The Pennsylvania State University

The Graduate School

Department of Bioengineering

DEFINED AND XENO-FREE CULTURE FOR HUMAN PLURIPOTENT STEM CELLS

A Thesis in

Bioengineering

by

Tzu Ting Lin

Submitted in Partial Fulfillment

of the Requirements

for the Degree of

Master of Science

May 2017

The thesis of Tzu Ting Lin was reviewed and approved* by the following:

Xiaojun Lian
Assistant Professor of Biomedical Engineering
Thesis Advisor

Justin L. Brown
Associate Professor of Biomedical Engineering

William O. Hancock
Professor of Biomedical Engineering
Head of Graduate Program

*Signatures are on file in the Graduate School

ABSTRACT

Owing to their ability to differentiate into all cell types in body, and therefore greatly impact the landscape of regenerative medicine and tissue engineering, the human pluripotent stem cells (hPSCs) including human induced pluripotent stem cells (hiPSCs) and human embryonic stem cells (hESCs) are of great interest to researchers in recent decades. One of the combating issues is the development of a robust stem cell culture environment to maintain stem cells without differentiation for long-term culture, in other words, to support their self-renewal and pluripotency. Moreover, because hiPSCs and hESCs have practical use in in-vivo study, for such a clinical application, it is necessary to establish a chemically defined, feeder-free culture system to maintain large scale undifferentiated stem cells in-vitro. Various methods have been proposed for maintaining large scale stem cell culture Some major considerations are: culture media, extracellular matrix, and environment cues. This study will be focused on developing and analyzing a new xeno-free stem cell culture medium, LaSR, for supporting long-term proliferation and pluripotency for both hiPSCs and hESCs to facilitate the basis of stem cell research.

TABLE OF CONTENTS

LIST OF FIGURES

LISTS OF TABLES

ACKNOWLEDGEMENTS

First, I would like to thank Dr. Xiaojun Lian for allowing me to conduct research in his lab, for teaching me lab techniques, for his help in the completion in my thesis, and for his guidance and counseling during my master's degree.

I would like to thank the committee members, Dr. Justin L. Brown and Dr. William O. Hancock, for agreeing to be a part of my thesis committee and their guidance in the completion of my thesis.

I would like to thank Lauren N. Randolph for her help with conducting the experiments, analyzing data, and teaching me the techniques necessary for success in the lab. Finally, thank you to my family in Taiwan for their support and comfort.

Chapter 1

Background

1.1 Human stem cells in culture

Since the first human embryonic stem cell (hESCs) line was described[1], several hundreds of stem cells lines including hESCs and human induced pluripotent stem cell (hiPSCs)[2] have been derived and investigated for their key signaling pathways and transcription factors which are related to self-renewal and lineage differentiation. Nowadays, three types of stem cells are commonly cultured in laboratory settings: ESCs, iPSCs, and adult stem cells such as mesenchymal stem cells. Stem cells hold promising hope for cell therapies and regenerative medicine with the ability to differentiate into all types of somatic cells.

Embryonic stem cells are stem cells derived from inner cell mass of an embryo. They are able to self-renew and be passaged for long term culture under appropriate culture conditions. The use of hESCs is hindered since the generation of embryonic stem cells requires destruction of blastocysts, which is controversial and facing ethical issues. Induced pluripotent stem cell are somatic cells reprogrammed back to pluripotent state by delivering certain sets of transcription factors such as Klf4, Oct4, Sox2, and c-Myc[2]. HiPSCs mimic hESCs with the ability to give rise to every cell type in the body. They also represent promising cell source

for therapeutic applications in the fact that patient-specific stem cells can be derived and applied[3]. Since hiPSCs can be derived from somatic cells, they can be made in a patient-specific manner, and bypass the need for embryo, which means each patient can have their own stem cell line for clinical application. Moreover, hiPSCs that have specific disease phenotype can contribute to develop personalized drug and understanding patient-specific basis of disease[3].

Adult stem cells are multipotent somatic stem cells found in differentiated tissues in human body, like blood or bone marrow. They are responsible for replenishing damaged or dying cells and regenerate damaged tissues from where they originate[4]. Unlike hESCs or hiPSCs, those cells only give rise to certain distinct cell types, which is termed multipotency[4]. In practice, these adult stem cells have limited self-renewal ability compared to hESCs and hiPSCs, and are very hard to be isolated from body.

Despite the different origins of the three types of stem cells, the current challenges with regards to stem cell culture conditions are roughly the same. Culture conditions dictate stem cell fate to some extent. In-vivo, hESCs are in a specialized dynamic microenvironment called stem cell niche[5]. In-vitro culture settings are supposed to mimic in-vivo stem cell niche to provide appropriate cues that govern stem cell fate. Cultured stem cells are subjected to an environment that main components are: medium, extracellular matrix, atmosphere, and cell-cell interaction. Each of these components combines to form a complicated network of signaling pathways that determine stem cell fate. The increasing interest in

optimizing stem cell culture methods has led to the evolution of culture conditions to meet the need of regenerative medicine and disease modeling.

1.2 Important factors involved in hPSCs culture

The proliferation of any type of mammalian cells in vitro relies on three key factors: cell culture media, extracellular matrix (ECM), and environmental cues[5] (Figure 1.1). HPSCs require different culture conditions from murine ESCs. Mouse embryonic stem cells (mESCs) rely on BMP4 and Stat3 signaling and leukemia inhibitory factors (LIF) to maintain self-renewal[6]. However, hPSCs depend on the interaction between different signaling pathways such as fibroblast growth factor (FGF-2), Noggin, Activin/Nodal, and TGF-β pathways in the absence of LIF[5,7,8,9,10,11]. Moreover, mESCs can be passaged and grow as single cell, but hPSCs lose viability after dissociation as single cell. Therefore, hPSCs are usually plated and grown as colonies. The intrinsic difference between mESCs and hPSCs leads to different consideration of culture conditions to support hPSCs proliferation in vitro[12]. To successfully culture hPSCs and maintain their pluripotency in vitro, it is critical to consider the combination of culture medium, extra cellular matrix, and environment cues. Ideally, a new stem cell culture protocol can be made based on one or in combination of the three contributing factors.

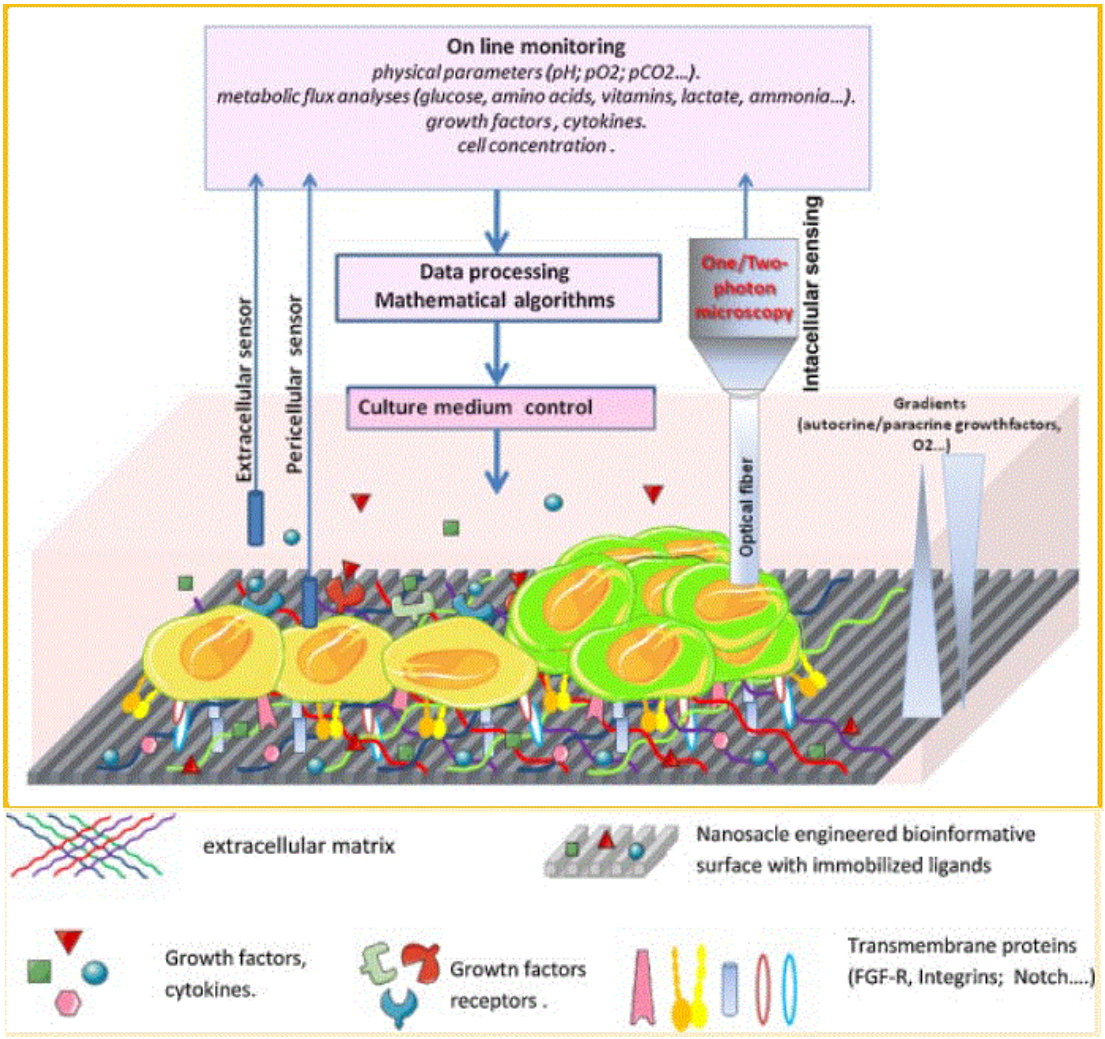

Figure 1.1 Stem cell culture environment. Culture medium, substrate, and environmental cues such as oxygen gradient are contributing to stem cell fate[31]. Figure adapted from Sanden et al. (31).

1.3 Culture medium

There is growing interest in optimizing stem cell culture, not only because cell culture is the basis of basic stem cell research in laboratory, but also due to the possible clinical applications of pluripotent stem cells in human body. Growth medium is one of the most critical components for stem cell culture, and it also has experienced dramatic evolution since it was first developed for human embryonic

stem cell culture[1]. In the beginning of stem cell culture, one of the biggest challenges was to determine the necessary components in a culture medium to support undifferentiated hPSCs. Researchers have been spending a lot of efforts trying to understand the key signaling pathways involved in the process for developing a robust culture medium.

