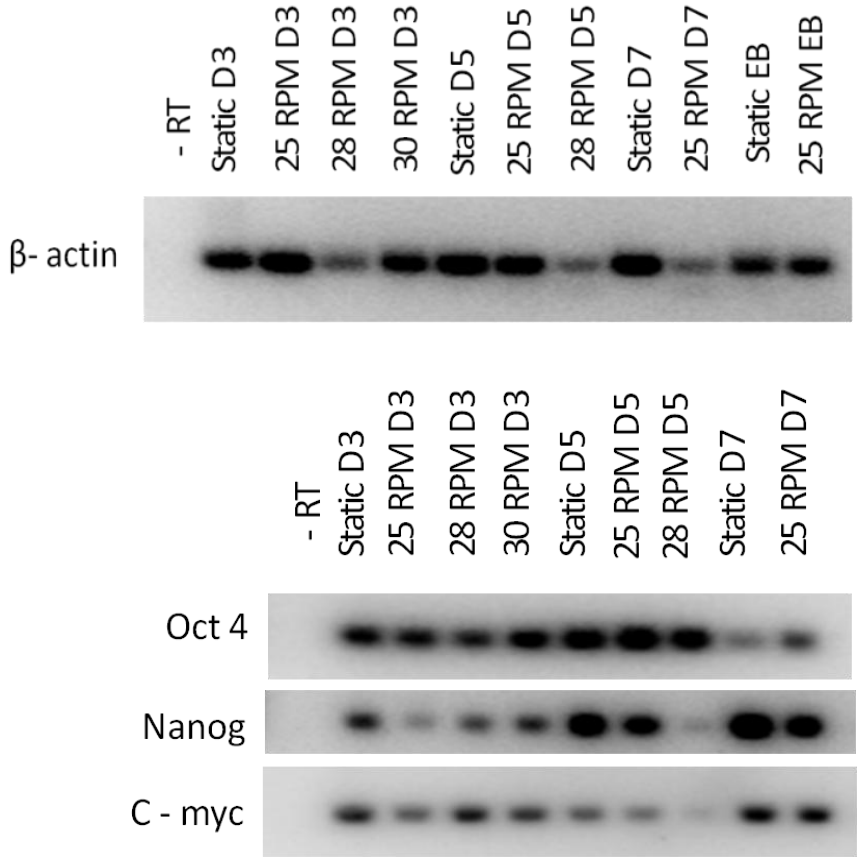


Figure 4.6 Reverse Transcription- Polymerase Chain Reaction analysis of pluripotency markers on days 3,5, 7 for 25, 28 and 30 RPM culture. β actin was considered as the house keeping gene

SSEA 1 and Nanog immunostaining was carried out after 7 days of culture at 25 RPM for cells fixed directly on carriers. Figure 4.7 demonstrates that miPS cell cultured on microcarriers in a spinner flask for 7 days at 25 RPM stained positive for both SSEA 1 and Nanog.

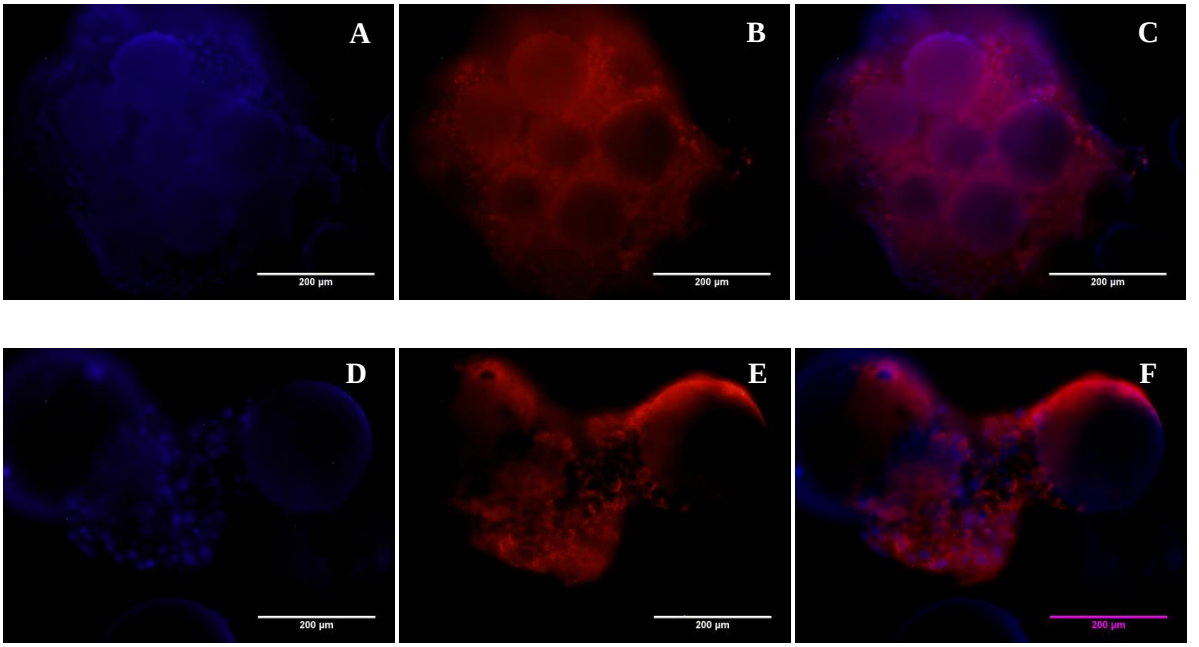
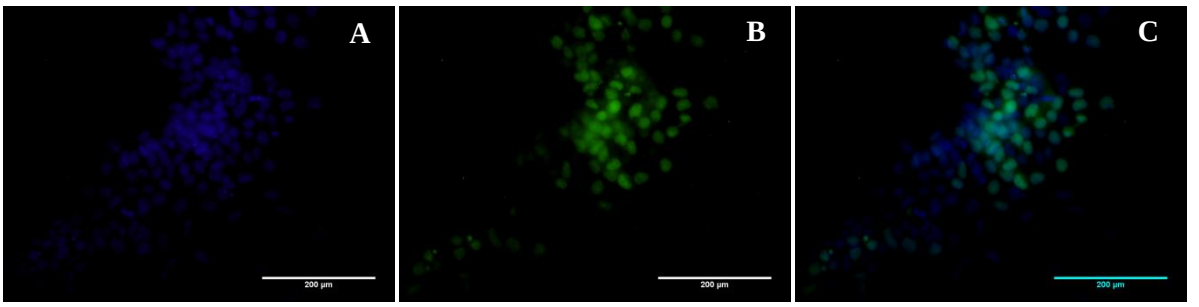


Figure 4.7 Immunostaining of fixed cells on microcarriers at the end of 7 days culture at 25 RPM. . Nuclei were stained blue with Hoechst. (A- C) - SSEA 1 staining, (D - F) - Nanog staining. Scale bar = 200µm

SSEA 1 staining was also done for replated cells after 7 days of spinner flask culture, along with Oct4- GFP live cell imaging. Cells from static culture were used as a control for comparison purpose in this case (figure 4.8, 4.9). Pluripotency in general was maintained by the cells although loss of SSEA 1 marker and Oct4-GFP was also observed, suggesting some loss of pluripotency for both the control and 25 RPM cultures.



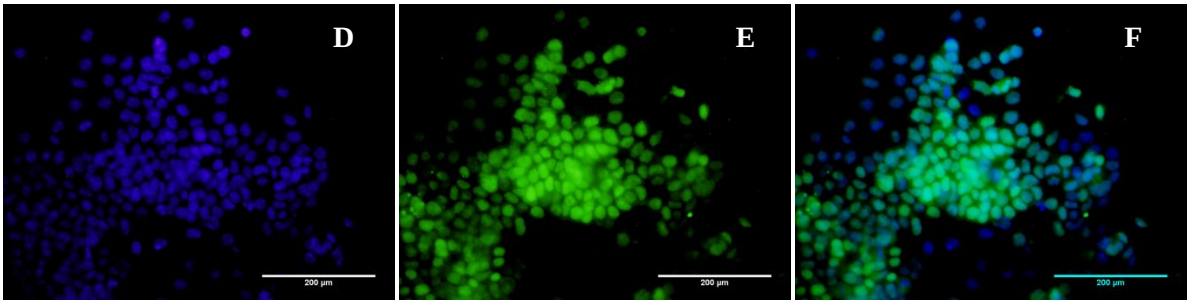


Figure 4.8 Oct4-GFP immunofluorescence of cells from day 7 culture at 25 RPM. .Cells were replated and imaged after 3 days of culture. Nuclei were stained blue with Hoechst. (A- C) - Static culture (control), (D - F) - 25 RPM culture. Scale bar = 200µm

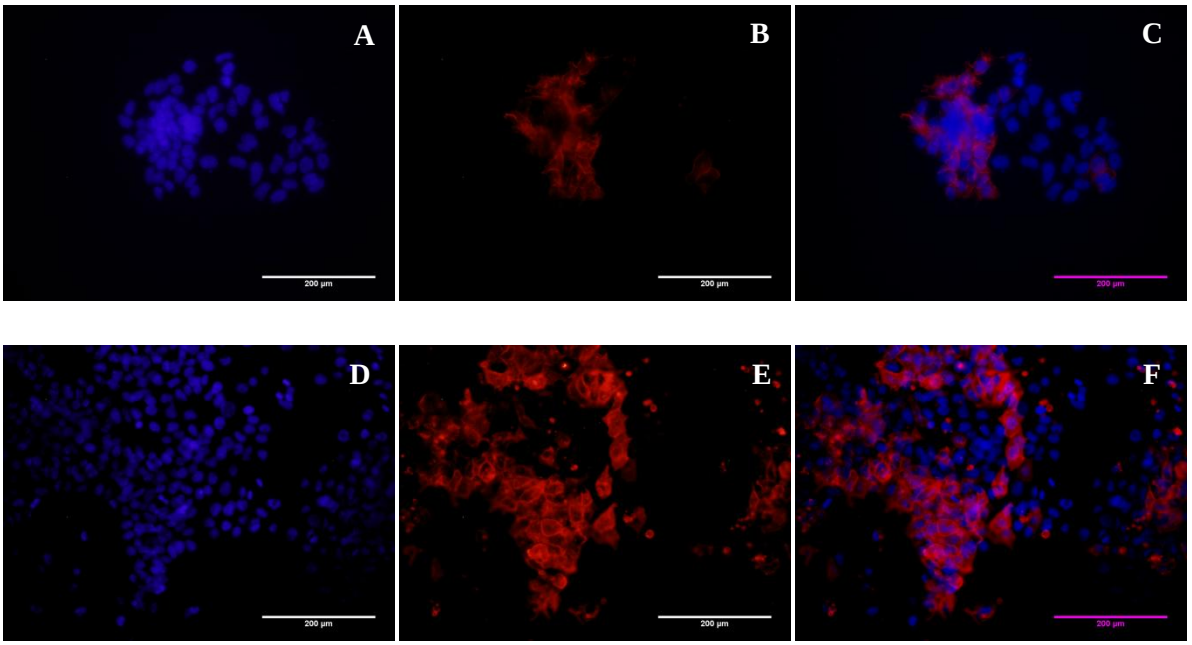


Figure 4.9 SSEA 1 immunostaining of cells from day 7 culture at 25 RPM. .Cells were replated and imaged after 3 days of culture. Nuclei were stained blue with Hoechst. (A- C) - Static culture (control), (D - F) - 25 RPM culture. Scale bar = 200µm

Quantitative analysis of SSEA - 1 fluorescence intensity using HCS array scan was done after 7 days culture at 25 RPM and was compared to static culture. Figure 4.10 shows average fluorescence intensity/ cell for SSEA 1 pluripotency marker. Although not highly significant, the intensity in the control culture was slightly lower than that of the 25 RPM culture cells.

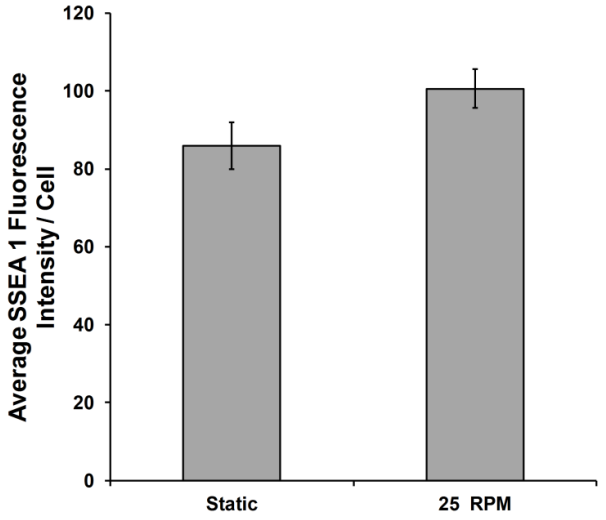


Figure 4.10 Quantitative analysis of pluripotency. (a) Comparison of average SSEA 1 fluorescence intensity/ cell between static culture and dynamic culture at the end of 7 days. Experiments were repeated for n=3 times and data represents mean±SEM

Flow cytometric analysis of Oct4 -GFP showed that around 96±2% cells for static culture and around 90±2% cells in case of dynamic culture (25 RPM) maintained the GFP fluorescence at the end of 7 days (Representative images shown in figure 4.11).

