Synthetic Hydrogel-Based 3D Culture System for Maintenance of Human Induced Pluripotent Stem Cell

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B.S., Kansas State University, 2014
B.S., Henan University of Technology, P. R. China, 2014

A THESIS

submitted in partial fulfillment of the requirements for the degree

MASTER OF SCIENCE

Department of Grain Science and Industry
College of Agriculture

KANSAS STATE UNIVERSITY
Manhattan, Kansas

2017

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Abstract

Human induced pluripotent stem cells (hiPSCs) are generated from human somatic cells using defined transcription factors. These cells possess characteristics very similar to that of human embryonic stem cells including the ability to differentiate into cell types of all three germ layers. HiPSCs show great potential in clinical researches like drug screening and regenerative medicine, that all require large amount of cells cultured under well-defined conditions. The most common culture methods used for hiPSCs are 2D culture methods using Matrigel or vitronectin coated culture plates or flasks. 2D culture methods require large surface area to produce the same amount of cells compared to 3D methods. In addition, cells cultured in 2D culture environment are far from that *in vivo*. In this study, we developed a robust 3D culture condition based on hiPSC-qualified PGmatrix (PGmatrix-hiPSC) hydrogel. This 3D culture system provide hiPSCs with well-defined, more *in vivo*-like environment that encapsulate cells in liquid rich hydrogel with appropriate oxygen supply that resembles the hypoxia condition *in vivo*. Two hiPSC lines grown continuously in PGmatrix-hiPSC showed higher total population expansion and higher viability, with more consistency compared to the same cell lines grown in 2D on Matrigel or Vitronectin-XF. After grown in 3D PGmatrix-hiPSC for over 25 passages, major pluripotency markers, such as Oct4, Sox2, Nanog, and SSEA4 are expressed in most hiPSCs examined by flow cytometry. RT-qPCR also confirmed adequate expression levels of major pluripotency related genes. In addition, karyotype analysis of hiPSC after 37 passages in 3D PGmatrix-hiPSC was found normal. The same hiPSC lines cultured continuously in parallel in 2D and 3D showed differences in gene expression and surface marker TRA-1-81 expression. These results indicated the 3D PGmatrix-hiPSC system is likely superior in maintaining hiPSC growth as well as

pluripotency. The findings also suggest that it is very important to study cells in 3D culture environment to better understand the mechanism of pluripotency maintenance.

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Acknowledgements

First, I would like to thank my advisor Dr. Xiuzhi Sun for all her guidance and advices throughout my master's program. She taught me how to approach a research problem and find possible ways to achieve my research goals. I also appreciate very much Dr. Sun's financial and tuition support that helped me accomplish my research.

I would like to thank Dr. Jianfa Bai for being my committee member and offered the help conducting PCR analysis. I would like to thank Dr. Bai's lab members, especially Dr. Xuming Liu who conducted most of the PCR experiments and taught me how to analyze the gene expression data.

I would also like to thank Dr. Ruby Tsai from Applied Stemcell Inc for being my committee member, and answering my questions about hiPSC and analysis of results during my study.

I would like to thank Dr. Guangyan Qi from Bio-material and Technology Laboratory for her constant support and timely help on PGmatrix systems.

I would like to thank Kaori Knights from Flow Cytometry Laboratory at College of Veterinary Medicine, who helped me throughout all flow cytometry experiments and took the time to answer all my questions.

Finally, I would like to thank my family and friends for their love and support throughout my study.

Chapter 1 - Literature Review

1.1 Development of *in vitro* Culture Systems for Human Pluripotent Stem Cells

Human pluripotent stem cells consist of human embryonic stem cells (hESC) and human induced pluripotent stem cells (hiPSC). These cells are able to differentiate into almost all types of somatic cells while self-renew indefinitely (Zhu and Huangfu, 2013). Different from ESCs that are obtained from early stage embryos, hiPSCs are generated from somatic cells using defined transcription factors: Oct3/4, Sox2, Klf4, and c-Myc (Takahashi et al., 2007). HiPSCs possess great potential in clinical research because it allows generation of patient-specific hiPSCs for drug testing and personalized therapies. Application of hiPSCs in clinical research requires generation of large amount of high quality hiPSCs in limited time and in a culture environment that is fully defined, or better, xeno-free. Therefore, successful *in vitro* culture of human pluripotent stem cells is of great significance for advanced research in disease modeling, drug screening, organ transplantation and so on.