It is very clear that culture medium dramatically influences cell proliferation and cell fate. Stem cell fate is always balanced between proliferation and differentiation. Therefore, the component a of stem cell culture medium is usually determined by its ability to support self-renewal and pluripotency. In other words, the medium is required to maintain a large-scale undifferentiated hPSCs as well as to promote their self-renewal. Stem cells from different sources are diverse. Different stem cell type might require distinct culture conditions owing to different signaling pathways. Thus, it is impossible to develop an universal stem cell culture medium.

Furthermore, some other general considerations are also required. Stem cell culture conditions must allow propagation of normal phenotype and karyotype cells. Culture conditions need to be well established and avoid any undefined matrix and medium component such as serum, for therapeutic applications. The earliest version of culture medium for human embryonic stem cell was composed of fetal bovine serum (FBS)[5], which is an undefined component secreted by mouse embryonic fibroblasts.

1.3.1 From feeder layer to defined culture system

When several hESC lines were first brought to culture, cells were maintained on feeder layers such as mouse embryonic fibroblasts (MEFs) in medium containing serum. Feeder cells secret growth factors and extracellular matrix to support stem cell growth. However, it is extremely difficult to determine which components are present in the medium due to the undefined secretion of these factors by different feeder cells. The preparation of feeder cells takes lots of time and efforts. Moreover, feeder cells and their secretion products might be a source of pathogens for stem cells which raises concerns when it comes to therapeutic applications. For example, it has been reported that immunogenic sialic acid (Neu5Gc) is present in the co-culture of hESCs and MEFs with serum replacements[13]. Thus, the undefined culture condition created by feeder cells impairs the potential use in clinical application, and must be replaced by more defined components.

Serum is also not fully defined since there are too many variabilities between different batches of serum products. Nowadays, more and more efforts have been focusing on the development of fully-defined medium by providing key growth factors or cytokines without the need of feeder cells (Figure 1.2).

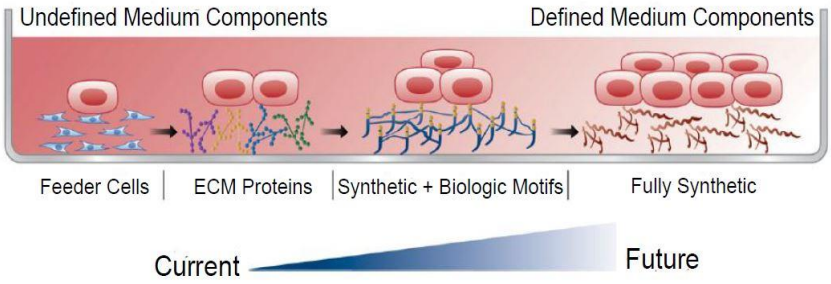

Figure 1.2 Evolution of stem cell culture condition. The in-vitro culture of stem hiPSCs has evolved from relying on feeder cells to defined, xeno-free medium components. Figure adapted from Villa-Diaz et al. (21).

1.3.2 Critical signaling pathways

Nowadays, serum-free medium supplemented with several growth factors and cytokines is used in most stem cell culture. One of the critical factors added to culture medium is bFGF, which facilitates to culture undifferentiated hESCs and hiPSCs[14]. Different cell lines may require different growth factors. For example, LIF is important for the proliferation of mouse ESCs, but has no effects for human stem cells[12]. Other soluble factors involved in stem cell culture include BMP family proteins, which can contribute to hESCs differentiation[15]. Nodal, activin A, and TGF-β are used to promote self-renewal of hESCs by the inhibition of BMP signaling[16]. Small molecules such as retinoic acid, hormones, and CHIR99021 are used in differentiation medium to promote stem cell differentiation towards certain pathway[5], likewise, there are many signaling molecules that can support undifferentiated stem cell population remaining undiscovered. Inhibition of GSK3 signaling has been shown to maintain pluripotency state of mouse and human embryonic stem cells[17].

1.3.3 Growth medium

Considering that cell culture is dynamic and changing due to the secretion and release of metabolites by cells, it takes plenty of time to achieve fully defined culture conditions. In recent years, researchers have been using more chemically defined medium to replace xenogeneic components. The first culture medium termed TeSR1 containing FGF-2, lithium chloride (LiCl), γ-aminobutyric acid

(GABA), TGF-β ,and pipeolic acid that supports feeder-free stem cell culture was developed by Thomson and colleagues[18]. More recently, Thomson and colleagues developed a chemically defined medium termed E8 medium, which is derived from TeSR containing 8 components and lacks serum albumin and β-mercaptoethanol. This E8 medium is reported to successfully support undifferentiated human stem cells including hESCs and hiPSCs during daily passages[19,20].

1.4 Extracellular matrix

Cell-matrix interaction plays an important role in regulating stem cell fate including self-renewal, differentiation, and pluripotency. Extracellular components include organic matrix derived from animal cells, hydrogel, matrix proteins, synthetic chemicals, and some are commercial available products[5].

1.4.1 Matrigel

Matrigel is now the most widely used coating on tissue culture plate for feeder-free stem cell culture. It is a basement membrane matrix composed of laminin, collagen, heparan sulfate proteoglycan, and undefined growth factors. However, Matrigel is produced from mouse Engelbreth-Holm-Swarm (EHS) sarcoma and ECM proteins derived from mouse embryonic fibroblasts[21], and therefore limits the clinical application of hPSCs. Thus, efforts have been focused on the study of ECM proteins to replace Matrigel for stem cell culture. It should be

noted that Matrigel cannot be used to culture clinical-grade hPSCs. Many different ECM have been reported to successfully culture hPSCs in vitro.

1.4.2 Alternative ECM proteins

Both natural proteins and synthetic biomaterials have been proposed recently trying to generate a defined hPSC culture environment. ECM proteins such as laminin, collagen and fibronectin have been proved to maintain hPSC self-renewal[21]. However, not all of them are suitable for hPSC cultivation. Stem cell culture typically involves two issues: first, the ability to promote proliferation, and, secondly, the ability to maintain pluripotency during cell culture. People have studied different types of ECM proteins such as collagen, fibronectin, and many other synthetic biomaterials. They are shown to be able to promote stem cell proliferation, however, they fail to support undifferentiated stem cell population, thus not an ideal candidate.

1.4.3 Laminin isoforms

Among different types of ECM, Laminin draws most attention because they are endogenously produced by both induced human pluriotent stem cells (hiPSCs) and human embryonic stem cells (hESCs) to support self-renewal and pluripotency of hPSCs. Laminin contains α, β, and γ chains. There are five α chains, three β chains, and three γ chains, and laminin is named by its various combination of each chain (α1-α5, β1-β3, γ1-γ3). The different isoforms are synthesized in different cell types and have distinct functions. It is shown that α5 laminins are endogenously

synthesized by hPSCs and significantly promote hPSCs proliferation and survival[22]. Laminin-521 therefore has been used for stem cell culture coating recently[23].

A recently study has shown that α-5 laminin isoforms synthesized by undifferentiated hPSCs play a key role in regulating hPSCs survival[22], which is consistent with previous findings including laminin-511 is an important regulator during embryo development[24], and laminin-521 can support ideal stem cell culture. Knocking down of *LAMA5* gene by shRNA and Cas9-mediated disruption results in reduced hPSCs survival and significantly increased apoptosis. Additionally, the self-renewal and survival were rescued by culturing the *LAMA5* deficient hPSCs on exogenously laminin coating. Based on those studies, laminin α-5 isoforms present good potential to support undifferentiated hPSCs in vitro.

1.5 Environmental cues

There are several environmental cues including cues from both physical and physiological environments which affect stem cells growth and differentiation. For example, temperature, humidity, cell density, rigidity of culture plate, osmosity, oxygen diffusion velocity, and multicellular associations[5]. Among these environmental cues, oxygen is the one of the most important factors that influences stem cell growth.

Cell are usually passaged and having medium change in a laminar flow hood, and being maintained in incubators under normoxic oxygen condition.

The physiological environment of mammalian embryo is hypoxic. Stem cell culture condition is supposed to mimic the in vivo environment in order to reconstruct stem cell niche. However, traditional stem cell culture has been implemented under normoxia (~21% O_2). Low O_2 tension can prevent spontaneous hESCs differentiation[25]. Moreover, physiological O_2 also facilitates hESCs recovery and reduces chances of abnormal chromosome. Therefore, optimal O_2 would be required to maintain healthy stem cells as well as helping the expansion of stem cell population without unwanted spontaneous differentiation.

1.6 Goal of this study

Owing to their ability to impact the landscape of regenerative medicine and tissue engineering, the human pluripotent stem cells including induced pluripotent stem cells and human embryonic stem cells are of great interest to researchers. One of the combating issues is the development of a good stem cell medium for prolonged culture and maintenance of the stem cells without further differentiation into any cell type. Because hiPSCs and hESCs have practical use in in-vivo study, it is important to be able to culture these cells in animal-contamination free medium to avoid the transmittance of animal-pathogens, and induce immunogenic responses. Therefore, this study considers the development of a xeno-free culture medium for hiPSCs and hESCs for long term culture and we analyze this new medium against existing xeno-free media, Nutristem. Glutamax modulation was also conducted for medium optimization.

Chapter 2

Methods

2.1 Maintenance of human iPSCs and human ESCs

Human embryonic stem cells (H9, H7)[1], and transgene and vector free human iPSCs (19-9-7)[2] were maintained at 37°C/5% CO_2 on Matrigel (Corning) coated tissue culture plates in LaSR medium. Matrigel plate was coated with a 1:100 dilution using DMEM/F12 (Life Technologies) and incubated at 37°C for 30 minutes prior to use. Cells were routinely passaged with 0.5mM EDTA in PBS. In brief, cells were washed twice with EDTA solution, then incubated with EDTA at 37°C for 10 minutes. Then EDTA was removed, and cell suspension was collected.

2.2 Cell counting

All experiments were done on 6-well tissue culture plates. Before starting cell counting, cells were adapted to the new medium for 1 passage. Cell were dissociated from the plate with 0.5mM EDTA on the day of counting, then 10 µl of cell suspension was subjected to hemocytometer for counting. Cell viability assay was performed by adding 10 µl of Trypanblue to the 10 µl cell suspension, then 10 µl of the mixed solution was subjected to hemocytometer for counting.