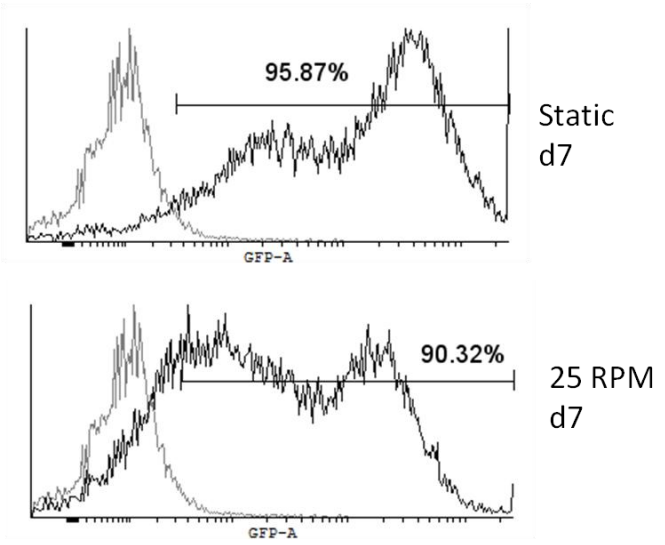


Figure 4.11 Representative images of live cell Oct4-GFP flow cytometry analysis at the end of 7 days culture. Cells were collected from static culture and from 25RPM dynamic culture

4.3.4 *In Vitro* Differentiation:

Spontaneous *in vitro* differentiation capability of the mouse iPS cells after dynamic culture was tested by EB formation using suspension culture method. Cells were collected at the end of 7 days culture and EBs were formed in low adherence culture plate using media without LIF. Day 10 EBs were collected for immunostaining and RT- PCR analysis. Cells from static culture were used as control. Cells from both static culture and dynamic culture (at 25 RPM) were able to form embryoid bodies.

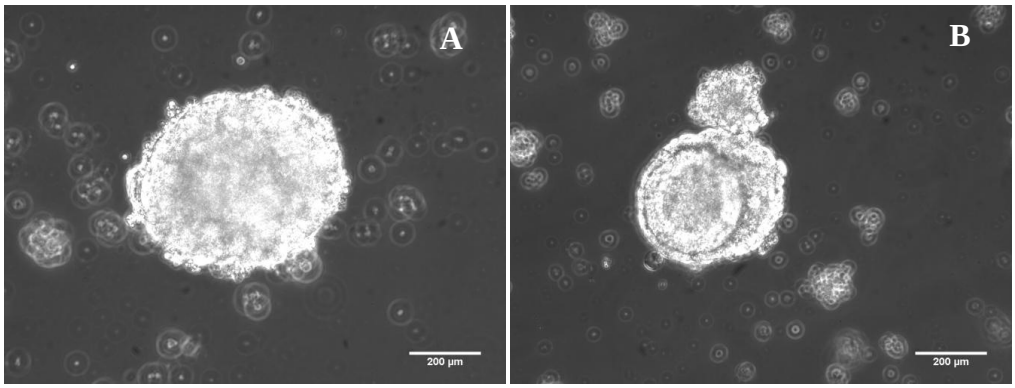


Figure 4.12 Representative images of day 6 embryoid bodies formed by spontaneous differentiation in suspension culture. (A) Static culture, (B) 25 RPM dynamic culture. Scale bar= 200µm

Figure 4.12 shows day 6 embryoid bodies (EB) formed from both static and dynamic culture. Multiple EB formation was observed. The EBs were collected on day 10 for analysis. Immunostaining and RT -PCR analysis were done for Nestin, Brachyury and FoxA2 to check the presence of cells from all three germ layers.

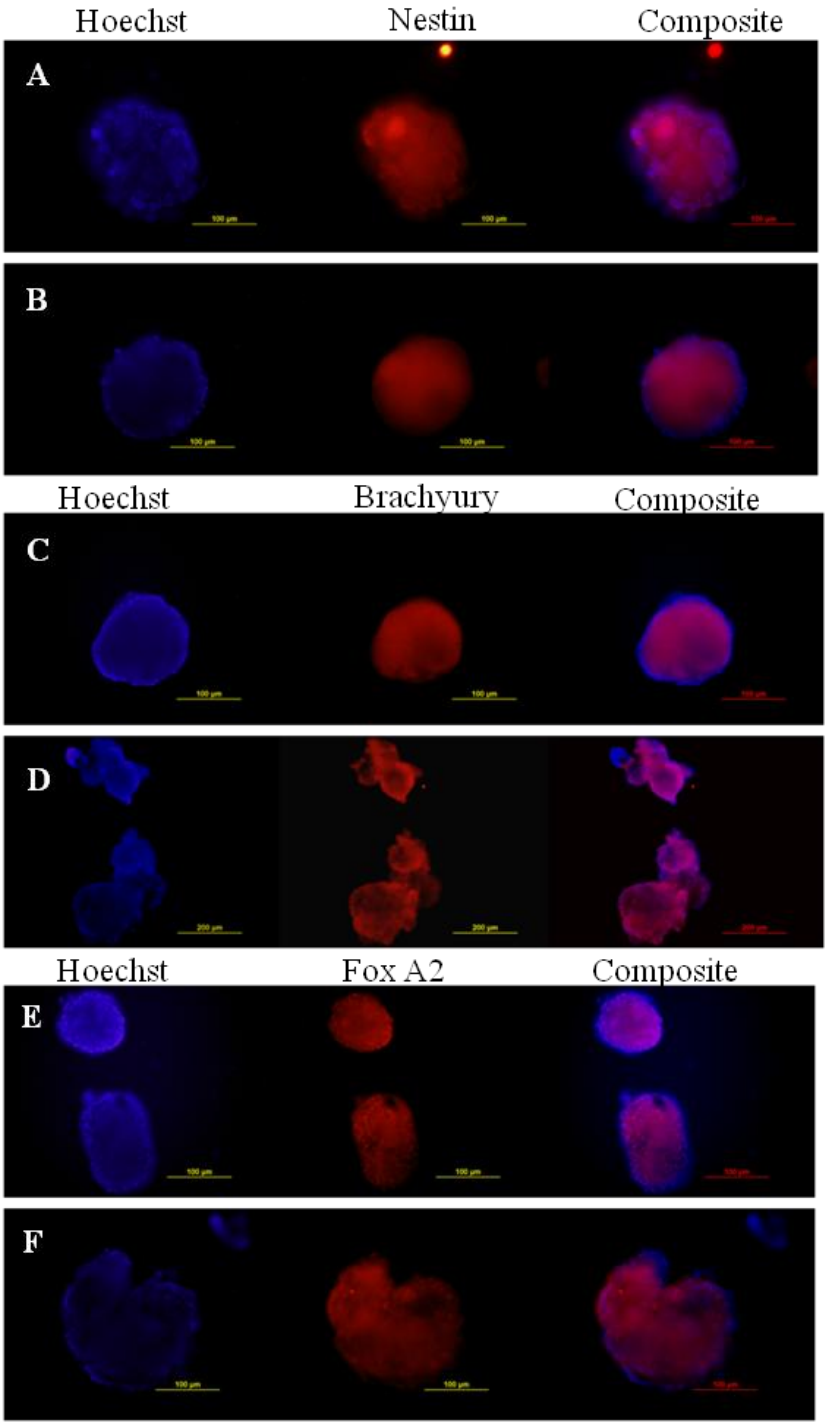


Figure 4.13 Embryoid body immunostaining for detection of 3 germ layers marker. (A,C, E) - EB from static culture, (B,D,F) - EB from 25 RPM dynamic culture. Scale bar = 200 µm

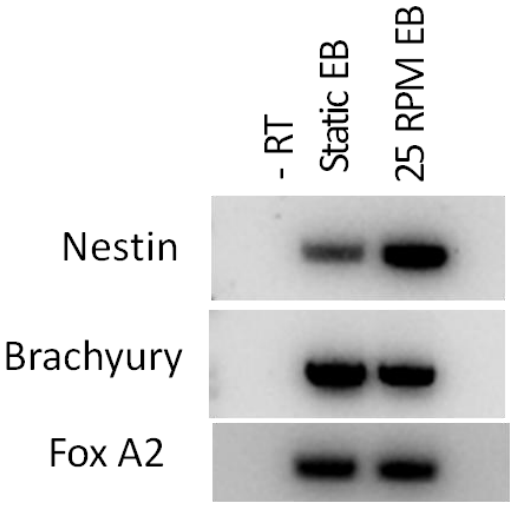


Figure 4.14 Reverse Transcription- Polymerase Chain Reaction for 3 germ layers marker on day 10 EBs; Ectoderm (Nestin), Mesoderm (Brachyury) and Endoderm (FoxA2). Static culture EBs was considered as control

RT- PCR analysis (Figure 4.14) shows the presence of all three germ layer markers at the end of 10 days. Immunostaining of EBs also shows the presence of 3 germ layer markers (figure 4.13). Both these analyses prove that the cells from dynamic culture retain the capacity to form cells of all three germ layers on random differentiation.

4.4 Discussion and Conclusion:

The ethical issues associated with the use of embryonic stem cells have led to extensive research in the field of induced pluripotent stem cells. Scaling up of pluripotent stem cell culture from the bench top level to a reactor system is an essential part in the use of these cells for different purposes. A spinner flask in conjunction with microcarriers has been widely used for large scale expansion of pluripotent stem cells. Efforts have been made for culturing of embryonic stem cells (both mouse and human) in such a set up since 2005[49] but to date, a uniform system with optimized parameters for the expansion of a specific cell type is yet to be achieved. The available literature on large scale expansion of ES cells varies widely in terms of the type microcarrier used, the agitation rate, etc. Also, the relevant studies solely concern the effect of a dynamic culture system on the cells and not on the microcarriers being used, even though it is obvious that the carriers themselves would have a threshold tolerance in terms of agitation. The use of

dynamic culture systems like spinner flasks for the expansion of iPS cells is still in its nascent stage and, similar to the ES cells, a uniform culture system is yet to be achieved.

Keeping all these in mind, this work was carried out to study the expansion of mouse iPS cells on Cytodex 3 microcarriers in a spinner flask. Studies were carried out to see the effect of different agitation rates not only on the attachment and expansion of iPS cells but also on the microcarriers themselves.

As expected, it was observed that the Cytodex 3 indeed had an agitation tolerance threshold above which there was extensive carrier breakage. 40 RPM was found to be the upper level of spin rate that these carriers were able to tolerate. Bead breakage was seen at and above 42 RPM. Since Cytodex 3 has been previously used at higher spin rates for cell expansion, it is possible that the effect of agitation rate on microcarriers is also dependent on the reactor set-up itself. It should also be kept in mind that the threshold agitation will vary with the type of microcarrier or attachment surface used. Hence, it is essential that similar studies for other carriers and different set-ups should be done for their future use in pluripotent stem cell expansion.

Based on the carrier study, RPMs of 40 and below were tested for iPS expansion. Agitation rates between 28 and 40 were unable to support long term cell attachment and expansion with cell detachment being directly proportional to the agitation rate. At 40 RPM, cells detached within 24 hrs while for 30 and 28 RPM, the cell number showed a steady decline, with complete detachment taking place between days 4- 6. 25 RPM on the other hand was able to support the attachment and expansion of the cells for 7 days. The above observations are interesting ones, since they show that the range of agitation speed which can support iPS cell expansion is very narrow, and hence calls for very precise control of speed in dynamic cultures for future work with such cell lines.

Preliminary experiments also showed that proper suspension of microcarriers was not achieved for spin rates at and below 20RPM.

Maintenance of pluripotency and differentiation capability of these cells during spinner flask expansion is highly important. 25 RPM dynamic culture showed positive results for OCt4, Nanog and c- myc in RT- PCR analysis. It was observed that the c-myc RT- PCR band for cells collected on day 5 from the dynamic culture at 28 RPM was almost negligible. This suggested

that although cells were present, their pluripotency was negatively affected. Immunostaining for 25 RPM culture at the end of the culture period showed positive staining for Nanog, SSEA- 1 and also showed positive Oct4-GFP fluorescence and work as further proof of pluripotency maintenance of the cells. The decrease in pluripotency markers observed in 2D, after spinner flask culture, could be due to the cells being replated on 2D gelatin coating and not a direct effect of dynamic culture itself. Quantification of SSEA -1 fluorescence intensity/ cell showed slightly higher intensity for cells from 25 RPM culture in comparison to the static culture system. All these data taken together show that long term expansion of mouse iPS cells at 25 RPM in a spinner flask does not affect cell pluripotency.

The ability to differentiate in-to cells of all three germ layers is an important property of pluripotent stem cells. Cells from dynamic culture and static culture (control) were spontaneously differentiated *in vitro* to check their differentiation capability. The mouse iPS cells from both static as well as dynamic culture were able to form embryoid bodies in suspension culture. Immunostaining, RT PCR analysis of EBs were done. Nestin, Brachyury and FoxA2 were used as markers for ectoderm, mesoderm and endoderm respectively. The EBs showed positive staining for Nestin, Brachyury and FoxA2 and positive bands were observed for the same markers in RT - PCR analysis proving the differentiation capability of the cells.

Taken together, the study confirms our hypothesis that dynamic culture of mouse induced pluripotent stem cell on Cytodex 3 microcarriers is viable. 25 RPM was found to be the optimum agitation rate for the expansion of miPS cells in spinner flask while maintaining all their essential characteristics. Our findings are in line with Bardy et al., who had also used a 25 RPM system for the expansion of hiPS cells and their neural differentiation on DE-53 microcarriers[60]. The group had used the same spinner flask set up but it is possible that the impeller height and adjustment may not have been the same. The present work also highlights the need for precise speed and set up control, and their extensive study prior to being used for dynamic culture of pluripotent stem cells.