1.1.1 2D Culture Systems

Early research found that these cells are adherent and can be grown on a feeder layer that consists of mouse embryo fibroblasts (MEFs) inactivated by g-irradiation or mitomycin C (Conner, 2001a). The MEFs not only secret extracellular matrix (ECM) proteins for stem cell attachment, but also secret various signaling proteins like growth factor that promote stem cell survival and growth. As live cells, MEFs vary from batch to batch and the substances they secret to the culture environment cannot be fully defined. Also, because of its xenogeneic origin, MEFs may trigger unusual immune response as well as cause mycoplasma contamination (Villa-Diaz et

al., 2013). These barriers hindered clinical application of human pluripotent stem cells and a better substrate is thus needed.

Initial search for MEF alternative focused on substrates from natural sources. The most successful one so far is Matrigel, which is an extract from Engelbreth-Holm-Swarm (EHS) mouse sarcoma. This product is temperature sensitive and forms a hydrogel under room temperature. It can be coated on commercially available tissue culture polystyrene (TCPS) plates as a thin layer that supports human pluripotent stem cell survival and growth. Matrigel has been shown to be compatible with a variety of human stem cell culture media, including those with defined composition, made it widely used as an alternative to MEFs. Unfortunately, it poses similar problems as MEFs because it is also from animal origin and does not have a consistent and defined composition. To overcome this limitations, it is beneficial to look into the composition of Matrigel in order to find clues to its functionality. An analysis showed that Matrigel contains large protein components like laminin, collagen IV and enactin, as well as a large number of low-molecular weight proteins that involved in cellular localization and various biological processes (Hughes et al., 2010).

Laminin, as one of the main components found in ECM as well as in Matrigel, has drawn much attention. It has been reported that laminin, when used in combination with MEF-conditioned media (MEF-CM), supported growth of undifferentiated hESC for at least 6 passages. The cells maintained expression levels of OCT-4 and hTERT similar to those cultured on Matrigel. Further investigation showed that hESCs grown on MEF, Matrigel or laminin all expressed high levels of α6 and β1 integrins that are specific for laminin binding, consistent with their observation that hESCs grow better on laminin than on fibronectin and collagen IV (Xu et al., 2001).

Laminin is a heterotrimer consists of alpha, beta, and gamma chain subunits. Various laminin isoforms have been identified that are expressed differently during development (Colognato and Yurchenco, 2000a). A study on laminin isoforms not only confirmed the high expression level of laminin-binding α6β1 integrin in hESC, but also identified recombinant human laminin-332, -511, -111 as substrates that support the growth of several hESC lines for 10 passages using MEF-CM. The researchers suggested that laminin isoforms correspond to α6β1 integrin would provide sufficient attachment for hESC (Miyazaki et al., 2008). Later study by the same group used truncated laminin 332 and 511 which were called laminin E8 fragments (LM-E8s). The two LM-E8s contain C-terminal regions of all three chains that include active integrin-binding site without any heparin-binding activity. Adhesion assay performed with H9 hESC and IMR90 derived hiPSC in mTeSR1 medium showed that the two LM-E8s supported cell adhesion in a dose-dependent manner and the adhesion can be greatly reduced through addition of antibodies against α6 and/or β1 integrins (Miyazaki et al., 2012). When plating completely dissociated hESCs or hiPSCs on LM-E8s in mTeSR1, it was observed that the cells quickly attached and spread without the addition of ROCK inhibitor Y-27632. Molecular analysis found that LM-E8s activated some signaling pathways promoting cell survival, but the pathways activated were different from those affected by Y-27632. Culture experiments showed that LM-E8s were compatible with mTeSR1 and StemPro media and supported the growth of three hESC lines and two hiPSC lines for at least 10 passages when plated as single cell suspension at a density of 50,000 cells per cm^2 (Miyazaki et al., 2012).