2.3 Flow cytometry analysis

To analyze Oct4 and Nanog expression, cells were dissociated into single cell suspension using 0.5mM EDTA in DPBS for 10 min at 37ºC and then fixed with 1% paraformaldehyde for 20 min. at room temperature. Cells were stained with primary and secondary antibodies in DPBS with 0.1% Triton X-100 and 0.5% BSA. FACS gating was based on the corresponding isotype antibody control.

2.4 Immunostaining

Cells were fixed with 4% paraformaldehyde for 15 min. at room temperature and then immunostained using primary and secondary antibodies in DPBS with 0.4% Triton X-100 and 5% non-fat dry milk. Nuclei were stained with Hoechst 33342. A Nikon TI Eclipse epifluorescence microscope was used for imaging analysis.

2.5 Statistical analysis

All results are represented as means ± SE. Students' T-test is used for comparisons between two groups.

Chapter 3

Results

In this chapter, we first compared LaSR medium to a commercially available stem cell culture medium, Nutristem, to evaluate LaSR medium in terms of the ability to support stem cell self-renewal. Next, we removed one of the components, Glutaxmax from LaSR, trying to examine the effect of Glutamax in the medium for supporting stem cell culture, and minimize the formula of LaSR. Last, we developed a xeno-free version of LaSR by replacing bovine serum albumin in the medium by human recombinant albumin, human serum albumin, and chemically lipid concentrate step by step. Cells cultured in different medium were analyzed for pluripotency markers by immunostaining and flow cytometry.

3.1 Comparison study between LaSR and Nutristem

LaSR basal medium was first developed by previous work to be used for endothelial differentiation[26]. Here, we added two growth factors together with Glutamax and vitamin C to the LaSR basal medium to form a new stem cell culture medium, which is termed LaSR. To evaluate its ability to support stem cell proliferation as well as maintaining stem cell pluripotency, we compare the proliferation rate of hiPSCs cultured in LaSR medium with the hiPSCs cultured in Nutristem (Biological Industries), which is designed to support long-term culture of hiPSCs and hESCs. HiPSCs were seeded onto a 6-well tissue culture plate

with the same seeding density on day 0 for each condition, and they were cultured in each condition for one passage to be adapted to the new medium before counting. Cells cultured in both conditions exhibited normal cell morphology, which includes uniform colonies of tightly packed cells and distinct colony edges (Figure 3.1).

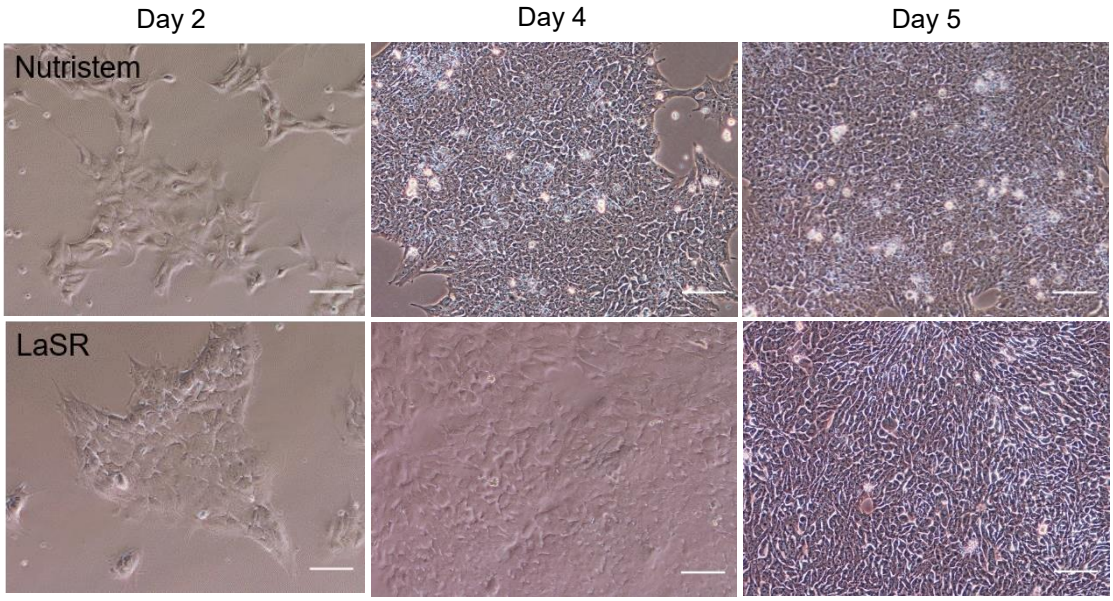

Figure 3.1 Micrographs of iPSC colonies in LaSR medium (Upper panel), and Nutristem medium (Lower panel) 2, 4, 5 days after seeding at passage 3. Scale bars are 100 µm.

Then cell counting was done on day 2, 4, 5 after seeding. The cell counting data have shown that hiPSCs' growth rates in LaSR were similar to those seen when cells were cultured in Nutristem (Figure 3.2). They reached the similar cell number at the end of each passage, and the growth curves fit what we expect to see for the hiPSCs. This suggests that LaSR has the same ability as Nutristem to support stem cells proliferation, and can be used for long-term cell culture.

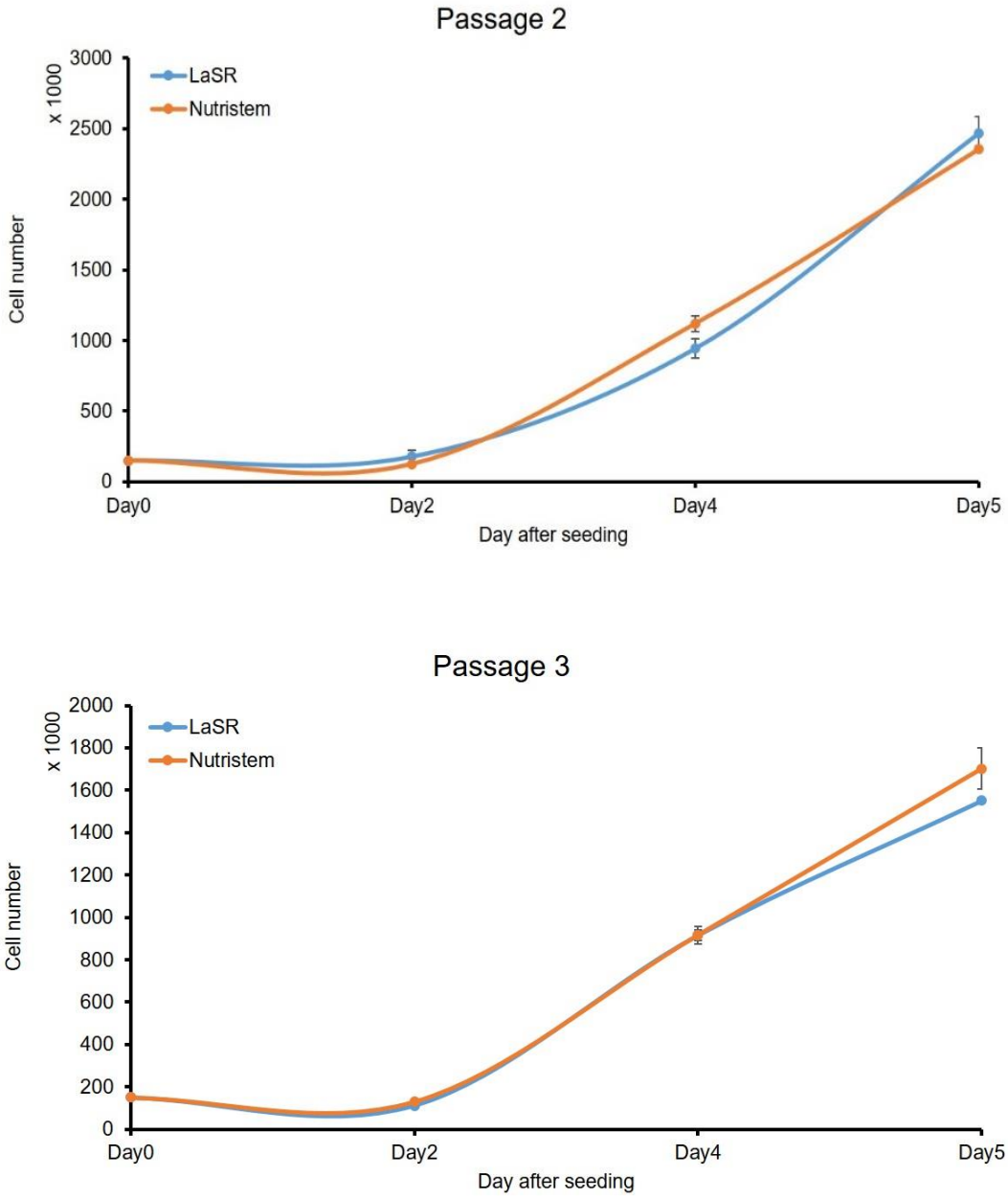

Figure 3.2 Growth curves of iPSC cultured in LaSR and Nutristem medium at passage 2 and 3. Cultures are seeded at 1.5 x 10^5 cells/well. Error bars represent standard error of the mean for each day. n=2.

3.2 Optimization of LaSR by removal of Glutamax

Glutamax is an improved direct substitute for L-glutamine, which is an important nutrient in cell culture. It is more stable than L-glutamine and does not degrade in aqueous solutions. To understand its effect in the regulation of stem cell proliferation, and try to simplify the formula of LaSR, we removed Glutamax from the LaSR medium, and compared the proliferation growth rate of cells cultured in LaSR medium with the cells cultured in LaSR without Glutamax medium. Again, the cells were cultured in each condition for one passage to be adapted to the new environment. The cell morphology was not affected by the removal of Glutamax (Figure 3.3).