Chapter 5 Effect of Lactate and pH on Expansion of Pluripotent Stem Cells

5.1 Introduction:

Lactic acid, a byproduct of glucose metabolism, has been reported to be a potential mammalian cell growth inhibitor for a long time [75]. The detrimental effect of lactate on mammalian cells can be due either to the accumulation of lactate in the media or due to the change in pH as a result of lactic acid accumulation. Until now, various studies have been performed to study the effect of both lactate and pH on different cell lines, such as hybridoma cells, BHK (baby hamster kidney) cells, mesenchymal stem cell, haematopoietic stem cells, etc [76, 80, 81, 86]. Brief studies in similar directions have also been undertaken on embryonic stem cells[43, 83]. The available literature on the effect of pH on mammalian cells mainly highlights two aspects - a decrease in cell proliferation for the majority of the cells and increased differentiation in some stem cells. However, the published data regarding the effect of lactate on various cells have certain inconsistencies. In addition, such studies on ES cells are very brief and none are reported for induced pluripotent stem cells.

The current chapter looks at the effect of pH and lactate individually on pluripotent stem cells in terms of cell proliferation, cellular metabolic activity and cell pluripotency.

Hypothesis:

Based on current literature on the effect of lactate and pH on mammalian cells, it is hypothesized that changes in lactate concentration and pH in the medium will significantly affects the growth and pluripotency of pluripotent stem cells. Although the exact reasons for their detrimental effects is not known, it has been suggested that an external change in pH results in a similar change in intracellular pH, which affects cell metabolism and proliferation. The presence of lactate ions has also been associated with negative effect on cell metabolism and proliferation.

5.2 Methods:

The following paragraphs include experimental methods used exclusively in this chapter.

5.2.1 Effect of Lactate on Pluripotent Stem Cells:

In order to check the effect of lactate exclusively on pluripotent stem cells, sodium lactate was added to the medium at specific concentrations between 0 and 4 mg/ml at an interval of 0.5mg/ml.

The experiments were carried out in 96 well multiwell plates with a cell seeding density of 1000 cells/well. Cells were incubated with 200 - 250 µl of media with different sodium lactate concentration for 3 days followed by assays to measure proliferation, pluripotency and cellular metabolic activity of cells.

All assay data were normalized with respect to control data (0 mg/ml lactate) and represented as fold change.

5.2.2 Effect of pH on Pluripotent Stem Cells:

In order to record the effect of a range of pH on cell growth, proliferation and pluripotency, a powdered DMEM formulation without sodium bicarbonate was used. Sodium bicarbonate was used to adjust the pH based on equilibrium bicarbonate ion ($[HCO_3^-]$, mM) concentration at 37°C. The relation between pH, bicarbonate ion concentration and partial pressure of CO_2 (pCO_2, mmHg) in the gas phase is given by the following equation[83, 130, 131]

$$log[HCO_3^-] = pH + log[pCO_2] - 7.543. \quad (6)$$

The partial pressure of 5% CO_2 at 37°C was taken as 36 mmHg. After adding the required amount of sodium bicarbonate, the medium was incubated overnight in an incubator in order to equilibrate it.

These experiments were carried out in a 48 well multiwell plate with a seeding density of 2500 cells/well. Cells were incubated with 1 - 2 ml of media for 3 days. After 72 hrs, assays for measurement of cellular metabolic activity, proliferation and pluripotency were undertaken.

All assay data were normalized with respect to control data (normal ES media) and represented as fold change.

5.3 Results and Observations:

5.3.1 Effect of Lactate on Pluripotent Stem Cells:

Based on the metabolic analysis of fibroblast conditioned media, a sodium lactate range of 0 - 4 mg/ml was chosen to study the effect of lactate on pluripotent stem cells. Sodium lactate was used to provide different lactate concentration without any change in pH. A feeder independent mouse embryonic stem cell line (Oct4B2) was used first since lack of a feeder layer made it possible to study the effect of lactate exclusively on ES cells. This was followed by similar set of experiments on feeder dependent mouse ES and iPS cell lines.

5.3.1.1 Effect of Lactate on Feeder Free Mouse ES Cells:

The effect of lactate on cell growth and pluripotency was initially tested on a feeder free ES cell line, Oct4B2.

5.3.1.1.1 Cell Growth and Proliferation:

Cell count after trypan blue staining was performed to estimate the cell number. Cell proliferation rate was measured by BrdU incorporation assay.

As seen in figure 5.1, viable cell number decreased steadily with increasing lactate concentration. The decrease in fold change in cell number became significant from a sodium lactate concentration of above 1.5mg/ml. At 4mg/ml of sodium lactate concentration, there was a final 3 to 4 fold decrease in the total viable cell number.

BrdU cell proliferation assay (Figure 5.2) followed a similar trend and showed a decrease in average BrdU- FITC fluorescence intensity/cell, suggesting that cell proliferation rate was also negatively affected by the addition of sodium lactate into the culture medium. The decrease in cell proliferation rate showed statistical significance from a sodium lactate concentration of 2.5mg/ml.

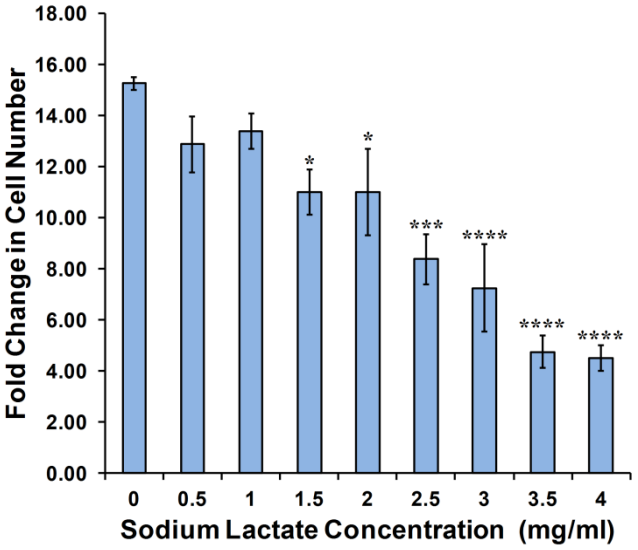


Figure 5.1 Fold change in Oct4B2 viable cell number with increasing sodium lactate concentration. Data represent mean±SEM of n=4 individual experiments

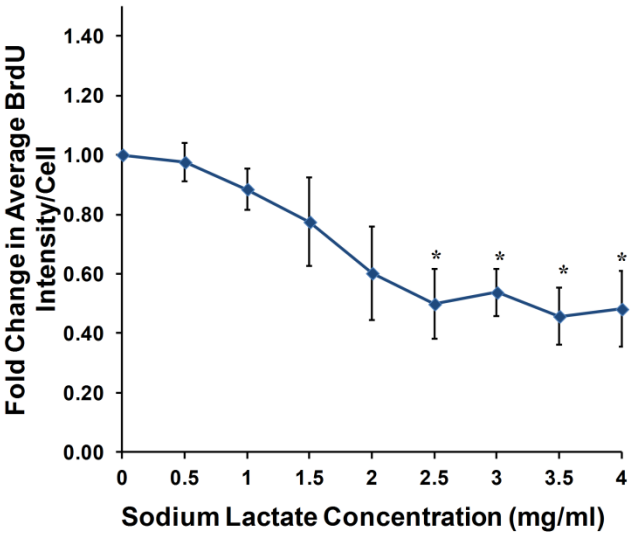


Figure 5.2 Fold change in average BrdU intensity/ cell for Oct4B2 with increasing sodium lactate concentration. Data represent mean±SEM of n=3 individual experiments

5.3.1.1.2 Cell Pluripotency Maintenance:

Pluripotency of the viable feeder free ES cells was estimated by quantifying SSEA 1 immunostaining and Oct4-GFP fluorescence. As is evident from figures 5.3 (A) and 5.3 (B), the addition of sodium lactate did not have a significant effect on the viable cell pluripotency.

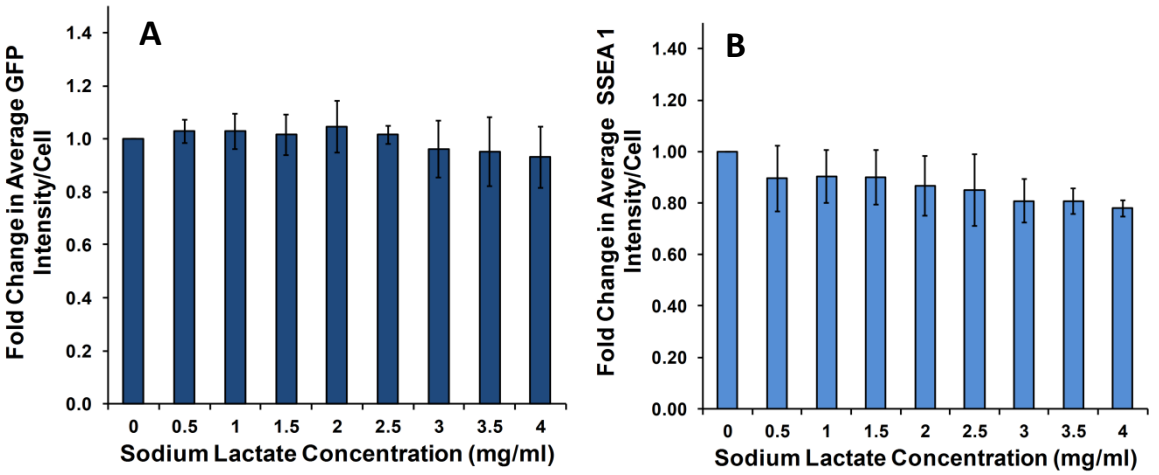


Figure 5.3 Fold change in pluripotency marker fluorescence intensity for Oct4B2 with increasing sodium lactate concentration. (A) Oct4- GFP, (B) SSEA -1. Data represent mean±SEM of n=3 individual experiments

5.3.1.1.3 Cell Metabolic Activity:

Most of the metabolic activity measuring assays measure the cells' mitochondrial activity. It is usually expected that the cellular metabolic activity is proportional to the cell viability and number. However, as seen in figure 5.4, in this case the Cell Titre Glo luminescence assay that measures the ATP content of the cells showed a relatively flat line despite the decrease in cell number and cell proliferation (figures 5.1, 5.2). The only explanation for these data is the possibility that there was an increase in the metabolic activity of the cells with increasing concentration of sodium lactate, even though the cell number and proliferation rate decreased. Based on this, we hypothesized that mouse ES cells were able to utilize the externally added lactate as a metabolite.

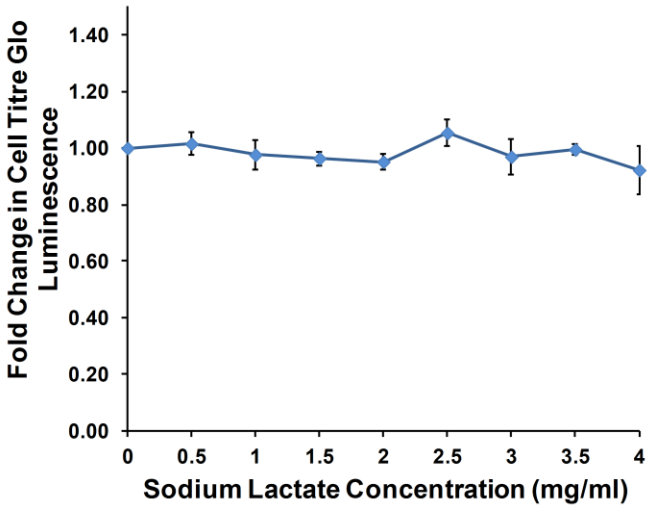


Figure 5.4 Fold change in Cell Titre Glo lumenescence as a measurement for cellular metabolic activity of Oct4B2 with increasing sodium lactate concentration. Data represent mean±SEM of n=3 individual experiments

5.3.1.1.4 HPLC Analysis of Lactate in Spent Media from ES Cell Culture:

In order to test our hypothesis of lactate usage by cells, HPLC analysis was carried out to measure the lactate concentration present in the spent media. Media collection was done at day 0, day 3 and day 4, followed by HPLC analysis. Figure 5.5 shows the result of lactate analysis in media collected from Oct4B2 ES cell culture.

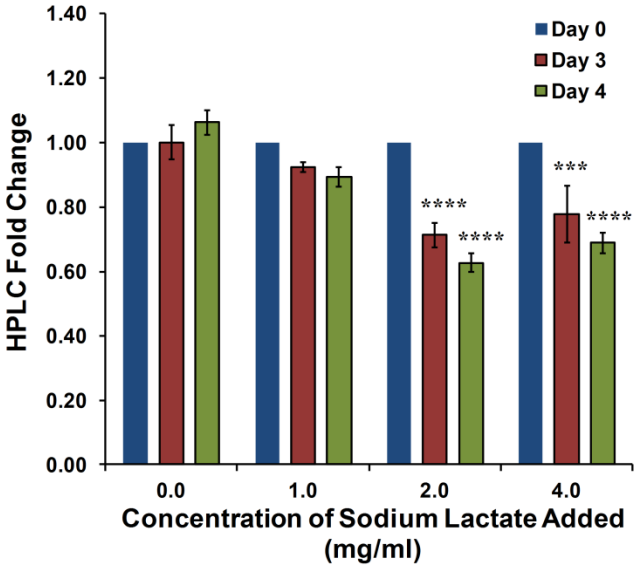


Figure 5.5 Fold change area under curve for HPLC analysis of spent media from Oct4B2 with increasing sodium lactate concentration. Data represent mean±SEM of n=3 individual experiments

As expected, it was observed that when no external sodium lactate was added to the culture, by day 4 lactate concentration in the media increased due to the metabolism of glucose. However, when sodium lactate was added externally, the lactate concentration in the media decreased with time. The decrease in lactate concentration was higher with increasing concentration of sodium lactate added.