Though Mizayaki et al suggested that laminin-521 would function as well as laminin-511, they didn't perform any experiment to confirm their assumptions. Another group took a closer look at the functionality of laminin-521 (Rodin et al., 2014) and showed that recombinant

human laminin-521 supported the growth of three hESC and two hiPSC lines in O3, mTeSR1 and TeSR2 media for at least 10 passages when plated as single cell without addition of Y-27632. It was claimed that laminin-521 as a substrate did not require Y-27632, but data showed that addition of Y-27632 resulted in better survival. As seen in previous study by Miyazaki et al, Rodin et al also observed cell migration towards each other in order to form aggregates for better survival, which did not happen to single cells plated on Matrigel. This phenomenon suggested that addition of E-cadherin might improve cell adhesion and survival since it is responsible for cell-cell adhesion. Experiments confirmed that when laminin-521 and E-cadherin was mixed between ratios 10:1 and 5:1, the most efficient colony formation was detected for two hESC lines tested. In addition, one of the hESC lines was grown on the mixture coating for 20 passages while able to maintain its normal karyotype and pluripotency (Rodin et al., 2014).

Study by Rodin et al showed that addition of E-cadherin would promote cell adhesion and survival. However, E-cadherin alone has been shown to serve as substrate for human pluripotent stem cell (Nagaoka et al., 2010). The group created a recombinant protein contains extracellular domain of E-cadherin and the IgG Fc domain. E-cad-Fc, commercialized as StemAdhere, was able to support growth of both hESC and hiPSC for more than 20 passages in mTeSR1 with a growth rate similar to that on Matrigel. However, cells interact with E-cad-Fc coating through surface E-cadherin, which is a common target of proteases. If passaging agents that have protease activities were used during harvesting, degradation of cell surface E-cadherin would case reduced plating efficiency (Nagaoka et al., 2010).

During study on Matrigel and laminin, which was mentioned previously, Xu et al (2001) also tested fibronectin as a possible substrate but found that though hESCs were able to form compact colonies, only few of them remained undifferentiated during culturing. A later study

found fibronectin is an effective substrate when used with their custom xeno-free, defined culture media called HESCO. The media is made by adding Wnt3a, FGF, insulin, transferrin, April/BAFF, cholesterol and human albumin into DMEM/F12. Two hESC lines was grown for at least 2 months (about 8 passages) on fibronectin with HESCO, and were found to maintain their undifferentiated states (Lu et al., 2006).

Besides fibronectin, vitronectin is another important cell adhesion protein present in plasma. Examination of integrin expression using three hESC/hiPSC lines showed that besides high levels of α6 and β1, some other integrin chains were also present including αVβ5, which binds to vitronectin and fibronectin. Integrin blocking assay of hESCs plated on different substrates showed that cell adhesion on vitronectin was not largely decreased when β1 integrin was blocked. Culture of three hPSC lines showed that recombinant vitronectin very well supported their growth for up to 12 weeks with mTeSR1, and maintained their pluripotency (Braam et al., 2008). Interestingly, another study found that the addition of a protein kinase C inhibitor, Gö 6983, and a histone deacetylase inhibitor (HDACI), trichostatin A made the conventional Knockout serum replacement media that was only comparible with MEF layer capable of supporting human pluripotent stem cells growth on vitronectin. This research tested two hESC lines and a hiPSC line derived from adipose stromal cell, and reached a conclusion that their custom media, named FXM3, performed equally or even slightly better than mTeSR1 (Kim et al., 2013). Recombinant vitronectin is now commercially available as Vitronectin-XF from Stemcell Technologies™, and is compatible with TeSR-E8 medium (Chen et al., 2011b).

While substrates derived from natural sources are able to serve as substrates for culture of human pluripotent stem cells, they are usually difficult to produce and some batch-to-batch variations is unavoidable. In order to find a more robust and cost efficient system, many

researchers turned to synthetic substrates. There are basically two types of synthetic substrates, polymers from compounds that do not exist naturally in human body, and peptide derived from naturally existed sequences.

In 2010, a synthetic polymer was reported to work as substrate for human pluripotent stem cells. It can be coated on to tissue culture plastics through surface-initiated graft polymerization. The resulting polymer, poly[2-(methacryloyloxy)ethyl dimethyl-(3-sulfopropyl)ammonium hydroxide] (PMEDSAH), formed a thin layer of hydrogel on which two lines of hESCs were successfully maintained for 25 passages with MEF-CM. Compatibility of this hydrogel with two defined media StemPro and mTeSR were also tested, unfortunately only one of the two hESC lines were able to survive 10 passages with StemPro medium (Villa-Diaz et al., 2010).