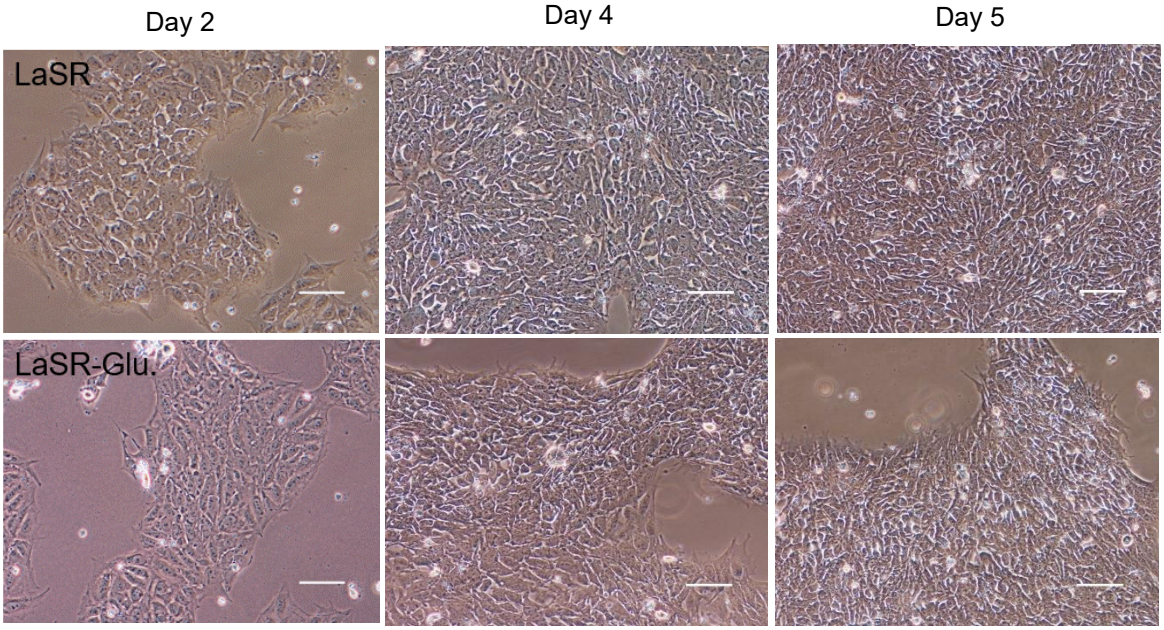

Figure 3.3 Micrographs of iPSC colonies in LaSR medium (Upper panel), and LaSR without Glutamax medium (Lower panel) 2, 4, 5 days after seeding at passage 6. Scale bars are 100 µm.

Cell counting was done on day 2, 4, 5 after seeding for 5 passages. The absence of Glutamax significantly affected cell proliferation rate (Figure 3.4, 3.5).

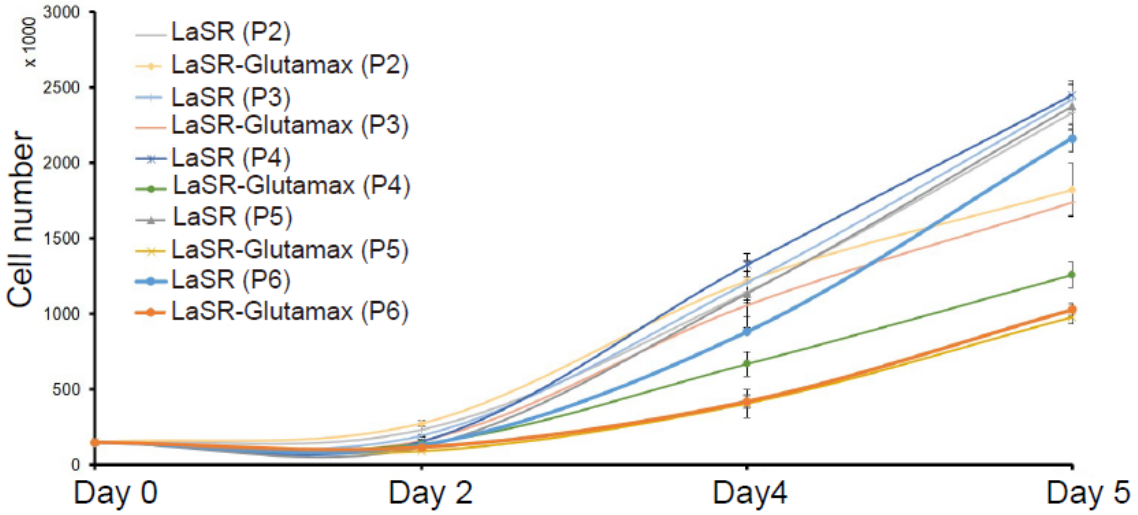

Figure 3.4 Growth curves of iPSC cultured in LaSR and Nutristem medium from passage 2 to 6. Cultures are seeded at 1.5 x 10^5 cells/well. Error bars represent standard error of the mean for each day. n=2.

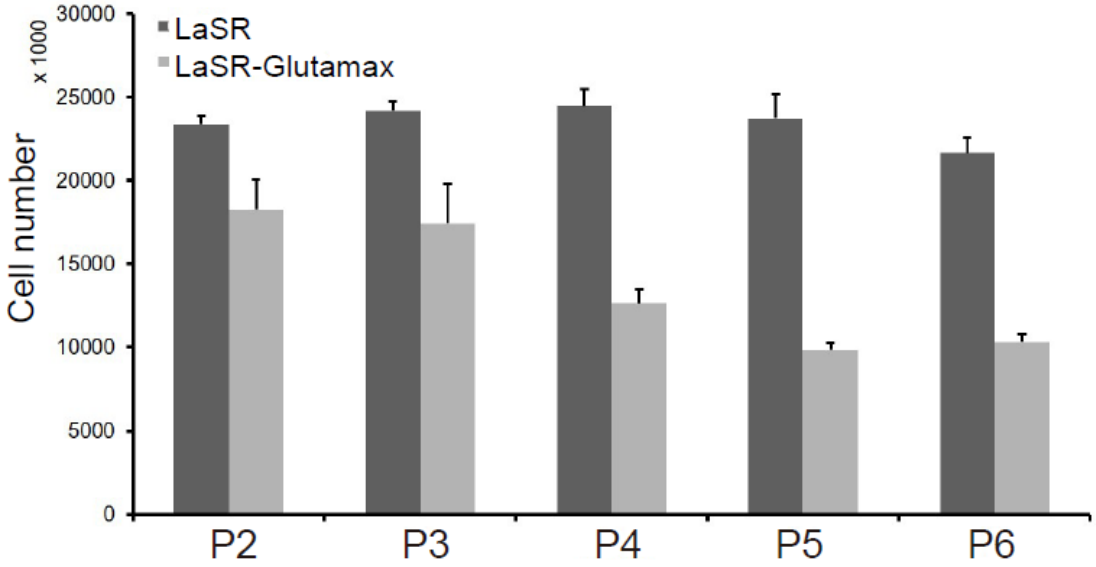

Figure 3.5 Cell counting number on day 5 of iPSC cultured in LaSR and LaSR without Glutamax medium. Error bars represent standard error of the mean for each day. n=2.

The cell numbers at the end of each passage for the cells cultured in LaSR without Glutamax medium were only about half of those cultured in LaSR. However, the removal of Glutamax did not affect cell survival at all (Table 1). This result shows that Glutamax is critical for the stem cell proliferation. It might have some beneficial effects to the cells, or it might cancel some toxic effects of other LaSR medium components. We then examined the pluripotency of cells by immunostaining against Oct4 for each condition. Data have demonstrated that both LaSR medium and LaSR without Glutamax medium supported undifferentiated human iPSCs culture (Figure 3.6).

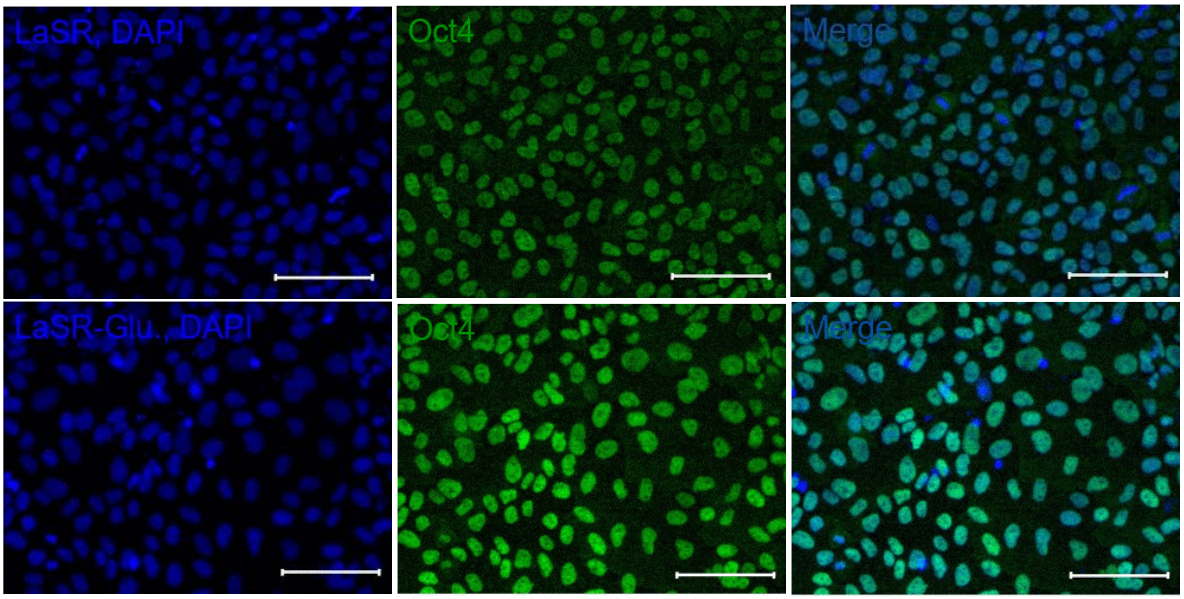

Figure 3.6 Immunostaining of pluripotency marker Oct4 (green) in iPSC cultured for 6 passages in LaSR (Upper panel), and LaSR without Glutamax medium (Lower panel). Individual cell nuclei were visualized using DAPI (blue). Scale bars are 100 µm.