5.3.1.2 Effect of Lactate on Feeder Dependent Mouse Pluripotent Cells:

In order to see if the results obtained with Oct4B2 ES cells are an anomaly or not, the same experiments were carried out on feeder dependent OG2 ES OG2 iPS cells.

5.3.1.2.1 Cell Proliferation:

Similar to the feeder independent ES cells, it can be seen in figure 5.6 that BrdU- FITC fluorescence intensity/cell decreased with increasing sodium lactate concentration, suggesting a decrease in cell proliferation. The decrease was however less sharp than the feeder independent cells and became significant at 3.5 mg/ml and beyond.

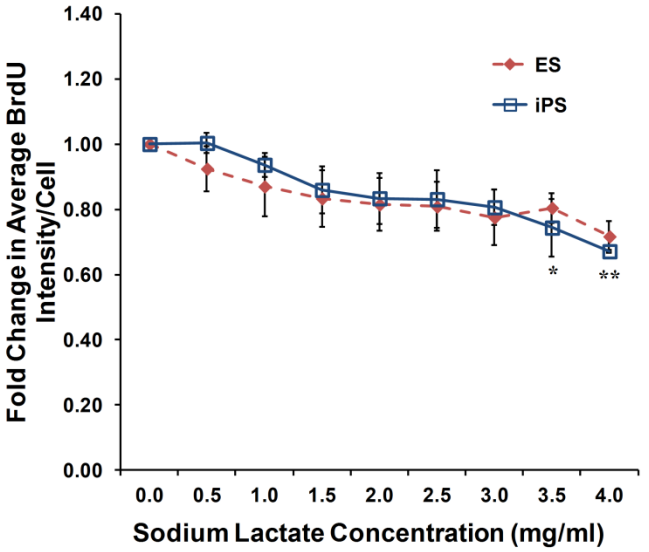


Figure 5.6 Fold change in average BrdU intensity/ cell for OG2 ES and iPS cells with increasing sodium lactate concentration. Data represent mean±SEM of n=3 individual experiments

5.3.1.2.2 Cell Pluripotency Maintenance:

SSEA 1 immunofluorescence and Oct4- GFP fluorescence intensity was quantified to estimate the effect of variation in lactate concentration on pluripotency of cells. It was observed that pluripotency of the feeder dependent ES and iPS cell lines was maintained even with increasing sodium lactate concentration in the media. Although there was a slight variation, there was no trend or significant change (figure 5.7).

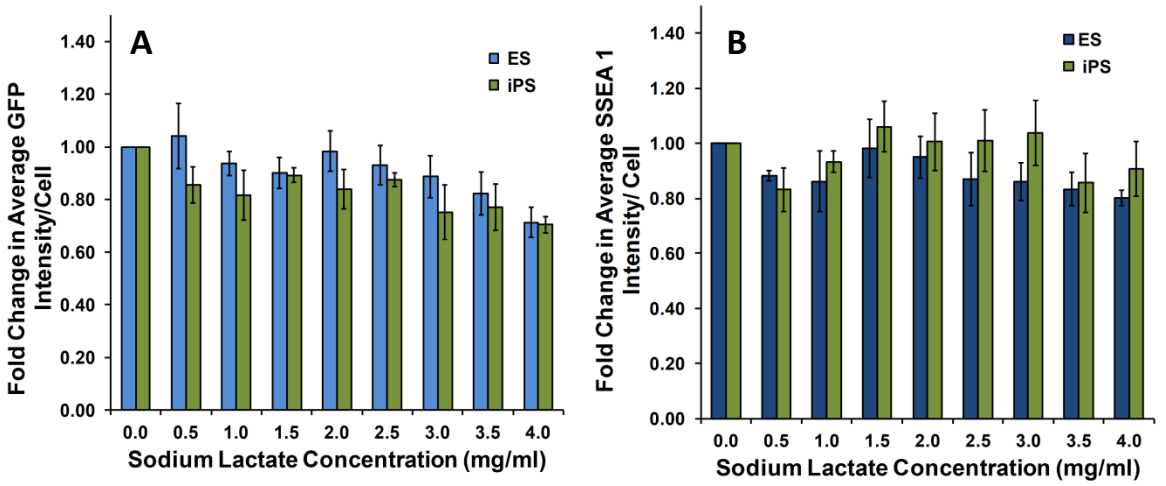


Figure 5.7 Fold change in average pluripotent marker intensity/ cell for OG2 ES and iPS cells with increasing sodium lactate concentration. (A) Oct4- GFP, (B) SSEA- 1. Data represent mean±SEM of n=3 individual experiments

5.3.1.2.3 Cellular Metabolic Activity:

Cellular metabolic activity measurement of OG2 ES and iPS cells by Cell Titre Glo assay also showed a trend similar to that of the feeder independent Oct4B2 ES cells. Figure 5.8 shows the fold change in luminescence for both the ES and iPS cells. The curve was relatively flat without any significant change in the luminescence value at any concentration with respect to the control value. These data were similar to those of the feeder independent ES cells, and were in line with our hypothesis of increased metabolic activity due to the usage of lactate along with glucose as a source of energy. HPLC analysis of spent media from both the cultures was carried out to verify our assumption.

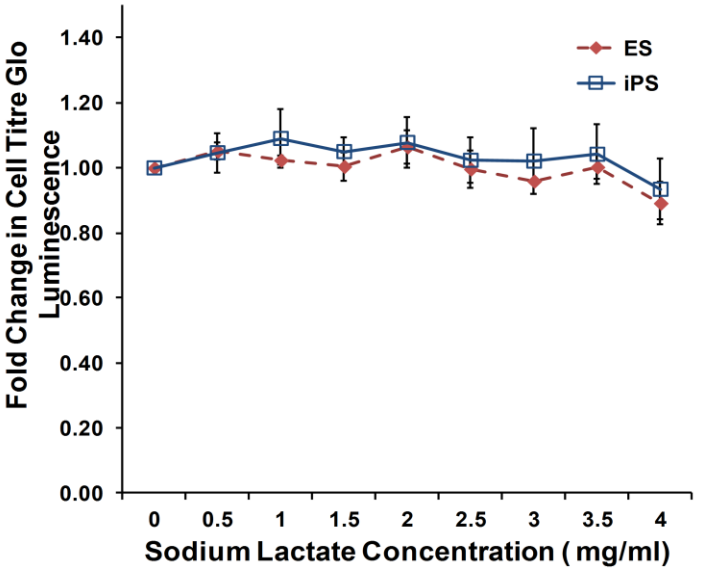


Figure 5.8 Fold change in Cell Titre Glo luminescnece for OG2 ES and iPS cells with increasing sodium lactate concentration. Data represent mean±SEM of n=3 individual experiments

5.3.1.2.4 HPLC Analysis:

HPLC analysis was carried out for spent media from feeder dependent ES and iPS cells. Figure 5.9 shows the fold change in HPLC data for the cell lines with respect to initial set up on day 0. A very slight increase in measured lactate concentration over time was observed for both ES and iPS cells in case of control set up (0 mg/ml sodium lactate). Similar to the feeder dependent ES cell, lactate utilisation was observed when sodium lactate was added to the medium suggesting that the feeder dependent ES and iPS cells were able to utilise externally added lactate.

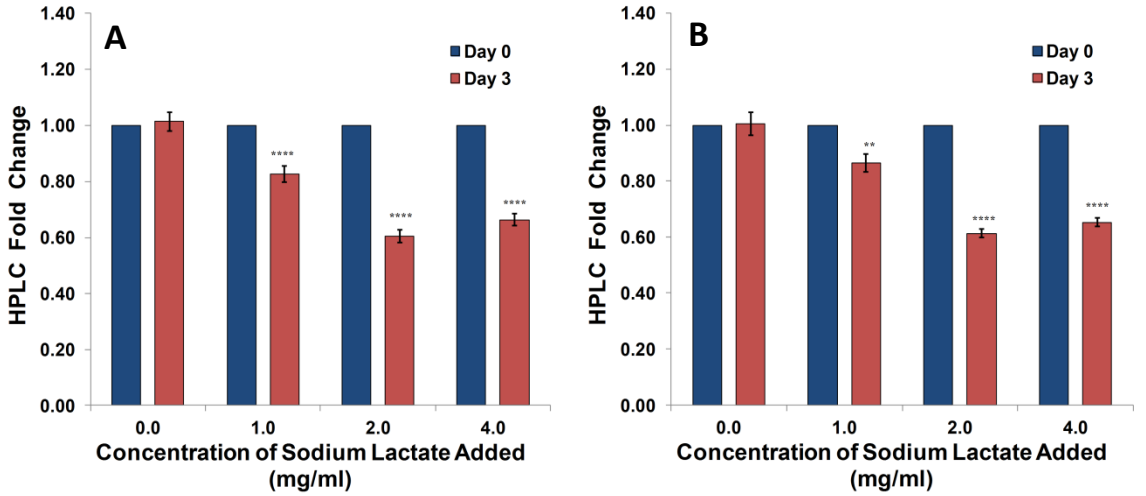


Figure 5.9 Fold change in area under curve for HPLC analysis of lactate in spent media from feeder dependent (A) OG2 ES, (B) OG2 iPS, with increasing sodium lactate concentration. Data represent mean±SEM of n=3 individual experiments

5.3.1.3 Conclusion:

In conclusion, externally added sodium lactate was found to be detrimental for growth and proliferation of all the three cell lines. Cell proliferation was significantly decreased with increasing sodium lactate, although the decrease was more in the case of the feeder independent cell line in comparison with the feeder dependent ones. This suggested that the feeder layer gave some protection to the pluripotent stem cells against the detrimental effects of external lactate. Pluripotency however, was not affected significantly for any of the cell lines in the case of externally added lactate. The cellular metabolic activity measurement assay showed a relatively flat line with increasing lactate concentration for all the three cell lines. Based on the observation, it was hypothesized that the pluripotent stem cells were able to adapt to externally added lactate and use it as an energy source along with medium glucose. HPLC analysis of spent media from the cultures showed a decrease in lactate concentration over time on external addition of lactate, validating our hypothesis that the pluripotent stem cells were able to metabolise externally added lactate as a source of energy resulting in higher metabolic activity.

5.3.2 Effect of pH on Pluripotent Stem Cells:

The effect of various pH levels on pluripotent stem cells was tested by using different concentrations on sodium bicarbonate. A 6- 8.5 pH range was chosen for this experiment and a normal ES media without any pH control or modification was used as the control set up. Similar to the previous experiments, both feeder independent and feeder dependent cells were used.

5.3.2.1 Cell Proliferation:

Both feeder independent as well as the dependent pluripotent stem cells were found to be highly sensitive to change in medium pH (figure 5.10). All three cell lines showed significant decrease in cell proliferation for both increase or decrease in pH in comparison to the control set up. Cell proliferation for feeder independent ES cells was best maintained at a pH of 7.5, while the feeder dependent cells were best maintained within a range of 7.0- 7.5. It was observed that the feeder independent Oct4B2 ES cells were more sensitive to pH change in comparison to the feeder dependent cells. Unlike the feeder dependent cells, even a slight drop of pH from 7.5 to 7.0 resulted in significant decrease in cell proliferation for Oct4B2 ES cells.

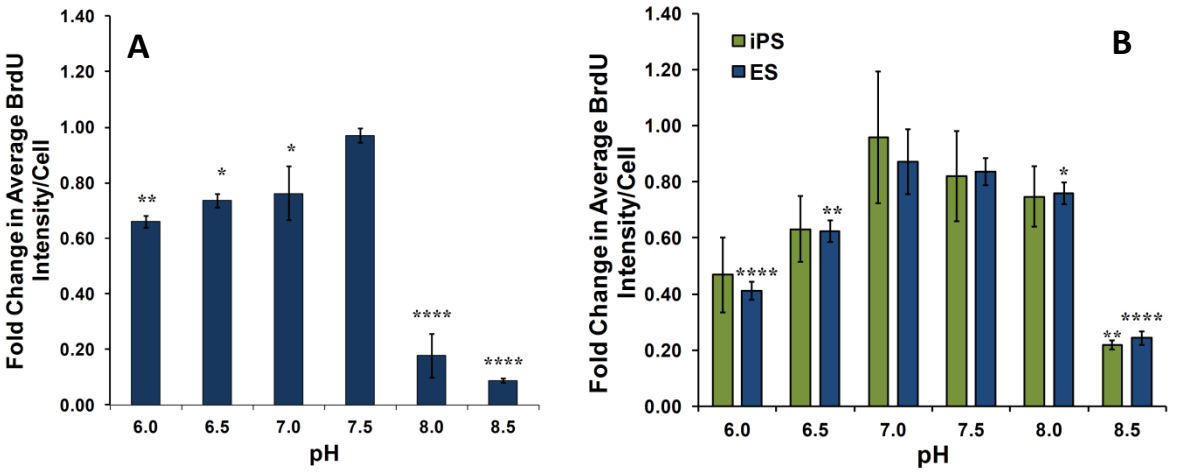


Figure 5.10 Fold change in average BrdU intensity/cell with change in medium pH. (A) Oct4B2, (B) OG2 ES and iPS cells. Data represent mean±SEM of n= 3 individual experiments

5.3.2.2 Cellular Metabolic Activity:

Cellular metabolism was measured using Cell Titre Glo assay kit and the results were represented as fold change with respect to normal ES media (control set up). As observed in figure 5.11, change in media pH severely affected the cellular metabolic activity of both feeder independent and feeder dependent pluripotent stem cells. Similar to cell proliferation, cellular metabolic activity of the feeder independent cells was also more sensitive to pH change in comparison to that of the feeder dependent pluripotent stem cells. Unlike the effect of lactate, the effect of pH on cellular metabolic activity and cell proliferation of the cells followed the same trend. A pH of 7.5 was found to be optimum for the feeder independent cells while for the feeder dependent ones the optimum range was between 7.0 and 7.5.