Another study used photoinitiated radical addition polymerization to form hydrogel coating in tissue culture polystyrene (TCPS) plates. A solution containing the monomer N-(3-Aminopropyl) methacrylamide hydro-chloride (APMAAm), a crosslinker and photoinitiator was added to plates and polymerized under UV light for gel formation. Two hESC lines, H1 and H9, with H9 contains hOct4 promoter driving GFP and Zeo, were grown on APMAAm gel and Matrigel with mTeSR1 containing 5μM ROCK inhibitor for at least 10 passages. The researchers claimed that though initial cell attachment on APMAAm was only 50~60% of that on Matrigel, the proliferation of both lines were faster on APMAAm, which is not exactly what was shown in their figures. Another unusual thing about this study was the immunochemistry results. H1 cells cultured on APMAAm and Matrigel had similar expression level of three pluripotency markers Oct-4, SSEA-4, and Tra-1-60. But for the H9 line, cells on APMAAm had much higher expression of SSEA-4 and Tra-1-60 compared to the extremely low levels from cells on

Matrigel, though Oct-4 levels were similar in the two. This made the study questionable especially the researchers did not give any explanations about this result. One important information obtained from this piece of work is the effect of BSA on cell adhesion to APMAAm. The designed experiments showed that bovine serum albumin (BSA) adsorbed onto the APMAAm gel improve cell adhesion in a dose dependent manner, but other components of complete mTeSR1 medium would compete with BSA for adsorption and thus cause slight reduction in cell adhesion compared to basal mTeSR1 with added BSA (Irwin et al., 2011).

Proteins adsorbed onto synthetic polymers can initiate cell adhesion and can sometimes mask the ability of the polymer itself to interact with cells. Brafman et al (2010) designed a high-throughput screening of a number of different polymers at different concentrations by depositing the polymer solutions onto a glass slide coated with acrylamide gel using a microarray printer. To eliminate the adsorption of ECM proteins from MEF-CM, a defined medium – StemPro that contains no ECM proteins was used for all cultures on tested polymers. Cells harvested from Matrigel were dissociated into single cells and plated at a density of 1.5×10^6, about 10~20 cells per spot. Culture of one hESC line identified one polymer that support proper cell adhesion and proliferation for at least five passages, as well as high Oct4 and Nanog expressions. The identified polymer - poly(methyl vinyl ether-alt-maleic anhydride) (PMVE-alt-MA) was evaluated for the growth of two hESC lines and one hiPSC line for five passages and were all able to maintain their morphology and pluripotency. Examination of cell growth on different concentrations of polymers revealed that PMVE-alt-MA support hPSC proliferation in a molecular weight dependent manner, with the highest proliferation occurring at 1.25×10^6 Da. This study also revealed that though human pluripotent stem cells attached to PMVE-alt-MA in

an environment that has no ECM proteins, the cells themselves would express more ECM proteins to promote cell adhesion and survival (Brafman et al., 2010).

Though the polymers mentioned above seemed to work well as substrates for human pluripotent stem cell culture, none of the cultured cells were examined in detail to see whether there was any change in gene expression that was unusual. Because these synthetic polymers do not exist in natural human tissue, they might trigger some unpredictable changes in cells that are hard to find. Another approach of synthetic substrates is to create short peptides whose sequences are derived from ECM proteins that already exist in human body.

To enable high-throughput screening, many studies conjugate their peptides to self-assembled monolayers (SAMs). One study used alkanethiol groups that forms SAMs on gold. This study checked sequences that are known to bind to various cell surface receptors including arginine-glycine-aspartic acid (RGD) and glycosaminoglycan-binding peptides. Their screening results showed that RGD was inconsistent in the maintenance of hESC using mTeSR1 and ROCK inhibitor Y-27632, but three heparin-binding peptides were good at maintaining hESC with high levels of pluripotency marker, with GKKQRFRHRNRKG being the best. This peptide derived from vitronectin were able to maintain two hESC lines for 2~3 months at the lowest concentration among all peptide screened. This interaction between heparin-binding peptide and hESC was mediated by cell-surface glycosaminoglycans (GAGs), and were able to support a proliferation rate similar to hESCs grown on Matrigel. Klim et al showed that this peptide can also be conjugated to other surfaces, like glass functionalized with bromoacetamide or to streptavidin-coated surfaces after biotinylation. All these systems supported hiPSC and hESC growth using mTeSR1 supplemented with Y-27632. Another experiment in this study combined biotinylated GKKQRFRHRNRKG and biotinylated cyclic RGD on the same streptavidin-coated

surface, which was able to maintain three hESC lines with high levels of pluripotency markers for 1~2 months without the addition of Y-27632, which indicated that simultaneous GAG and integrin interactions would be more effective in human pluripotent stem cell maintenance (Klim et al., 2010).