3.3 Development of Xeno-free LaSR medium

Besides the ability to support robust undifferentiated stem cell population, we are also interested in developing a defined, xeno-free version of LaSR medium for clinical applications. We took a step-by-step approach to replace the undefined component in the LaSR medium. First, we replaced bovine serum albumin (BSA) by human recombinant albumin (HRA), and compared growth rate of human iPSCs cultured in LaSR -BSA medium with cells cultured in LaSR supplemented with HRA. Cells were seeded at a density of 1.5 x 10^5 cells/well in a 6-well plate. Then we counted the cell number on day 2, 4, 5 after seeding. Cells maintained in LaSR supplemented with 0.5 mg/mL HRA degenerated two days after medium was changed. In addition, removal of BSA significantly decreased the growth rate of human iPSCs (Figure 3.7).

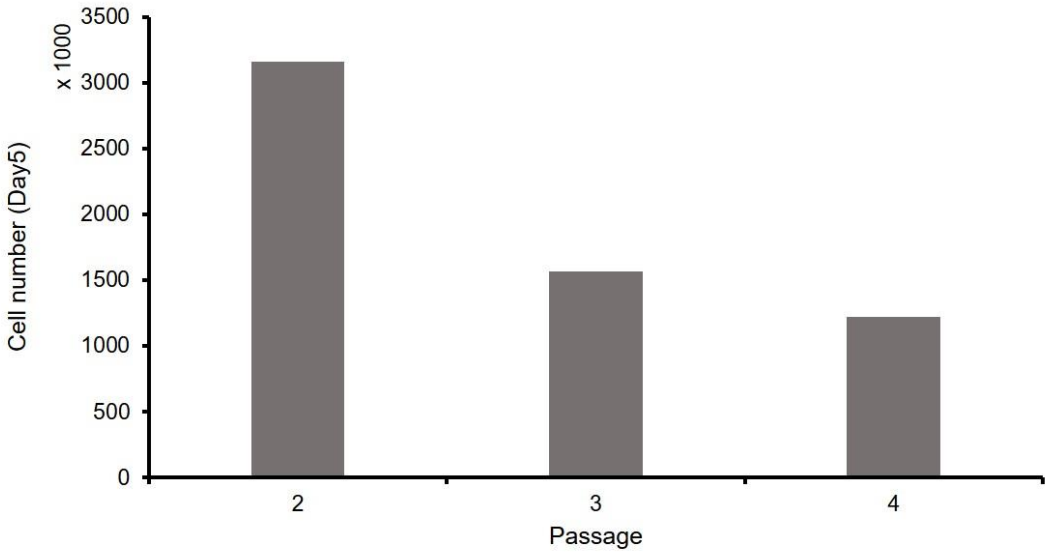

Figure 3.7 Cell counting number on day 5 of iPSC cultured in LaSR -BSA medium.

To re-examine the effect of HRA, we added 0.25 mg/mL, 0.5 mg/mL, and 2.5 mg/mL HRA back to the cells cultured in LaSR -BSA medium on day 2 after seeding in passage 4. 5 days after addition of HRA, tremendous cell death was observed. The cell morphologies were different from those maintained in LaSR including smaller colonies and many dead cells floating around (Figure 3.8). This suggests that HRA might be toxic to human iPSCs and does not support stem cell culture.

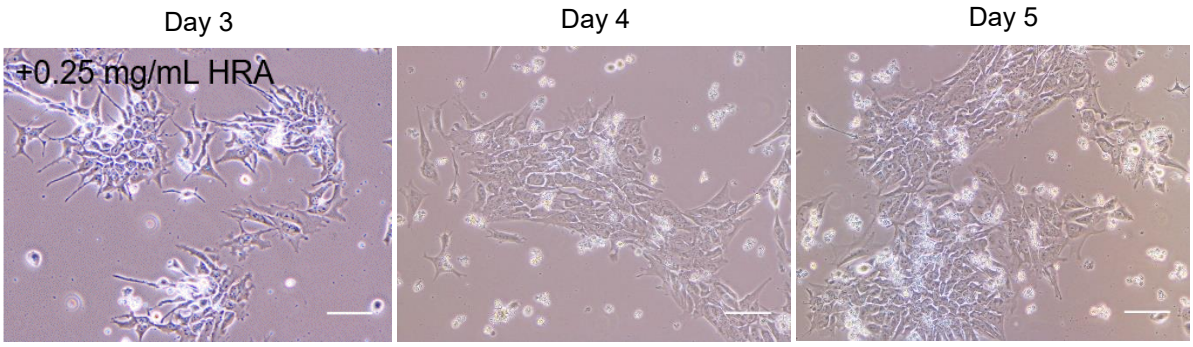

Figure 3.8 Micrographs of iPSC colonies in LaSR -BSA medium supplemented with 0.25 mg/mL HRA, 3, 4, 5 days after seeding at passage 3. Scale bars are 100 µm.

Next, we tested another human albumin substitute, which is human serum albumin (HSA) in the effort to replace BSA from the LaSR medium. We cultured human iPSCs and human ESCs in the LaSR medium in the absence of BSA, but supplemented with 0.4 mg/mL HSA. The cell morphology was not affected by the addition of HSA (Figure 3.9).

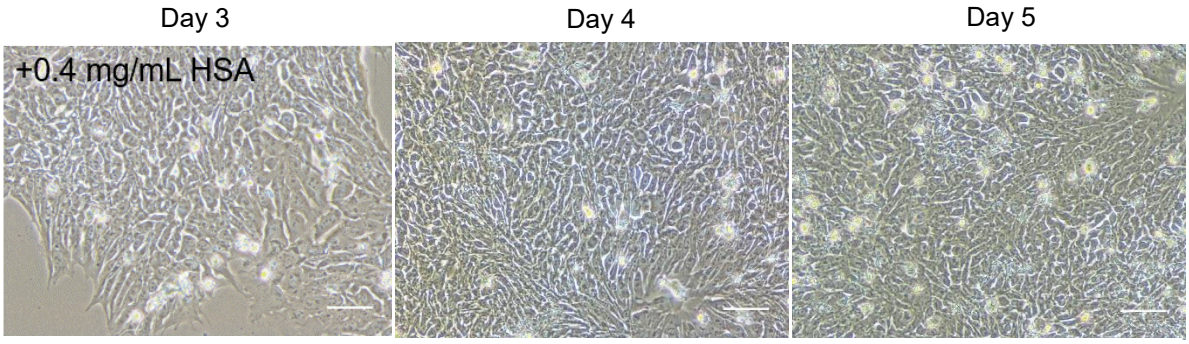

Figure 3.9 Micrographs of iPSC colonies in LaSR -BSA medium supplemented with 0.4 mg/mL HSA 3, 4, 5 days after seeding at passage 3. Scale bars are 100 µm.

Cell counting was done on day 2, 4, 5 after seeding for 5 passages. Cells exhibited robust proliferation comparable to cells cultured in normal LaSR (Figure 3.10).

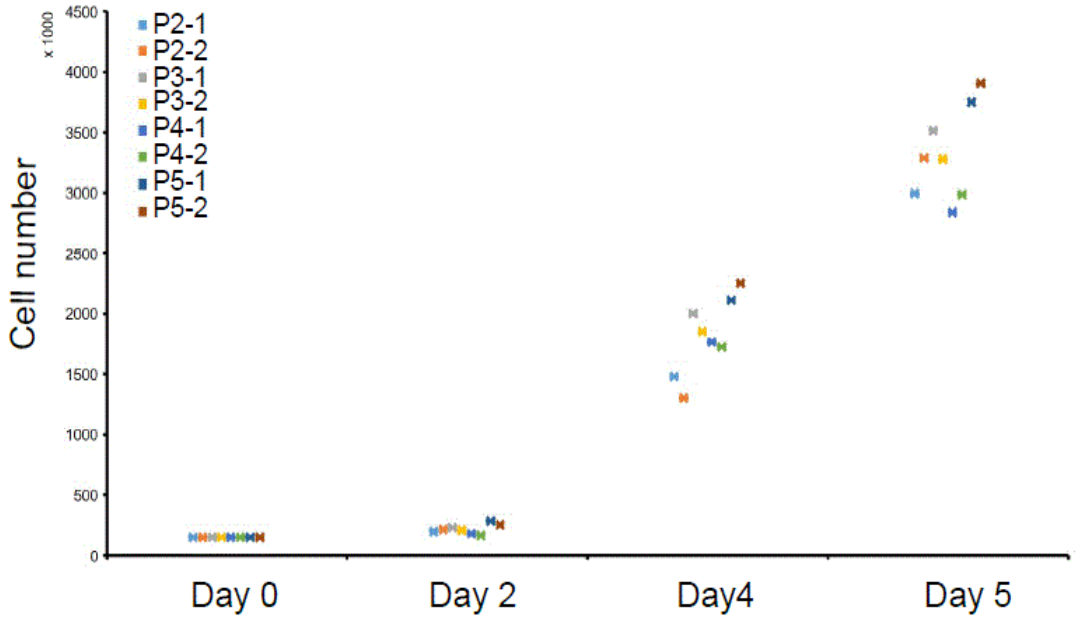

Figure 3.10 Cell number of iPSC cultured in LaSR -BSA medium supplemented with 0.4 mg/mL HSA on day 0, 2, 4, 5, from passage 2 to 5. Cultures are seeded at 1.5 x 10^5 cells/well. n=2.

The cells were further cultured to passage 14 to test whether LaSR HSA medium supports long-term hiPSCs cultivation. Immunostaining against Oct4 and Nanog in passage 6 and 14 confirmed the cells cultured in this medium were sill pluripotent (Figure 3.11).