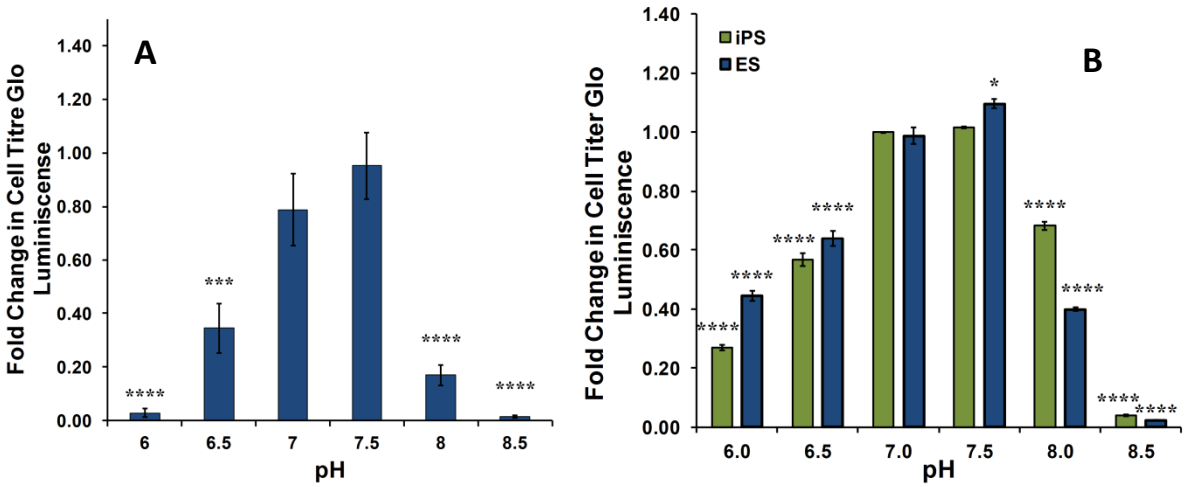


Figure 5.11 Fold change in Cell Titre Glo luminescence with change in medium pH. (A) Oct4B2, (B) OG2 ES and iPS cells. Data represent mean±SEM of n= 3 individual experiments

5.3.2.3 Cell Pluripotency:

Cell pluripotency was quantified by measuring fluorescence intensity of Oct4-GFP and SSEA 1 immunofluorescence. Unlike the effect of externally added sodium lactate, increase or decrease in medium pH beyond 7.5 were found to be detrimental to cell pluripotency for all the three cell lines. Both SSEA1 and Oct4-GFP fluorescence were significantly decreased when the medium pH was changed (figure 5.12). A pH of 7.5 was found to be optimum for pluripotency maintenance of both feeder independent as well as feeder dependent cells.

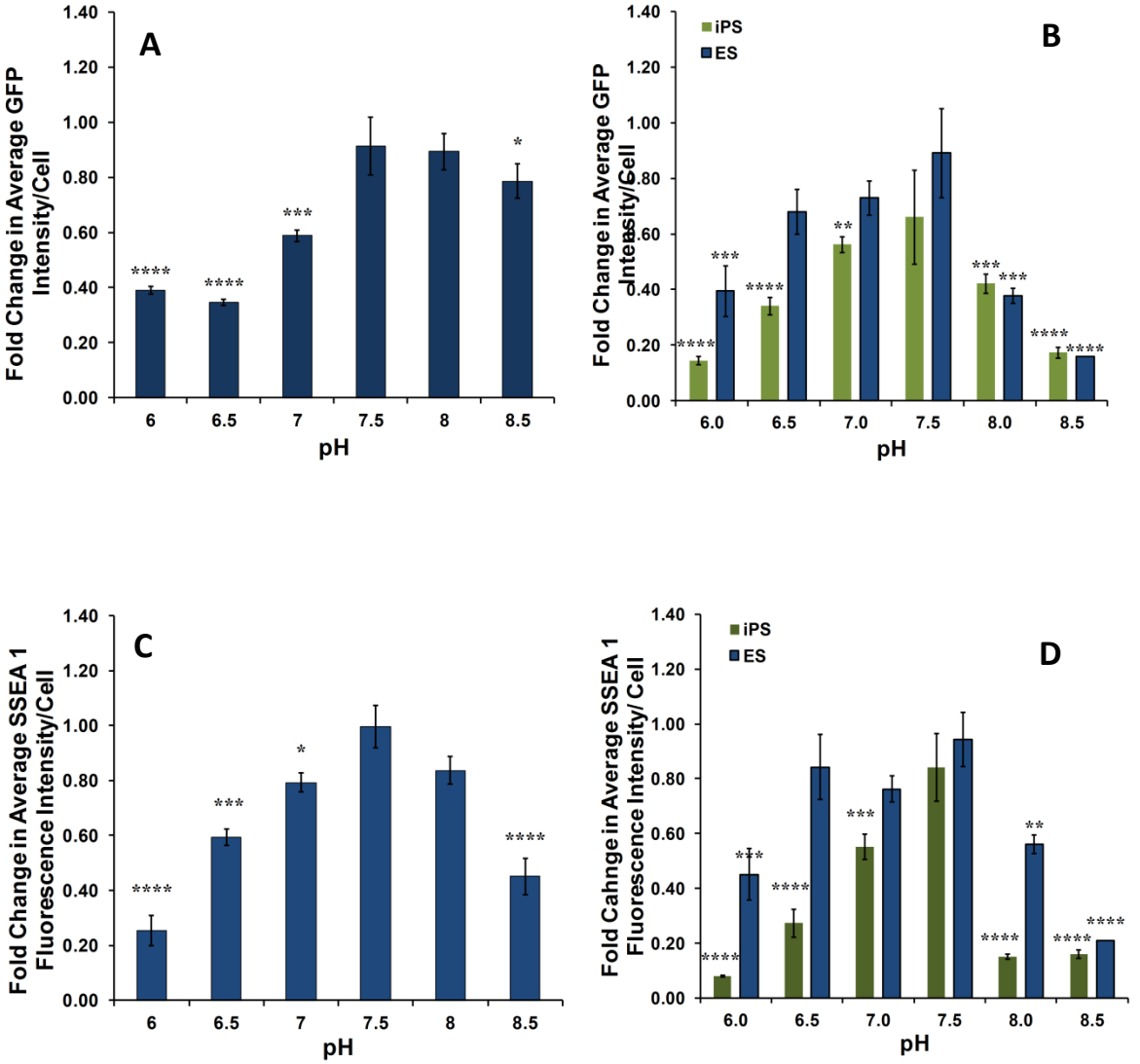


Figure 5.12 Fold change in pluripotent marker intensity/cell with change in medium pH. (A) Oct4 - GFP of Oct4B2, (B)Oct4-GFP of OG2 ES and iPS cells, (C) SSEA-1 of OctB2 and (D) SSEA- 1 of OG2 ES and iPS cells. Data represent mean±SEM of n= 3 individual experiment

5.3.2.4 Conclusion:

The effect of lactate on pluripotent stem cells was found to be much more severe in terms of cell proliferation, cellular metabolic activity and pluripotency when compared to the presence of excess lactate in the medium. Both the feeder dependent and feeder independent pluripotent stem cell lines behaved in a similar fashion in terms of cell proliferation, pluripotency and cellular metabolic activity. The feeder independent ES cells were found to be more sensitive to change in

medium pH. It is possible that the feeder layer provides some sort of protection to the pluripotent stem cells against the detrimental effects of pH change. A ph of 7.5 was found to be the optimum for the feeder independent ES cells while a pH range of 7.0 to 7.5 was found to be optimum for the feeder dependent ES and iPS cells. Away from the optimum pH, increase as well as decrease in pH was found to be harmful for the cells.

5.4 Conclusion:

Lactic acid has been reported to be one of the major waste products in mammalian cell culture, produced mainly during glycolysis. The influence of lactic acid on cells can be explained mainly by change in pH or effect of lactate ions. To date, a number of papers have reported the detrimental effects of both pH change and lactate ion accumulation on mammalian cells. However, very few studies have been performed to elucidate the effect of lactate on pluripotent stem cells, with only mouse embryonic stem cells being subjected to such studies. Also, the current literature has various discrepancies within it. The present study looked at the effect of lactate on cell proliferation, pluripotency and cellular metabolic activity on three different mouse pluripotent stem cell lines in a bid to determine general trends.

It was observed that there was no significant change in pluripotency of mouse pluripotent stem cells on addition of exogenous sodium lactate. This was observed with not only the feeder free ES cells but also the feeder dependent ES and iPS cells. These data supported the previously published literature, which had shown that presence of lactate does not affect pluripotency of mouse ES cells [43].

Similar to Ouyang et al.'s observation, total cell number for feeder free mouse ES cells decreased with increase in lactate concentration [43]. The decrease in cell number became significant from a sodium lactate concentration of 1.5 mg/ml and the significance degree of the change in cell number increased with increasing lactate concentration. Extending the scope of the work, it was seen that the cell proliferation rate for feeder free mouse ES cells also decreased with increasing sodium lactate concentration. It became significant once the sodium lactate concentration reached 2.5mg/ml. Experimentation with feeder dependent mouse ES and iPS cells to see the effect of lactate on their proliferation rate showed a similar decreasing trend in cell proliferation

rate although the change was not as significant as observed with the feeder free cells. The decrease in cell proliferation for iPS cells showed statistical significance only when the sodium lactate concentration reached a value of 3.5mg/ml. It is possible that the feeder cells were in some way responsible for protecting the cells from the detrimental effect of lactate.

Cell Titer Glo assay was carried out to measure the cellular metabolic activity. This assay measures ATP generation by the cells. In most cases, the cellular metabolic activity is proportional to cell proliferation and cell number. However, when this assay was carried out for the feeder free ES cells as well as feeder dependent ES and iPS cells, it was observed that there was no change in the cellular metabolic activity for any of the cells. These data were in stark contrast to the cell proliferation results. Based on the cell proliferation and cellular metabolic activity data, it was hypothesized that these three cell lines are able to utilize exogenously added lactate as a source of energy along with glucose.

In order to support the hypothesis, HPLC analysis of the spent media from the feeder free ES cell culture with sodium lactate concentrations of 1mg/ml, 2 mg/ml and 4mg/ml was carried out. ES media with no sodium lactate (0 mg/ml) was used as control. The feeder free ES cells were chosen for this due to their relative ease of handling. As expected, it was observed that when no sodium lactate was added to the media, the lactate concentration in the media increased over time, suggesting that lactate was being produced by the cells as a metabolic by-product. However, when sodium lactate was added to the culture, we observed a decrease in the fold change in measured lactate concentration over time. The decrease was significant at a sodium lactate concentration of 2mg/ml and 4mg/ml. These data support our hypothesis of lactate utilization by the mouse ES cells. Similar observations were also made with the feeder dependent ES and iPS cells.

Although relatively less studied, alteration of cell metabolism due to externally added lactate and utilization of lactate as a preferred energy source have been reported earlier for a number of cell lines. Recombinant CHO cells have been shown to have increased erythropoietin production on addition of exogenous sodium lactate, although there was a decrease in specific cell growth and glucose consumption [132]; that study suggested that the high concentration of sodium lactate led to oxidation of lactate to pyruvate at a high rate by the LDH (lactate dehydrogenase) enzyme. This resulted in an accumulation of NADH and high concentration of pyruvate. The energy thus

generated, however, was used for amino acid and protein synthesis. Studies on lactate metabolism by CHO cells have also been carried out by Zagari et al.; they reported that consumption of lactate by the CHO cells was associated with an increased mitochondrial activity and oxygen consumption [133]. The same group also identified the malate- aspartate shuttle as a key factor in lactate consumption by CHO cells [134]. Interestingly, a positive effect of lactate on mouse ES cells has also been reported. It has been shown that media enriched with lactate increases colony number and size for feeder dependent ES cells[84]. They suggested that lactate gets converted to Acetyl CoA, which can either be used in mitochondrial metabolism or increased gene expression via histone acetylation. The HPLC data that show decrease in lactate concentration are consistent with these data and hypothesis.

In contrast, the present findings on the effect of change in pH on mouse pluripotent stem cells were consistent with the available literature. Cell proliferation, cellular metabolic activity as well as pluripotency of the mouse pluripotent stem cells were significantly decreased with any change in the media pH towards acidity or alkalinity. A pH of 7.5 was found to be optimum for the feeder independent cells while a pH between 7.0 and 7.5 was best suited for the feeder dependent pluripotent cells.