Another similar study examined peptide sequences derived from laminin using similar method as Klim et al. One peptide RNIAEIIKDI was identified to support hESC adhesion and growth using MEF-CM, though long term culture stability was not mentioned. This study also discovered that the peptide interact with cells through ligand binding instead of surface charges, and required high ligand density for optimal adhesion and cell survival (Derda et al., 2007).

Similarly, one study designed synthetic peptide-acrylate surfaces (PAS) that are fabricated by first depositing carboxylic acid containing acrylate onto culture vessel surface then conjugate amine-containing peptides (Melkoumian et al., 2010). Several types of peptide sequences derived from bone sialoprotein (BSP-PAS), vitronectin (VN-PAS), long or short fibronectin (sFN-PAS or lFN-PAS) and laminin (LM-PAS) was examined using two hESC lines with X-VIVO10 medium supplemented with growth factors. Though all sequences tested contain RGD motif, results showed that only VN- and BSP-PAS were able to support hESC adhesion and growth at a similar extent to Matrigel. Further testing found that for BSP-PAS to function as well as VN-PAS and Matrigel, high peptide density was required. Long term culture results confirmed that hESCs can be cultured on the two PAS for more than 10 passages while maintaining their pluripotency. To expand the use of PAS, Melkoumian et al checked the compatibility of the two surfaces with other defined culture media, they concluded that mTeSR1, Knockout serum replacement medium, and TeSR2 were all effective for the maintenance of hESCs on PAS. The findings from this study was commercialized as Synthemax® by Corning.

All the synthetic peptides mentioned above were derived from existing ECM proteins and tried to mimic the function of those ECM proteins by interacting with human pluripotent stem cells in the same or similar way. Integrins, as the main receptor, is in the center of cell adhesion and survival pathways. Another approach to optimize culture conditions for human pluripotent stem cells was to find soluble substances that could stimulate pathways to promote cell survival, and ROCK inhibitor Y-27632 is a perfect example (Watanabe et al., 2007). A group of researchers adopted this approach to search for short peptides and found angiopoietin-1 (Ang-1) as their subject of study. This is a secreted glycoprotein that usually act as ligand for Tie2 receptor on endothelial cells, but Ang-1 was also found binding to integrins on cells that do not express Tie2. The study used a highly conserved Ang-1 sequence QHREDGS first as culture media supplement. This supplementation not only improved colony formation during single cell passaging, but also promoted cell survival during long term culture (5 passages) without any negative effect on pluripotency maintenance. Furthermore, the QHREDGS peptide can be conjugated to PEG hydrogel at different concentration and was shown to promote hiPSC adhesion in a dose-dependent manner. Though the group did not evaluate the potential of this PEG-QUREDGS as a culture substrate for human pluripotent stem cell, their results suggested that this was possible since the interaction between the peptide and cells were mediated by β1 integrins, which is the same as laminin (Dang et al., 2014).

There are some other studies that tried to modify glass for plastic surfaces with different methods to improve culture of human pluripotent stem cells. These methods include amine modification (Kolhar et al., 2010), plasma etching (Mahlstedt et al., 2010), UV-treatment (Saha et al., 2011), and modification of roughness and nanotopographic properties (Chen et al., 2012b). While none of the substrates created in these studies would function well for human pluripotent

stem cell culture on its own without coating or adsorption of ECM proteins, they would provide some useful information on optimizing physical properties of culture environment that promote cell adhesion and survival.

1.1.2 3D Culture Systems

The systems mentioned above are all about culturing pluripotent stem cells in a 2D environment. 2D culture allows easy access to the cells and its environment, thus is good for some experiments that require timely adjustment to the cell or its environment. However, cells are grown in a 3D environment in nature. Except for the substances that exist in soluble form, cell survival and growth also relate to the interactions that take place in a 3-dimensional way between cells and their environment, which is largely different from what can be provided by 2D culture systems. Therefore, there is a need to create a more *in vivo*-like environment that allows cells to reside in a more natural state.