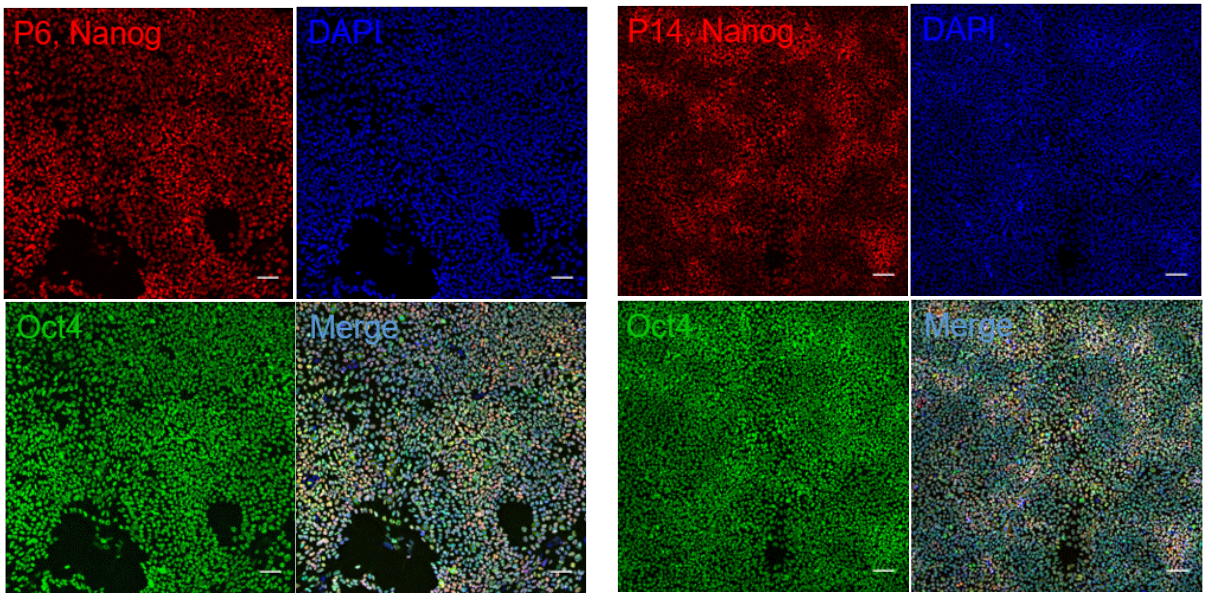

Figure 3.11 Immunostaining of pluripotency marker Oct4 (green) and Nanog (red) in iPSC cultured in LaSR +HSA medium at passage 6 (Left panel) and passage 14 (Right panel). Individual cell nuclei were visualized using DAPI (blue). Scale bars are 100 µm.

Flow cytometry was also performed to quantify the percentage of double-positive cells against Oct4 and Nanog. 79.1%, 79.3% of the cells were double-positive in passage 6 and 14, respectively (Figure 3.12). Overall, the data show that LaSR HSA medium can maintain long-term stable undifferentiated stem cell culture.

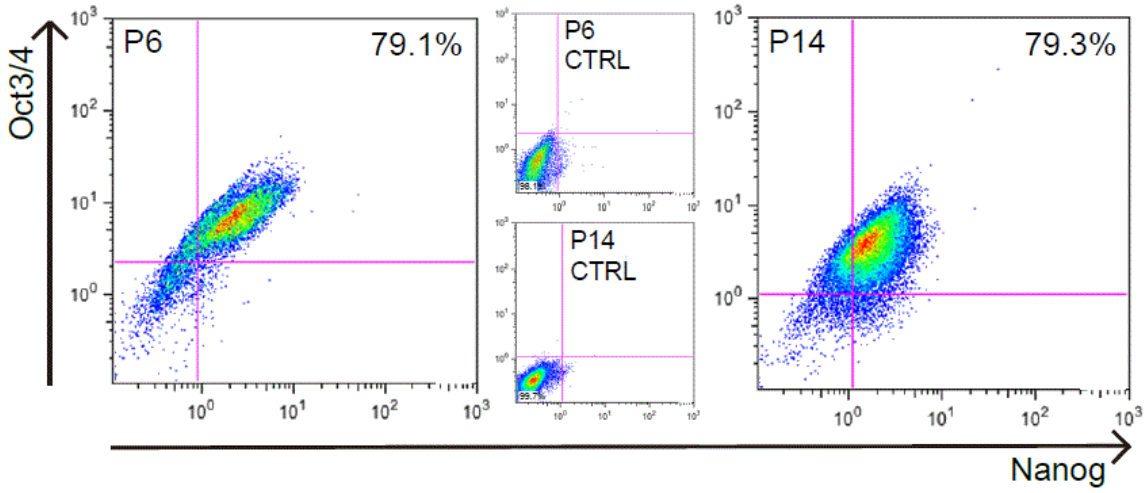

Figure 3.12 Flow cytometry analysis of Oct4 (y-axis) and Nanog (x-axis) expression in iPSC cultured in LaSR +HSA medium at passage 6 (Left panel) and passage 14 (Right panel).

3.4 Chemically defined lipids concentrate as a medium supplement

However, serum albumin varies from batch to batch, and what kinds of molecules present in the serum are unknown. Although we identified a human source albumin that can be supplemented in LaSR medium for long-term stem cell culture, the undefined components in the serum still hamper the possible clinical application of stem cells cultured in this medium. We sought to replace BSA from the medium by chemically defined lipid concentrate (Invitrogen). It is a combination of saturated and unsaturated fatty acids designed for a wide variety of applications (Table 2). We first cultured the human iPSCs in LaSR -BSA medium in the presence of different dilutions of lipids to test which dilution is most suitable for supporting stem cell culture. Each well had the same seeding density (150K cells/well) on day 0, and we compared the number of cells at the end of each passage.

It was obvious that lipids did have some beneficial effects to the cells by comparing the control with cells supplemented with lipids in terms of morphology and cell number (Figure 3.13). Immunostaining against Oct4 was performed, and cells cultured in LaSR +lipids medium were still pluripotent until passage 8 (Figure 3.14). Interestingly, randomly differentiation was observed for the cells cultured in the highest dilution of LaSR +lipids medium (1:100) starting from passage 5.

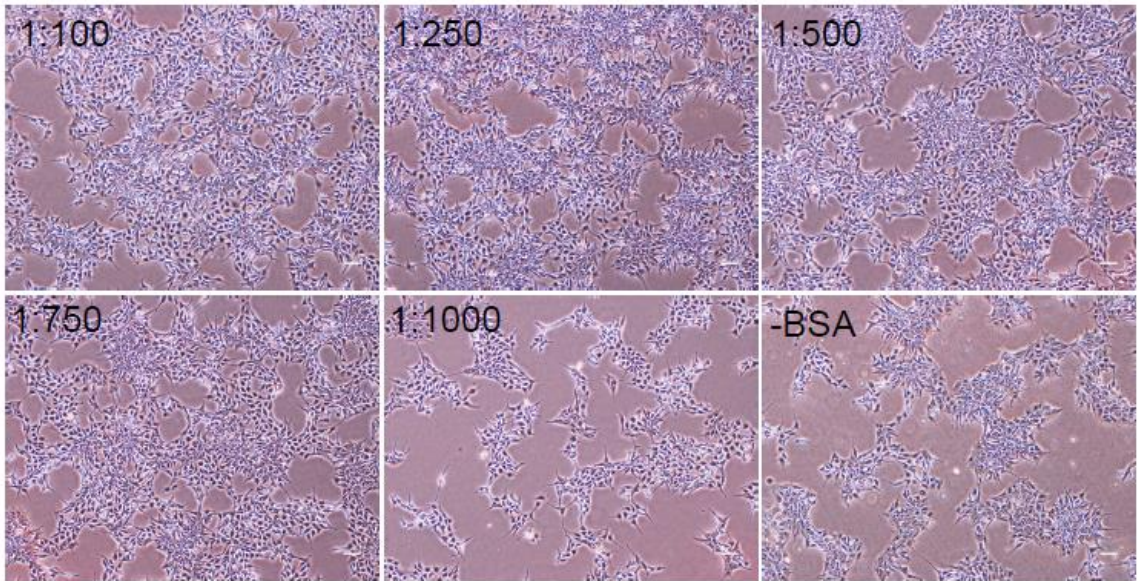

Figure 3.13 Micrographs of iPSC colonies in LaSR -BSA medium supplemented with 1:100, 1:250, 1:500, 1:750, 1:1000 dilution of lipid concentrate, and control (LaSR -BSA) 2 days after seeding at passage 4.

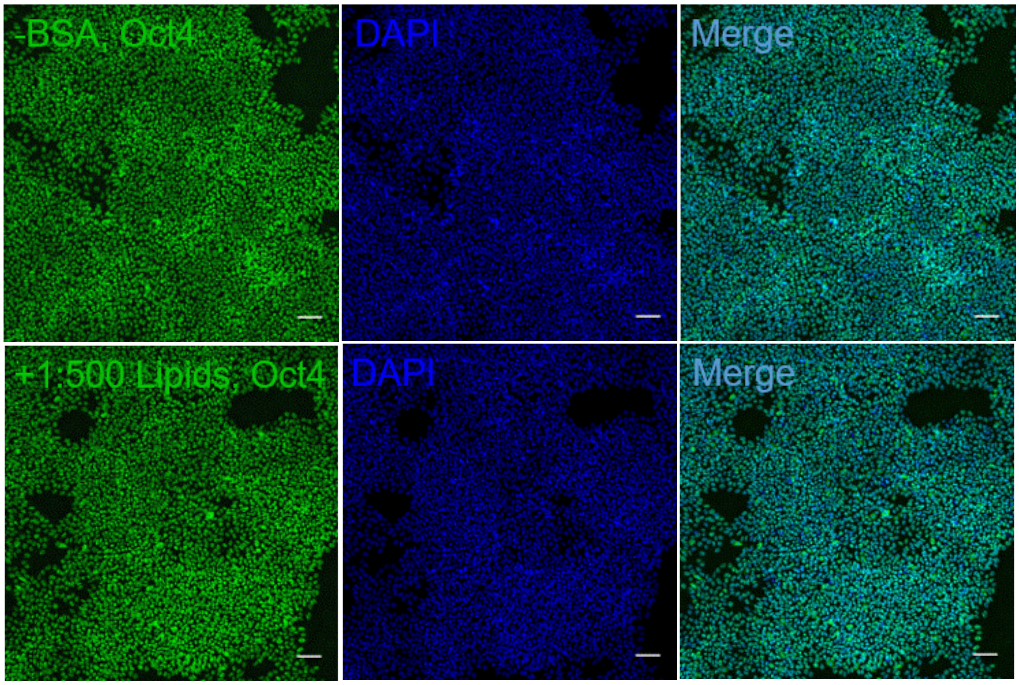

Figure 3.14 Immunostaining of pluripotency marker Oct4 (green) in iPSC cultured in LaSR -BSA medium and LaSR -BSA +1:500 dilution lipids medium at passage 8. Individual cell nuclei were visualized using DAPI (blue). Scale bars are 100 µm.

To re-examine the beneficial effects of lipids on proliferation rate of stem cells, we decided to choose 1:250 dilution of lipid concentrate as our target, which is also the same concentration as HSA we used. We compared the growth rate of cells cultured in LaSR -BSA medium with those cultured in LaSR +lipids medium. Each condition started with the same amount of hiPSCs, then cell counting was done at the end of each passage (day 4) to see whether addition of lipids can rescue cells in the LaSR without BSA medium or not. The cells cultured in the LaSR +lipids medium exhibited uniform cell morphology across the whole well (Figure 3.15).