In conclusion, the current chapter has presented the effect of exogenously added lactate and change in media pH on mouse pluripotent stem cell culture. Corroborating our hypothesis, it was observed that pluripotent stem cells are extremely sensitive to any change in media pH, which affects the cell growth, viability, proliferation and pluripotency. As per the hypothesis, it was expected that both cell proliferation and pluripotency would be affected by externally added lactate. However, the data deviated slightly from our hypothesis, and it was seen that only cell proliferation, and not the pluripotency, was negatively affected by increase in lactate concentration. As a surprising and new finding, cellular metabolic activity measurement and spent media analysis suggest that the cells were able to utilize the lactate as a source of energy. These studies suggest that careful consideration of media pH and involved metabolites are of utmost importance for proper growth and maintenance of pluripotent stem cells.

Chapter 6 Fibroblast Conditioned Media Analysis and Comparison with Theoretical Modeling

6.1 Introduction:

Pluripotent stem cells have the capacity for unlimited self renewal and differentiation into all cell lineages. Traditionally, these cells are co cultured on a 'feeder layer' of mouse embryonic fibroblast cells. Various other cell lines, like human fibroblasts, have also been used as 'feeder layer' to avoid cross species contamination problem[20]. Further advancement in the field led to the use of specialized commercial media or feeder cells conditioned media, along with suitable matrices, for the growth, expansion and pluripotency maintenance of the cells[28]. The fibroblast conditioned media is hypothesized to contain a plethora of unknown growth factors and cytokines needed to culture the pluripotent stem cells *in vitro*. Efforts have been made to identify and classify the different protein factors secreted by the fibroblast cells[69, 135]. For example, proteomic analysis of human neonatal fibroblast conditioned media showed it to be a complex mixture of growth factors, extracellular matrix proteins and differentiation factors[69]. However, very little information is available regarding metabolite components of conditioned media. Recently in 2011, MacIntyre et al. published a complete characterization of inactivated human foreskin fibroblast conditioned media using NMR spectroscopy[136]. However, very little or no data are available for the metabolite components of mouse embryonic fibroblast cells, which is the most commonly used feeder cell line.

Information regarding metabolic components, especially lactate concentration of feeder cells, is of utmost importance since it has been reported to be one of the potential growth inhibitors for mammalian cells, including pluripotent stem cells [43, 75, 137].

In order to nullify the detrimental effect of lactate, conditioned media is mixed with fresh media before being used for embryonic stem cells. However, an exact or approximately optimised ratio of conditioned: fresh media is not available.

Over time, analytical methods, such as chromatography and NMR spectroscopy, have been used to generate metabolic profiles of various cell lines like CHO, BHK, Hybridoma cells, mesenchymal stem cells [138-141]

Experimental procedures of any kind will have errors due to experimental assumptions, manual handling and many other reasons. Use of mathematical modeling is a common way for validating the accuracy of experimental data. A comparative study between experimental data and theoretical prediction has been done for metabolic analysis of various cell lines[138, 139]. According to Sidoli et al., mathematical models can be classified as structured or unstructured, segregated or unsegregated, and deterministic or stochastic [142].

The mathematical model used here assumes the fibroblast population to be homogenous in nature whose behavior can be described by an average cell. Hence the model is unstructured, unsegregated and deterministic in nature[139].

6.2 Materials and Methods:

6.2.1 Metabolite (glucose and lactate) Analysis of Fibroblast (SNL) cell line:

Metabolite analysis for mouse embryonic fibroblast (SNL cell line) was performed experimentally using High Pressure Liquid Chromatography (HPLC). The experimental data were also compared with a simple theoretical model prediction using MATLAB.

6.2.1.1 Experimental Procedure:

Mouse Embryonic Fibroblast (MEF) cell line, SNL, was obtained from the laboratory of Prof Allan Bradley, UK. The cells were maintained in standard MEF media and were grown in tissue culture treated T- 25 flasks (Himedia, Mumbai, India). On reaching 80% confluency, the cells were passaged using trypsin/EDTA, plated on 24 wells plate at a seeding density of ~ 50,000 cells/ cm^2 (around 10x 10^4 cells/ well) and incubated for about 10 hrs in a CO_2 incubator at 37°C for the cells to adhere properly. After incubation, the cells were washed twice with PBS to remove any non adherent cells. 1 ml of fresh MEF media was added to each well (~0.5ml/cm^2) and incubated. The time point for fresh media addition was considered as 0 hrs. Conditioned media were collected at regular intervals, centrifuged at 3000xg for 10 minutes to precipitate out any cell debri. The supernatant was collected for metabolite analysis. Cell counts were done in triplicates for each time point using hemocytometer.

Glucose and lactate concentration estimation was undertaken using HPLC. Aminex HPX-87H cation exchange column (BioRad) was used with 5mM H_2SO_4 as the mobile phase at a flow rate of 0.6ml/min. The temperature was fixed at 60°C. Lactic acid was determined using a UV detector (214nm) while glucose was estimated using a RI detector.

Standard Curve:

Standard curves for glucose and lactic acid concentration were prepared by serial dilution of their solutions. The ability of the instrument to separate and detect glucose and lactic acid was tested using mixture of pure glucose, pure lactic acid and mixture of standard media and lactic acid.

6.2.1.2 Theoretical Modeling:

The theoretical modelling of fibroblast metabolism was conducted using ODE 45 solver in MATLAB. The following equations were used for this purpose[139].

$$\frac{dX_v}{dt} = X_v.(\mu - \mu_d) \quad (1)$$

$$\mu = \mu_{max}.\frac{X_g}{K_g + X_g} \quad (2)$$

$$\frac{dX_m}{dt} = \pm q_m . X_v - k_{md}.X_m \quad (3)$$

$$\frac{dX_d}{dt} = X_v.\mu_d \quad (4)$$

Doubling time was estimated using the equation –

$$t_d = (t_2 - t_1) \times \{\frac{\log(2)}{\log(q_{2/q_1})}\} \quad (5)$$

Notations:

X_m = Total moles of a metabolite in total volume (mol)

where m = Metabolite referring to glucose, glutamine or lactate

X_v = Total cell number in total volume (cells)

X_g = Total moles of glucose in total volume (mol)

X_l= Total moles of lactate in total volume (mol)

μ_{max} = Maximum specific growth rate (hr^{-1})

μ= Specific growth rate (hr^{-1})

μ_d= Death rate (hr^{-1})

t_d= doubling time (hr)

q_m = Specific metabolite reaction rate (mol/cell/hr)

q_g =Specific consumption rate of glucose (mol/cell/hr)

q_l = Specific production rate of lactate (mol/cell/hr)

q_{gl} = Specific consumption rate of glutamine (mol/cell/hr)

K_g= Monod constant of glucose (mol)

q_1 = Cell number at time t_1

q_2 = Cell number at time t_2

k_{md} = Degradation rate constant of metabolite (hr^{-1}) (Applicable only for glutamate, glutamine or other amino acids that degrade over time).

Assumptions for mathematical model:

1) Visual assessment using trypan blue showed very few dead cells. Hence cell death was taken to be negligible and μ_d= 0.

2) As per literature degradation of glucose and lactate is negligible. So k_{md}= 0 for glucose and lactate profile study [139]

Values taken for mathematical model:

From Experiment:

$X_v(initial)$= 7x10^4

$X_g(initial)$= 0.017

$X_l(initial)$= 1.6x 10^{-4}

μ_{max} = 0.01925

t_d= 36 hrs

q_g = 3.65x 10^{-10}

q_l = 1.64x10^{-9}

From Literature:

K_g= a) 0.004 [139, 143, 144] , b) 0.006 [145]

q_{gl} = 2*10^{-12} [139]

k_m = 0.0064 (for glutamine only) [139]

Validation of experimental data with the modeling was only done for glucose and lactate with negligible cell death. To check the validity and robustness of the model, glucose consumption, lactate production and cell number was estimated on incorporating cell death term. Theoretical glutamine consumption was also estimated.

6.3 Results and Observations:

Metabolic profiling of fibroblast conditioned media was carried out using HPLC methodology and was compared with theoretical estimation of the same. Cell count was also done at every

time point across the experimental timeline in triplicates and compared to theoretical data as well.

The parameters like Monod constant and cell death rate were changed and/or incorporated in the model to check the model's robustness. Initial standard curve graphs for glucose and lactate measurement using HPLC method had R^2 values around 0.99 (Figure 6.1).

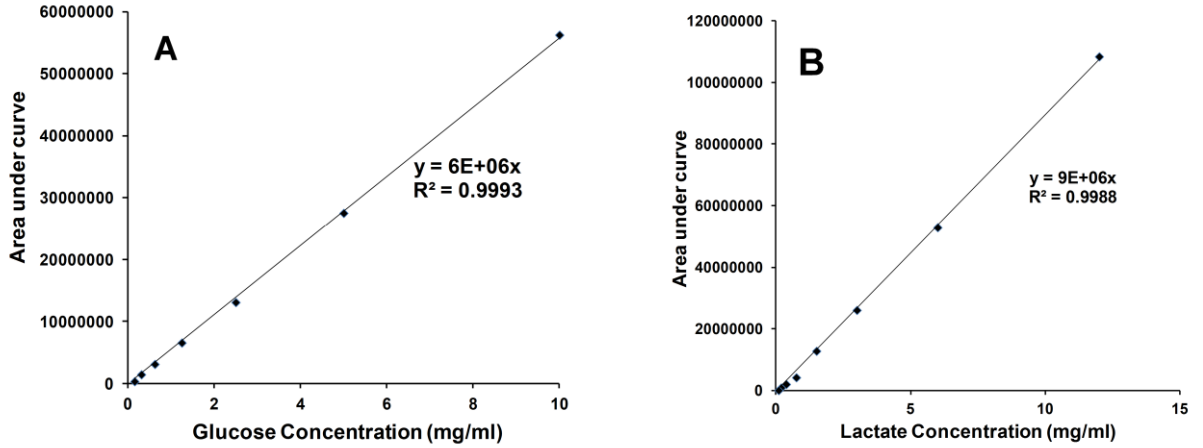


Figure 6.1 Standard curves for glucose and lactate concentration measurement using HPLC. Area under curve data were plotted against known concentrations of glucose and lactate. (A) Glucose concentration, (B) Lactate concentration

6.3.1 Cell Count:

Viable cell density was estimated at all the time points using trypan blue assay. Mouse embryonic fibroblasts cell number expanded after a very short lag phase and resulted in a four fold increase in viable cell number at the end of 72 hours (Figure 6.2 A, B). Based on trypan blue assay, cell death at all time points was assumed to be negligible for the theoretical modeling. The theoretical curve was also seen to fit the experimental data. To check the robustness of the modeling, the cell death estimation term (μ_d) was incorporated in the model (Figure 6.2 C). It was observed that a μ_d value of zero best suited our experimental data and supported our assumption of negligible cell death.

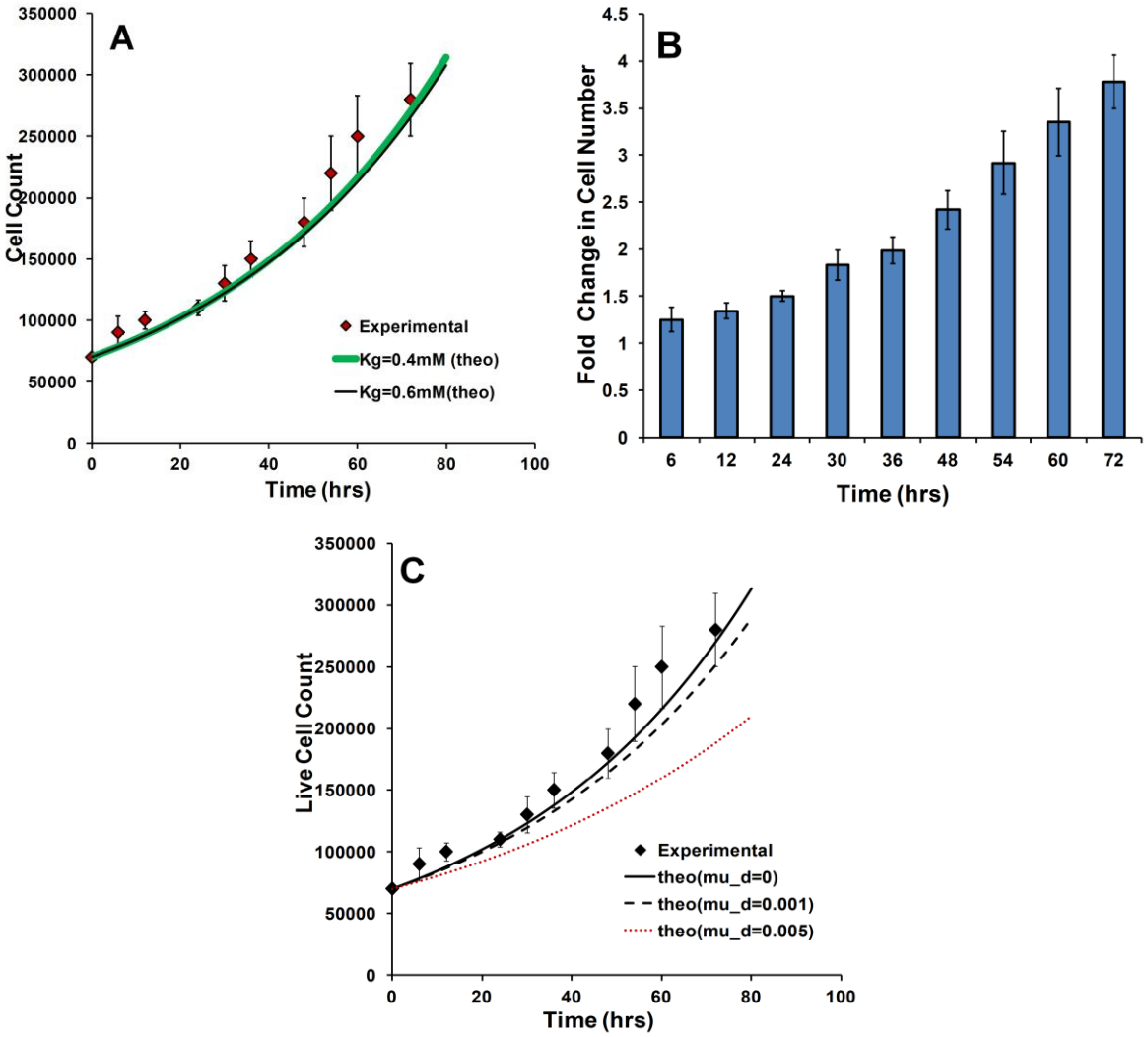


Figure 6.2 Mouse Embryonic Fibroblast cell count. (A) Comparison of experimental cell count with theoretical estimation at different values of Monod constant, (B) Fold increase in cell number, (C) Comparison of experimental cell count with theoretical estimation

6.3.2. Metabolite Analysis:

The main aim of this study was to study the metabolic profile of the MEFs to analyse the consumption of glucose and formation of lactate by the cells over time. HPLC analysis of spent media collected at regular interval was carried out. As expected, glucose concentration decreased and lactate concentration increased in the medium with time. Glucose concentration went down from 3.05mg/ml to 2.03mg/ml (0.011 moles) in 72 hours while the lactate concentration reached a final value of 2.23mg/ml (0.025 moles) in the same time (Figure 6.3 A, B). Interestingly, the

experimental data suggested that the 0 hr control medium also showed the presence of a very little amount of lactate ($1.6x10^{-4}$ moles) in it; this may be due to the animal serum present in it.