In studies that use SAM for short peptide screening, scientists had proved that those short peptides are transferable (Klim et al., 2010). To take advantage of this property, Derda et al (2007) designed a hydrogel by adding an amphiphile tale to their synthetic peptide RNIAEIIKDI. Though not specified, this added tale would self-assemble under certain condition and result in a hydrogel that presents the peptide at high density. It was briefly mentioned that the group managed to grow hESC in this gel undifferentiated and maintained its pluripotency.

Study by Derda et al. showed the possibility of 3D culture for human pluripotency stem cell, but little information was provided about the 3D system they created. In the meantime, another study chose hyaluronic acid to construct hydrogel for hESC 3D culture. This study found that hyaluronic acid (HA) was internalized into hESC cells during culture, and was involved in

maintenance of undifferentiated hESC on MEF layers. Therefore, they added methacrylate groups to HA for hydrogel formation. The formed hydrogel did support hESC growth without differentiation, as well as maintaining their pluripotency (Gerecht et al., 2007). This study was one of the early explorations for synthetic 3D systems. Though it seemed to work, several problems remained: Formation of hydrogel requires exposing the monomer-cell mixture under UV light; release of cells needs treatment with hyaluronidase; and the gel would degrade overtime because of internalization of HAs into the cells cultured. More importantly, hESCs were cultured in 3D for only one passage before transferred to 2D for genetic evaluation. This approach clearly ignored long term effect of those short UV and hyaluronidase exposures.

Though hyaluronic acid was involved in hESC maintenance, it was not a good target for hydrogel construction. Another approach adopted natural polymers that allow more gentle process for cell seeding and harvesting. AlgiMatrix® is a product offered by ThermoFisher Scientific that consist of pure alginate. Though this product was only being used for tissue and tumor modeling, researchers were able to create a 3D scaffold for hESC by modifying the product. Their study reported the construction of a porous scaffold using chitosan and alginate, because both polymers have structures similar to that of glycosaminoglycan (GAG), an important ECM protein. By ionically bind the amine group of chitosan with the carboxyl group of alginate and freeze them in 24-well plate, the resulting cylindrical scaffold had relatively uniform pore size, as well as higher compressive and tensile modules than pure alginate scaffolds. When used for hESC culture, the scaffold was cut into thinner pieces and placed into 24-well plate, while cell suspension was added. Cells were cultured with Knockout Serum replacement medium, and the proliferation rate, as reported, was limited mainly by pore sizes at later stage of 21-day culture. After three continuous passages, hESCs seemed to maintain their

pluripotency. However, long term culture stability was not tested in the study and cell lost during harvest might be a concern as less than 90% of cells were collected during cell recovery from 3D (Li et al., 2010b).

While the chitosan-alginate scaffold was able to support hESC growth, its relatively complex harvesting procedure caused noticeable cell loss. A new study in 2013 addressed this issue by creating a temperature sensitive hydrogel that can turn into liquid state at low temperature. This hydrogel is commercialized as Mebiol Gel and consists of poly (N-isopropylacrylamide)-co-poly(ethylene glycol) (PNIPAAm-PEG). Both hESC and hiPSC lines were tested by encapsulation in 3D as single cell or cell cluster with two growth media: mTeSR1 and E8. Results showed that when supplemented with 10 μM ROCK inhibitor, cell clusters in E8 medium with a seeding density of 1×10^6 cells/mL had the greatest expansion in a 4~5-day culture. The total culture length vary from around 15 up to about 60 passages for different cells lines, all cell lines exhibited high express level of Oct4, differentiation and teratoma assay using one cell line cultured in 3D for 35 passages confirmed its pluripotency (Lei and Schaffer, 2013).

3D culture systems using hydrogel have the potential to be applied to suspension culture, which allows large scale production of cell bank. As indicated by Lei and Schaffer (2013), gel with cells already encapsulated in it can be extruded into fibers and suspended in media, though it was not clear whether the system involved spinner.