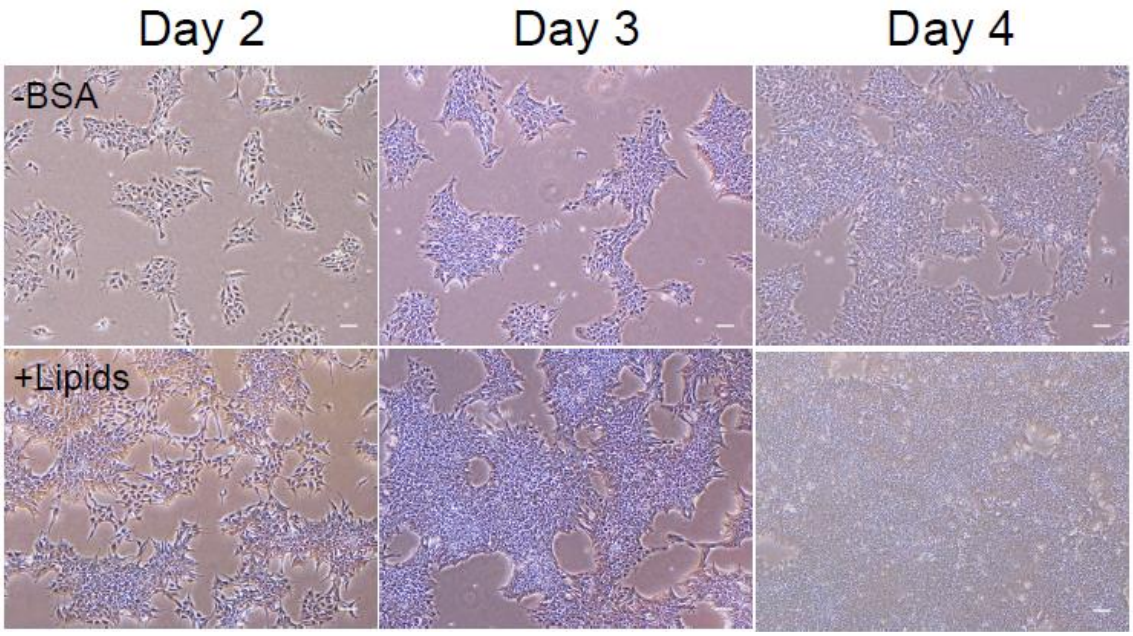

Figure 3.15 Micrographs of iPSC colonies in LaSR -BSA medium (Upper panel), and LaSR -BSA supplemented with 1:250 dilution of lipid concentrate (Lower panel) 2, 3, 4 days after seeding at passage 4. Scale bars are 100 µm.

Moreover, we found that the cell number significantly increased in the presence of lipids over 4 passages, showing lipids did have some beneficial effects on cell proliferation (Figure 3.16).

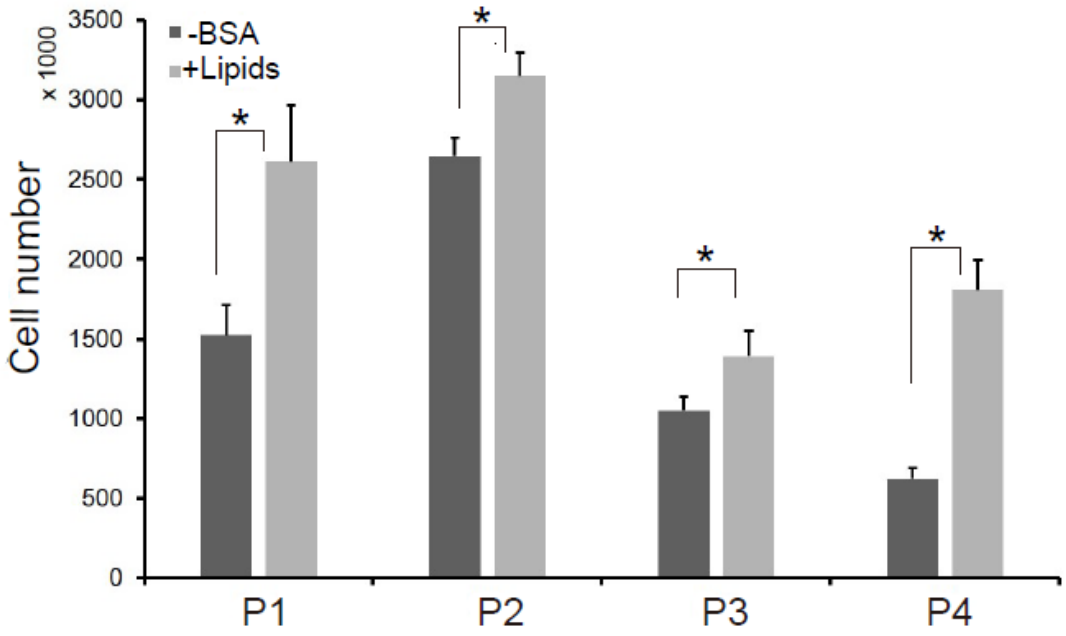

Figure 3.16 Cell counting number on day 5 (passage 1, 2), and day 4 (passage 3, 4) of iPSC cultured in LaSR -BSA medium versus LaSR + 1:250 dilution of lipid concentrate. Error bars represent standard error of the mean for each passage. *P < 0.05, LaSR -BSA versus LaSR -BSA+lipids; Student's *t* test. n=3.

Immunostaining against Oct4 and Nanog for the cells in LaSR-BSA and LaSR +lipids was performed, and both conditions were double-positive for the pluripotency markers (Figure 3.17).

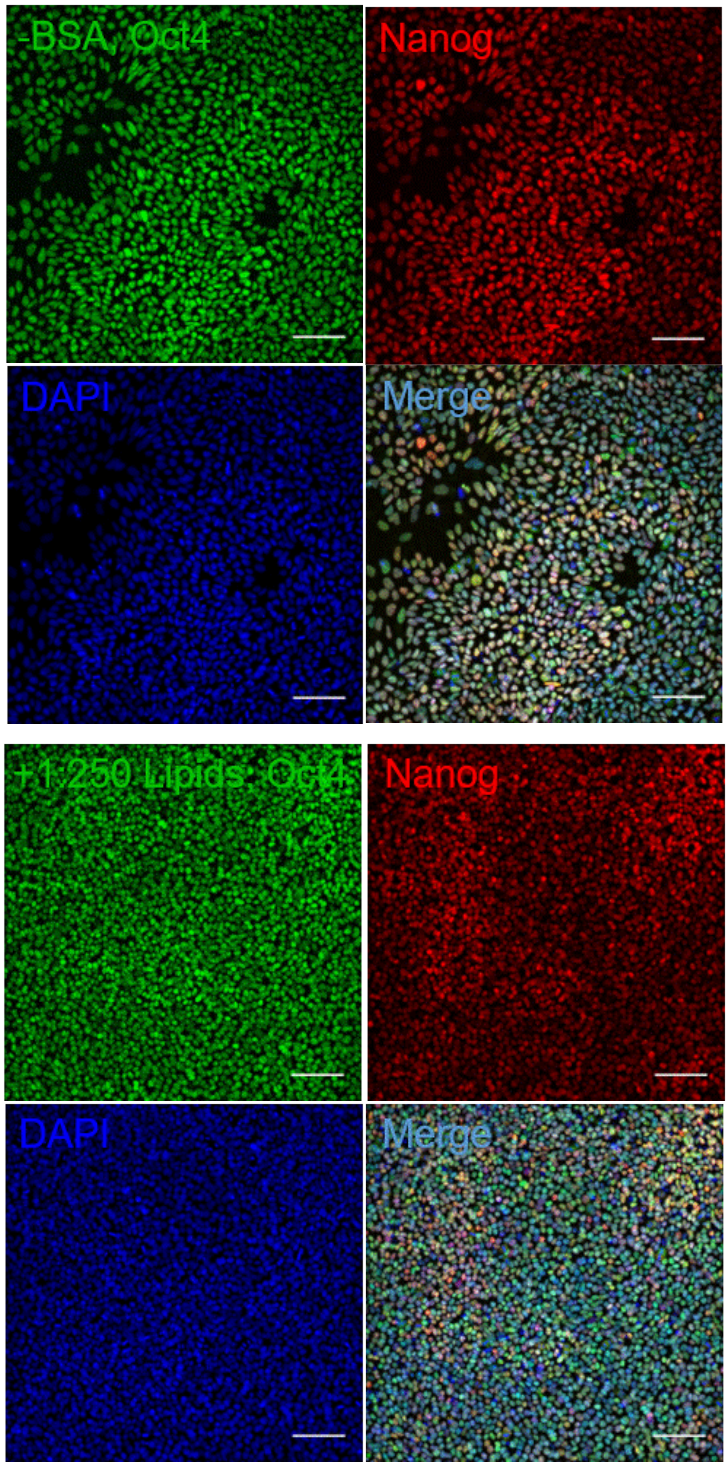

Figure 3.17 Immunostaining of pluripotency marker Oct4 (green) and Nanog (red) in iPSC cultured in LaSR -BSA medium and LaSR -BSA + 1:250 dilution of lipid concentrate. Individual cell nuclei were visualized using DAPI (blue). Scale bars are 100 µm.

Chapter 4

Discussion, Conclusion, and Future Work

4.1 Discussion

Previous study has claimed Glutamax can decrease blastocyst apoptosis and prolong the shelf life of culture medium to at least 1 year[27]. By removal of Glutamax from LaSR, we have shown Glutamax is an essential component in our medium, cells without Glutamax gradually lose their ability to proliferate as normal. A research group has proposed that glutamine is essential for survival of human iPSCs, because their energy production relies mainly on glutamine oxidation[28]. Our initial hypothesis was that the deprivation of Glutamax will make human stem cells enter dormant state, where they stop proliferation but still maintain their life, and can be awakened simply by the addition of Glutamax into culture medium. This concept of dormant state could facilitate the knowledge of maintaining stem-cell-derived progenitor cells. More work needs to be done to investigate the initiation of dormant state solely by small molecules for the human stem cells.