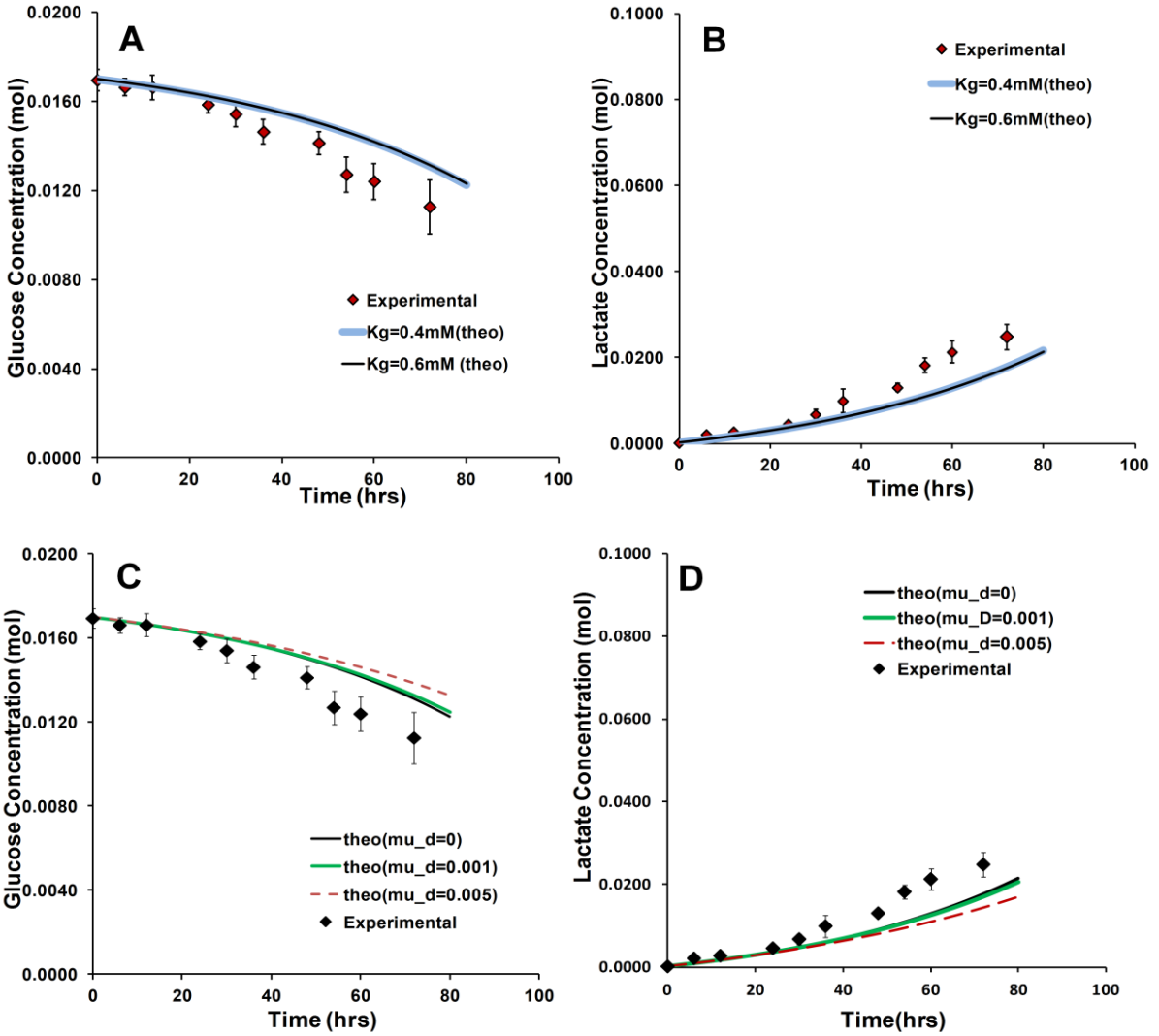


Figure 6.3 Metabolic analysis. (A) Comparison of experimental glucose consumption with theoretical estimation at different Monod's constant, (B) Comparison of experimental lactate production with theoretical estimation at different Monod's constant, (C) Comparison of experimental glucose consumption with theoretical estimation at different cell death rate, (D) Comparison of experimental lactate production with theoretical estimation at different cell death rate. Experimental data represent Mean±SEM (n=4)

The value of Monod's constant is an important parameter for theoretical estimation and modeling of glucose and lactate metabolism. Since the Monod's constant value was taken from the

literature, more than one reported value was tested to see its effect glucose and lactate concentrations. It was observed that changing the value of Monod's constant had no effect on the theoretical metabolic curve of glucose and lactate (Figure 6.3 A, B).

Similar to the theoretical curve for viable cell count, different values of the death constant were used for theoretical glucose and lactate concentration estimation curves. The overall curve trend remained the same in both cases (Figure 6.3 C, D).

A theoretical curve for glutamine consumption was also obtained from the same modeling (figure 6.4). Glutamine consumption modeling suggested that its depletion was faster than glucose and hence it may be necessary to replenish glutamine while using the conditioned media. However, detailed experiments need to be done in order to reach any valid conclusion.

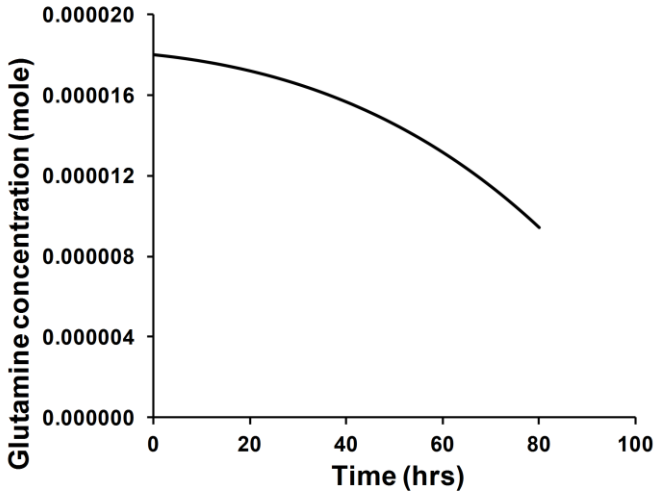


Figure 6.4 Theoretical estimation of glutamine consumption

6.4 CONCLUSION:

HPLC analysis of spent media from mouse embryonic fibroblast cells was carried out for estimation of glucose consumption and lactate production over time. Glucose concentration went down from 3.05mg/ml to 2.03mg/ml in 72 hours while a lactate accumulation of 2.23 mg/ml occurred in 72 hours. Together with the results from the previous chapter, the current data

on metabolic analysis would be of immense help in an optimized use of conditioned media for expansion of pluripotent stem cells.

Simple mathematical modeling was also carried out for prediction of the metabolism trend beyond 72 hours if needed.

The mathematical model was able to predict the trend of cell count, glucose consumption, lactate production and glutamine consumption. The concentration of glutamine decreased more than that of glucose, suggesting a replenishment of the same prior to its use as conditioned media. However, more experiments need to be done to study the actual effects of glutamine depletion and to optimize a threshold glutamine concentration to avoid detrimental effects on the cells. Incorporation of cell death rate in the modeling was also done. As expected, increase in cell death rate showed decrease in glucose consumption rate as well as lactate formation rate. It was also observed that changing the value of Monod constant did not have any detectable effect on glucose consumption, lactate production or cell count.

Chapter 7 Conclusion and Future Perspectives

7.1 Conclusion:

A survey of the literature concerning the theme of this thesis highlighted the fact that the large scale expansion of pluripotent stem cells is dependent on several tightly controlled intrinsic and extrinsic parameters. Extensive work has been carried out to see the effect of one or more of these parameters on different embryonic stem cell lines, but there is a dearth of similar studies on induced pluripotent stem cells. It was also observed that the obtained results were cell line dependent to some extent.

Based on this survey, a number of experiments were designed and carried out in an effort answer the primary objectives highlighted in *chapter 1* of this thesis. Based on the observations and results, the following overall conclusions can be made:

1. *Chapter 3* looks at the suitability of certain novel biomaterials both in 2D and 3D culture for expansion of mouse embryonic stem cells. The first set of experiments tested the suitability of tropoelastin in 2D. It was observed that, although the material was able to maintain the cell attachment and pluripotency, the cell expansion rate was slow and the net cell number yield was very low in comparison to the widely established growth surfaces gelatin and Matrigel. The morphology of the cell colonies was also found to be different. Since we were looking for a material that would give high cell yield, tropoelastin was not found to be a suitable material.

   In the next work, we selected a material PMVE- MA, which has already been shown to support embryonic stem cell growth in 2D, and used it to obtain a novel 3D scaffold of PMVE - MA and gelatin. The aim was to see if the scaffold was able to support the attachment, expansion and pluripotency of mouse embryonic stem cells. Experimental results proved that the novel scaffold was able to support the attachment, long term expansion and pluripotency of mouse embryonic stem cells. However, being a macroporous structure, the cells were embedded inside the scaffolds, posing a problem during cell harvesting and limiting its usage to some extent.

The third part of this work summarises preliminary experiments to compare the attachment and expansion of pluripotent stem cells on commercially available Cytodex 3 and Hillex II for use in a subsequent work. Cytodex 3 showed better results in comparison to Hillex II for both mouse embryonic and induced pluripotent stem cells in terms of cell attachment and final cell yield, making Cytodex 3 a viable choice of cell expansion surface for the next work.

A comparison of the 3D structures (scaffolds and microcarriers) suggests that the difficulties in cell extraction from within the scaffolds make the use of microporous or non porous beads more suitable when the aim is to generate a large number of pluripotent stem cells and/or their derivates *in vitro*.

2. Carrying forward the preliminary results from the previous chapter, *chapter 4* looks at the expansion of mouse induced pluripotent stem cells in a spinner flask culture using Cytodex 3 microcarriers.

   Initially, spin rate optimization for microcarriers' structural integrity maintenance as well as proper suspension showed that RPMs below 20 resulted in the carriers settling down, while RPMs above 40 resulted in bead breakage. These results highlighted the importance of similar optimization for other 3D structures prior to their use in dynamic culture systems.

   Based on the above optimization, spin rates within the range of 25-40 were tested for the attachment, long term expansion and pluripotency maintenance of the cells. It was observed that spin rates above 30 RPM resulted in instant cell detachment from the microcarriers followed by cell death. It was also seen that, although RPMs of 28 and 30 supported cell attachment initially, long term cell expansion was not possible.

   A spin rate of 25 RPM supported long term attachment, expansion, pluripotency maintenance and spontaneous differentiation capability of induced pluripotent stem cells. Since lower spin rates did not support proper suspension and mixing of microcarriers, 25

RPM was taken to be the optimum spin rate for long term culture of mouse induced pluripotent stem cells in our set up.

3. *Chapter 5* looks at two important physicochemical parameters, pH and medium lactate concentration. A wide range of pH and lactate concentration were tested on pluripotent stem cells, both embryonic as well as induced. The use of both these cell types allowed us to generalise the effect to some extent.

   Both embryonic as well as induced pluripotent stem cells were highly susceptible to any change in pH, whether towards acidity or alkalinity. Cell attachment, proliferation and pluripotency were all affected by pH change.

   It was also observed that the absence of a feeder layer made the cells more susceptible to changes in pH. A pH range of 7.0 to 7.5 was found to be optimum for the feeder dependent ES and iPS cells, while feeder independent ES cells showed optimum growth at a pH of 7.5. The presence of the same optimum pH range for both ES and iPS cells suggested that the effect of pH on pluripotent stem cells may be independent of the cell line and type of pluripotent stem cell.

   The study on the effect of externally added lactate on pluripotent stem cells showed some unexpected and interesting results. Proliferation rate, final cell density for both embryonic and induced pluripotent stem cells were affected by increasing lactate concentration in media. However, no significant change in pluripotency of the cells was observed.

   Analysis of cellular metabolic activity showed some interesting results for both the cell types. In stark contrast to the cell proliferation and expansion data, results obtained from Cell Titre Glo assay showed no change in the cellular metabolic activity of the cells with increasing lactate concentrations. We hypothesized that the pluripotent stem cells were able to use lactate as a source of energy along with glucose, resulting in an increasing metabolic activity of the cells even though the cell number and proliferation rate

decreased. In order to validate our hypothesis, estimation of lactate concentration in spent media from the culture was carried out using HPLC. It was observed that on addition of lactate externally, the total concentration of lactate in the media decreased with time for all the cell lines used, supporting our hypothesis of lactate utilization by the cells. Although relatively new for pluripotent stem cells, usage of lactate as an energy source has been reported for various other cells like CHO cells, rat spermatids and certain cancer cell lines[146-148].

4. The MEF cells are the most commonly used feeder cells for pluripotent stem cell culture. Conditioned media from feeder cells have been widely used to avoid the usage of feeder layer directly as well as the expensive growth factors like LIF. In most cases, conditioned media is mixed with fresh media prior to its usage to decrease the concentration of waste materials like lactate and to replenish the essential nutrients, like amino acids and glucose. However, there are no ratios or concentrations for this and it varies between every group.

   The last body of work in *chapter 6* looks at the metabolic analysis mouse embryonic fibroblast conditioned media. Such an analysis will be of immense help in optimizing the working concentration and mixing ratio of fibroblast conditioned media: fresh media for culturing of pluripotent stem cells.

   HPLC analysis of the conditioned media collected at different time points showed an increase in lactate concentration over time, reaching around 2.23 mg/ml in 72 hrs. Glucose concentration decreased and reached a value of 2.03mg/ml. Interestingly, the presence of a very small amount of lactate was found in the fresh media and can be attributed to the serum present in it.

   Mathematical modeling was undertaken and validated by the experimental data; the predictions for glucose and lactate concentration were in line with the experimental values. An estimation of glutamine concentration suggested that this amino acid gets

depleted much faster than glucose. However, experimental validation of the depletion is required before reaching any specific conclusion.

The data obtained in *chapter 5* and *6* are important in optimizing conditioned media collection and its usage for pluripotent stem cell expansion.

Overall, the thesis highlights the importance of studying and optimizing parameters like adhesion surface, pH, lactate concentration, etc, for pluripotent stem cells. Similar optimized values and cell behaviour in case of properties like pH and lactate for both embryonic and induced pluripotent stem cells suggest that initial optimization for other parameters using ES cells can be applied directly to iPS cells. The work also demonstrated that long-term feeder depletion of cells results in their being extra sensitive to certain culture parameters. The importance of precise and in depth study of biomaterials and 3D structure integrity in dynamic culture prior to their use in 3D cell culture is highlighted too. In conclusion, it can be said that similar optimization experiments for other parameters along with comparative studies between ES and iPS cells for the same is of utmost importance.

7.2 Future Perspective:

The field of stem cell bioengineering is huge and the parameters involved are numerous. The results and conclusions reported in this thesis are capable of acting as the basis for a number of related studies in future within this area.

1. . The novel PMVE- MA and gelatin scaffold was suitable for culture of mouse ES cells but problems in cell extraction from the scaffold are a hindrance to its future use. Finding an effective method for harvesting the cells from the scaffolds can open up avenues for further use of this scaffold. This can be done by either making specific modifications to the scaffold or by finding a universal method for cell extraction from within the scaffold.

2. Dynamic culture studies show that the structural integrity of 3D structures is dependent on the spin rate. Similar studies for other available 3D structures, both commercial and

novel, would be valuable. Also, studies to see if other flow patterns like parallel flow and radial flow also have similar effects on 3D structure would be of interest to many.

3. An in depth metabolic cycle analysis and genomic analysis for pluripotent stem cells in order to understand the lactate utilization process can also be performed. Comparison to the other cell types with similar properties may present some interesting results.

4. Studies related to the effect of conditioned media collected at different time points on pluripotent stem cells will help in optimizing the collection and use of the same. Testing of different mixing ratios for fresh media and conditioned media, along with the effect of the essential nutrients and growth factors in the medium, would be of considerable help in terms of large scale expansion of pluripotent stem cells.

5. Comparative studies involving different permutations and combinations of multiple parameters can be carried out too. These studies would be important in mimicking the *in vivo* 'niche' of the cells, since in a practical situation, different parameters act together and even affect each other.

Appendix

The appendix chapter compiles the protocols and/or results of experiments carried out for different characterization and optimization processes during the research but are not directly related to the objectives within the thesis chapters.

A. Alkaline Phosphatase Assay

Alkaline phosphatase is a phenotypic marker for pluripotent stem cells and is routinely used for identifying and characterizing embryonic stem cells and induced pluripotent stem cells.

Methods:

The cells were cultured on to 35mm tissue culture dish and allowed to attain a semi confluent state. Alkaline phosphatase assay kit was procured from Millipore. The test was carried out as per manufacturer's instructions. Briefly, the reagents were prepared by mixing Fast Red Violet with Naphthol AS-BI phosphate solution and water in a ratio of 2:1:1. The cells were fixed using 4% paraformaldehyde for 1-2 minutes. The fixative was aspirated and the cells were washed twice with the rinse buffer (0.1% tween 20 in PBS). The premixed reagent was added such that it covered the entire area and was incubated in dark at room temperature for 15 minutes. The staining solution was then rinsed and the cells were washed with the Rinse buffer. The cells were then covered with PBS and AP positive colonies (red colonies) were counted.

Results:

Alkaline phosphatase staining showed pluripotent cells as red coloured colonies under the microscope, depending on the degree of pluripotency. The completely differentiated cells lacked any colour. All the three pluripotent stem cell lines used were alkaline phosphatase positive in nature. Figure A.1 shows a representative image for alkaline phosphatase staining of feeder independent Oct4B2 embryonic stem cells.

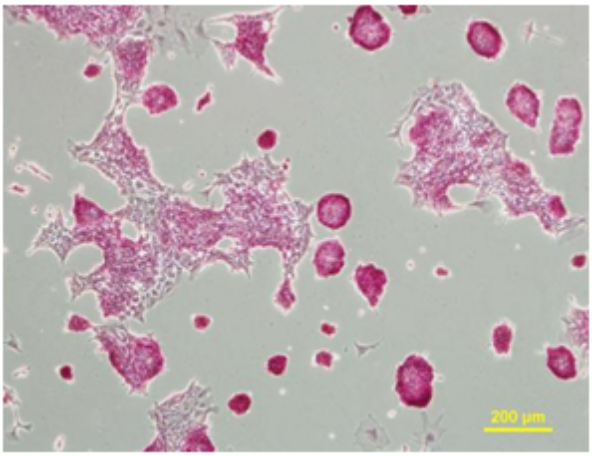


Figure A. 1 Alkaline Phosphatase staining ofOct4B2 mouse embryonic stem cells. Pluripotent colonies stain red. Scale bar: 200µm

B. Optimization of Cell replating for the purpose of HCS Instrument Analysis:

Fluorescence measurement for the analysis of cell proliferation (BrdU incorporation) and pluripotency maintenance (SSEA 1 staining and presence of OCT-GFP) was done using the ArrayScan High Content Analysis instrument. However, due to the pluripotent stem cells' property of growing in colonies, the instrument's scanning and analysis capabilities were hampered. This problem was solved by trypsinization of the cells and replating it at the end of the experiment followed by cell fixation. The fixation time was decided based on cell attachment and fluorescence intensity of the Oct4 tagged GFP for the cells. For this purpose, Oct4B2 ES cells were fixed at different time points (between 1- 8 hrs) after trypsinization and the GFP intensity was measured using ArrayScan.

As shown in figure A.2, it was observed that the GFP intensity rose sharply after 2 hours of attachment and it became almost constant after 5 hours. Visual inspection also showed that the cells were properly attached by 5 hours. Hence, for the future experiments with ArrayScan, cell fixation was done between 5-8 hours of replating the cells.

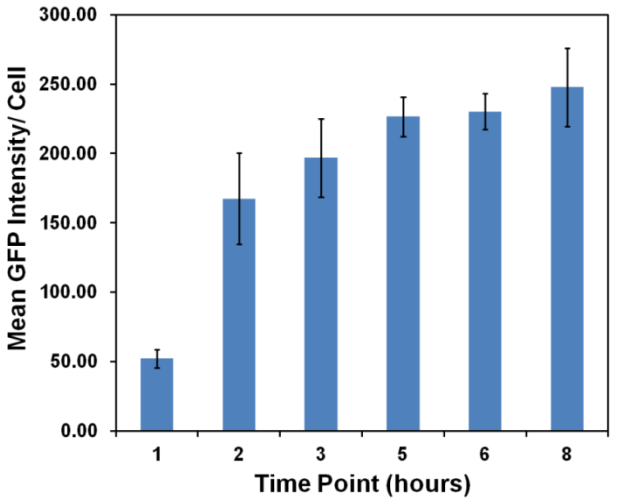


Figure A. 2 Mean GFP intensity/cell at different time points after cell plating

C. Optimization of BrdU Addition Time Point:

For the BrdU incorporation assay, initially cells were plated on to optilux plates with clear bottom and opaque wall, followed by addition of BrdU solution 18-24 hrs prior to cell staining. Two difficulties were faced in this initial experimental set-up. Firstly, as seen in the previous paragraphs the efficacy of the High Content Screening and Analysis (Thermo Fisher Scientific) instrument is dependent on its ability to identify and analyse individual cells. Since the pluripotent cells grow in colonies, the instrument was unable to segregate the cells. This problem was solved by trypsinizing the cells and replating them, as mentioned in the previous section.

Secondly, during initial experiments, it was observed that the presence of BrdU in culture for a long time was detrimental for ES cells. Hence, an optimization experiment was carried out to find out an ideal time period for BrdU addition that would facilitate its uptake by the cells without causing any harm to them. BrdU solution was added to Oct4B2 ES cell culture at different time points, followed by staining as per the protocol mentioned in *chapter 2*. BrdU-FITC fluorescence intensity was measured for BrdU incorporation at different time points using ArrayScan.

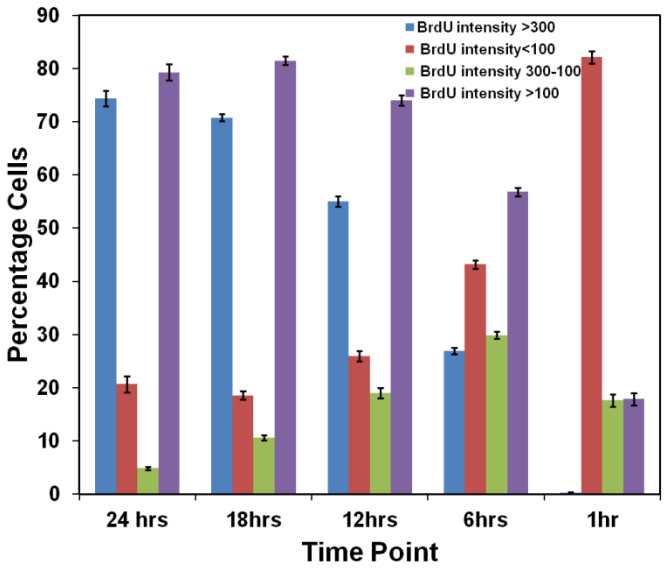


Figure A. 3 BrdU-FITC fluorescence intensity measurement for BrdU added at different time points

The BrdU uptake of mammalian cells was dependent on the cell cycle. It was observed (Figure A.3) that for feeder independent Oct4B2 mouse ES cells, more than 50% of the cells showed high BrdU- FITC intensity after 12 hrs of BrdU addition. The percentage of cells showing high BrdU- FITC intensity was almost equal for 18hrs and 24 hrs time points. Based on these data, BrdU was added to the the experimental set ups 18-24 hrs prior to its termination for all experiments..

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List of Publications

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- Chhabra, H.; **Gupta, P.**; Verma, P.; Jadhav, S.; Bellare, J.(2014), 'Gelatin–PMVE/MA composite scaffold promotes expansion of embryonic stem cells', Material Science and Engineering C, 37, 184-194.

- **Gupta, P.**; Verma, P.; Hourigan, K.; Bellare, J.; Jadhav, S.(2012), 'Metabolic analysis of fibroblast conditioned media and comparison with theoretical modeling', World Academy of Science, Engineering and Technology, International Science Index 72, 6(12), 266–270.

- **Gupta, P.**; Ismadi, M.; Verma, P.; Fouras, A.; Jadhav, S.; Bellare, J.; Hourigan, K. (2014), 'Optimization of agitation speed in spinner flask for microcarrier structural integrity and expansion of induced pluripotent stem cells', Cytotechnology (DOI:10.1007/s10616-014-9750-z).

- Ismadi, M.; **Gupta, P.**; Fouras, A.; Verma, P.; Jadhav, S.; Bellare, J.; Hourigan, K. (2014), ' Flow characterization of spinner flask for induced pluripotent stem cell culture application', PLOS One (DOI: 10.1371/journal.pone.0106493).

Poster Presentation

- **Gupta, P.**; Jadhav, S.; Bellare, J.; Hourigan, K.; Verma, P.(2012), ' Comparison of tropoelastin with gelatin and matrigel as attachment matrix for mouse embryonic stem cells', World Congress on Biotechnology, Hyderabad, India, 4th to 6th May 2012.

To be Submitted

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