We found that the addition of HRA cannot support stem cell culture, whereas HSA can successfully support long-term stem cell culture. The major difference between the two human sourced of albumin is that HRA is a purely recombinant protein that only contains albumin itself. However, HSA is derived from human serum, and undefined lipids or other unknown molecules may be collected as well. It has been suggested that albumin-associated lipids promote human ESCs self-renewal, not the albumin itself[29]. The result of this study is consistent to what we observed when we tested both HRA and HSA in the role of regulating stem cell proliferation, and explains the reason why HRA fails to maintain stem cell self-renewal.

Random differentiation was observed when cells were cultured in the LaSR supplemented with 1:100 dilution of lipid concentrate, which is the highest concentration we have tested. This may suggest that the high concentration of lipid concentrate could activate some signaling pathways related to stem cell differentiation. The concentration of culture medium supplements must be tested carefully to avoid unwanted side effects such as random differentiation and karyotype abnormality.

4.2 Conclusion

We have successfully demonstrated that both human iPSCs and human ESCs cultured in LaSR medium exhibited comparable growth with cells cultured in Nutristem, which is a widely used serum-free medium for stem cell culture. In addition, cells maintained pluripotency and normal morphology through long-term cell culture. The cost of stem cell culture medium could be reduced by our LaSR medium, which has less growth factor and cytokine components. This advantage will facilitate the research of stem cell-based therapies for a variety of diseases.

The xeno-free LaSR medium was developed by replacing BSA by lipid concentrate. Our data show the XF-LaSR has the ability to maintain hiPSC self-renewal and pluripotency through long-term passage, which are the two most important characteristics of stem cells. This medium can be used to culture stem cells that are meant to be used for clinical applications. Due to the low cost and robust ability to support stem cell proliferation, we have been using regular LaSR medium for all routine culture in the lab. In other words, we have developed two

versions of LaSR: regular LaSR and XF-LaSR. They can serve for different purposes. If xeno-free and chemically defined culture environment is not required, regular LaSR can be used for routine cell culture for a lower cost of medium, whereas the XF-LaSR is a defined medium that can be used to generate clinical-grade human stem cells.

4.3 Future Work

As we have shown, HRA does not support stem cell proliferation, and causes rapid cell death no matter what concentration we use. This is surprising to us since HRA is not a toxic molecule to stem cells. Even though HRA itself is not able to promote stem cell proliferation, it should not kill the cells. More work needs to be done to investigate the signaling pathways triggered by the treatment of HRA in order to fully understand the mechanisms involved. This knowledge may allow us to get better understanding of HRA for developing a new medium.

Extracellular matrix is also a critical factor impacting stem cell fate. For the generation of clinical-grade human stem cells, it is required to consider both the medium component and the ECM we used to culture the cells. Matrigel is a most

widely-used ECM protein for routine stem cell culture in-vitro. However, it contains undefined molecules from animal source. More work needs to be done to find a more appropriate ECM protein which can allow long-term propagation of human stem cells. It has been reported that Laminin isoforms can be used to culture stem cells in-vitro. However, the cost of Laminin is considerable. The cost for maintaining daily cell culture would be much higher if Laminin is used as a substrate. Chemically-defined synthetic material such as hydrogel may be a good alternative[30] since they are stable and made from standard procedures. Most importantly, synthetic substrates are fully defined and exhibit very little variation from batch to batch. Further considerations of the existing methods, medium, ECM components are required to generate clinical-grade human stem cells and their derivative tissues for the purpose of regenerative medicine and potential drug screening models.

APPENDIX

Table 1. Average cell viability values (LaSR vs. LaSR-Glutamax)

	Passage 2	Passage 3	Passage 4	Passage 5	Passage 6
LaSR	88%	92%	87%	86%	85%
LaSR-Glu.	96%	91%	85.5%	87%	86%

Table 2. Formulation of Chemically Defined Lipid Concentrate (Invitrogen)

Components	Concentration (mg/L)
Arachidonic Acid	2
Cholesterol	220
DL-alpha-Tocopherol Acetate	70
Ethyl Alcohol 100%	N/A
Linoleic Acid	10
Linolenic Acid	10
Myristic Acid	10
Oleic Acid	10
Palmitic Acid	10
Palmitoleic Acid	10
Pluronic F-68	90000
Stearic Acid	10
Tween 80®	2200

BIBLIOGRAPHY

1. Thomson, J. A. *et al.* Embryonic stem cell lines derived from human blastocysts. *Science* **282,** 1145–7 (1998).
2. Takahashi, K. *et al.* Induction of Pluripotent Stem Cells from Adult Human Fibroblasts by Defined Factors. *Cell* **131,** 861–872 (2007).
3. Qin, Y. & Gao, W. Q. Concise Review: Patient-Derived Stem Cell Research for Monogenic Disorders. *Stem Cells* **34,** 44–54 (2016).
4. Eyler, C. E. *et al. Adult Stem Cells*. *Cell* (2011). doi:10.1007/978-1-61779-002-7
5. Chen, K. G., Mallon, B. S., Mckay, R. D. G. & Robey, P. G. Cell Stem Cell Human Pluripotent Stem Cell Culture: Considerations for Maintenance, Expansion, and Therapeutics. *Stem Cell* **14,** 13–26 (2014).
6. Evans, M. J. & Kaufman, M. H. Establishment in culture of pluripotential cells from mouse embryos. *Nature* **292,** 154–156 (1981).
7. James, D., Levine, A. J., Besser, D. & Hemmati-Brivanlou, A. TGFbeta/activin/nodal signaling is necessary for the maintenance of pluripotency in human embryonic stem cells. *Development* **132,** 1273–1282 (2005).
8. Vallier, L., Alexander, M. & Pedersen, R. a. Activin/Nodal and FGF pathways cooperate to maintain pluripotency of human embryonic stem cells. *J. Cell Sci.* **118,** 4495–4509 (2005).
9. Wang, G. *et al.* Noggin and bFGF cooperate to maintain the pluripotency of human embryonic stem cells in the absence of feeder layers. *Biochem. Biophys. Res. Commun.* **330,** 934–942 (2005).
10. Xiao, L., Yuan, X. & Sharkis, S. J. Activin A maintains self-renewal and regulates fibroblast growth factor, Wnt, and bone morphogenic protein pathways in human embryonic stem cells. *Stem Cells* **24,** 1476–1486 (2006).
11. Xu, R.-H. *et al.* Basic FGF and suppression of BMP signaling sustain undifferentiated proliferation of human ES cells. *Nat. Methods* **2,** 185–190 (2005).
12. Ginis, I. *et al.* Differences between human and mouse embryonic stem cells. *Dev. Biol.* **269,** 360–380 (2004).

13. Martin, M. J., Muotri, A., Gage, F. & Varki, A. Human embryonic stem cells express an immunogenic nonhuman sialic acid. *Nat Med* **11,** 228–232 (2005).
14. Xu, C. *et al.* Feeder-free growth of undifferentiated human embryonic stem cells. *Nat. Biotechnol.* **19,** 971–974 (2001).
15. Xu, R.-H. *et al.* BMP4 initiates human embryonic stem cell differentiation to trophoblast. *Nat. Biotechnol.* **20,** 1261–1264 (2002).
16. Rao, B. M. & Zandstra, P. W. Culture development for human embryonic stem cell propagation: Molecular aspects and challenges. *Curr. Opin. Biotechnol.* **16,** 568–576 (2005).
17. Ding, S. & Schultz, P. G. A role for chemistry in stem cell biology. *Nat. Biotechnol.* **22,** 833–840 (2004).
18. Ludwig, T. E. *et al.* Derivation of human embryonic stem cells in defined conditions. *Nat. Biotechnol.* **24,** 185–187 (2006).
19. Chen, G. *et al.* Chemically defined conditions for human iPS cell derivation and culture. *Nat. Methods* **8,** 424–429 (2011).
20. Chen, G., Hou, Z., Gulbranson, D. R. & Thomson, J. A. Actin-myosin contractility is responsible for the reduced viability of dissociated human embryonic stem cells. *Cell Stem Cell* **7,** 240–248 (2010).
21. Villa-Diaz, L. G., Ross, A. M., Lahann, J. & Krebsbach, P. H. Concise review: The evolution of human pluripotent stem cell culture: From feeder cells to synthetic coatings. *Stem Cells* **31,** 1–7 (2013).
22. Laperle, A. *et al.* ??-5 Laminin Synthesized by Human Pluripotent Stem Cells Promotes Self-Renewal. *Stem Cell Reports* **5,** 195–206 (2015).
23. Lu, H. F. *et al.* A defined xeno-free and feeder-free culture system for the derivation, expansion and direct differentiation of transgene-free patient-specific induced pluripotent stem cells. *Biomaterials* **35,** 2816–2826 (2014).
24. Domogatskaya, A., Rodin, S., Boutaud, A. & Tryggvason, K. Laminin-511 but not -332, -111, or -411 enables mouse embryonic stem cell self-renewal in vitro. *Stem Cells* **26,** 2800–9 (2008).
25. Ezashi, T., Das, P. & Roberts, R. M. Low O2 tensions and the prevention of differentiation of hES cells. *Proc. Natl. Acad. Sci. U. S. A.* **102,** 4783–8 (2005).
26. Lian, X. *et al.* Efficient differentiation of human pluripotent stem cells to endothelial progenitors via small-molecule activation of WNT signaling. *Stem Cell Reports* **3,** 804–816 (2014).

27. Zhao, M. H., Kim, N. H. & Cui, X. S. GlutaMAX prolongs the shelf life of the culture medium for porcine parthenotes. *Theriogenology* **85,** 368–375 (2016).
28. Tohyama, S. *et al.* Glutamine Oxidation Is Indispensable for Survival of Human Pluripotent Stem Cells. *Cell Metab.* **23,** 663–674 (2016).
29. Garcia-Gonzalo, F. R. & Belmonte, J. C. I. Albumin-associated lipids regulate human embryonic stem cell self-renewal. *PLoS One* **3,** 1–10 (2008).
30. Higuchi, A. *et al.* Long-term xeno-free culture of human pluripotent stem cells on hydrogels with optimal elasticity. *Sci. Rep.* **5,** 18136 (2015).
31. Van Der Sanden, B., Dhobb, M., Berger, F. & Wion, D. Optimizing stem cell culture. *J. Cell. Biochem.* **111,** 801–807 (2010).