Suspension culture has been around for large scale production of some somatic cells, and researchers have been trying to use this system with human pluripotent stem cells. Common solutions involved growing hPSC as cell aggregates (Chen et al., 2012a), or on microcarriers coated with Matrigel or ECM proteins (Chen et al., 2011a). In both case, vulnerable hPSCs were exposed directly to shear stress, which would negatively affect cell viability. In addition, signs of

cell agglomeration were shown in both studies, which would lead to differentiation. An interesting approach suggested adding an outer layer to protect cells from these problems. Microencapsulation with porous polymer would still allow access to soluble nutrients in the media while decreasing harmful shear force and agglomeration. One study applied alginate microencapsulation to single cell, cell aggregates and cells attached to microcarriers that were grown in suspension culture with MEF-CM. For single cells, this technique did not help much to promote survival, but viability of the latter two showed some improvement. (Serra et al., 2011) Though suspension culture seemed to be the best solution for large scale production, current suspension systems for human pluripotent stem cells are still having limitations. Except for issues of cell agglomeration and shear force mentioned above, another problem with these suspension culture systems is that it often involves coating with Matrigel and using undefined media. In addition, studies on suspension culture of hPSCs often ignore evaluation of long term culture stability. Instead, cells were usually cultured for a long period of time for only one passage.

1.2 Effect of Various Factors on Maintenance of Human Pluripotent Stem Cells

No matter it is at their most natural state inside live tissue or *in vitro* environment, human pluripotent stem cells (hPSCs) including human embryonic stem cells (hESCs) and human induced pluripotent stem cells (hiPSCs) are surrounded by a very complicated environment. Cells interact with their surroundings by constantly send and receive signals in various forms. These signals can be physical characteristics of the environment, soluble factors from blood or culture media, as well as signaling molecules produces by stem cells themselves. Different signaling pathways are activated by different signals, resulting in cell responses to environmental

changes – adhesion, growth, differentiation, or death. In order to develop a successful 3D culture system for human pluripotent stem cells, it is necessary to understand what environmental factors will have an effect on cells, what signaling pathways are essential to grow stem cells and maintain their pluripotency, and how these major signaling pathways are affected by various signals.

1.2.1 Major Signaling Pathways

No matter what interactions cells have with their environment, the consequences were fundamentally resulted from activation or inhibition of certain signaling pathways. To better understand effects of environmental factors, we need to first elucidate the major signaling pathways related to survival and expansion of human pluripotent stem cells.

Shown in Figure 1 are major signaling pathways in cancer and embryonic stem cells. Specifically, MAPK/ERK, PI3K/AKT, and TGFβ signaling pathways are the most important ones in human pluripotent stem cells. Though NF-κB signaling plays an important role in immune response and other cellular activities, its role in human pluripotent stem cells remains unclear (Dreesen and Brivanlou, 2007). Results from recent study suggested potential functional role of NF-κB signaling as its activity level decrease during hiPSC differentiation, and siRNA knockdown of this pathway resulted in down regulation of Oct3/4 and Nanog expression without affecting cell morphology and colony formation (Takase et al., 2013).

Mitogen-activated protein kinase (MAPK) signaling pathway responds to a wide range of signals from various stimuli and can result in proliferation, differentiation, apoptosis, as well as inflammatory responses. The MAPK cascade includes a series of protein kinases that relay signals received to different effectors through phosphorylation. The phosphorylated effectors

would then function in the cytosol or translocate to nucleus to change protein functions or gene expressions that lead to different biological responses (Morrison, 2012). The MAPK pathway consists of three parts that relay signals through different MAPKs: ERK 1/2, p38, and JNK/SAPK. Pathways involving the three kinases respond to different activators and have different effectors, but they are tightly related to each other and form a small MAPK signaling network through cross-talks happen at various levels. When it comes to the more specific MAPK/ERK pathway, it is activated primarily through receptor tyrosine kinases (RTKs). G protein-coupled receptors (GPCRs) have also been reported to activate MAPK/ERK, which was mediated by stimulation of RTKs (Zhang and Liu, 2002).

PI3K/AKT is a highly conserved signaling pathway. In the simplest form, PI3K is activated through receptor tyrosine kinases (RTKs). The activated PI3K then phosphorylates PIP_2 to PIP_3, which binds PKB/AKT for activation. This activation is partial but is sufficient to promote protein synthesis and cellular proliferation. When fully activated, PKB/AKT can mediate more cellular functions in addition to proliferation (Hemmings and Restuccia, 2012). PI3K signaling also contributes to cell migration as it regulates the accumulation of PIP_3, which can promote actin polymerization when reaches certain level, as well as affect cytoskeletal activities (Devreotes and Horwitz, 2015). PI3K/AKT pathway has been reported to be essential for the survival of human pluripotent stem cells. It has been reported that the use of various AKT inhibitors would result in high levels of apoptosis (Hossini et al., 2016; Romorini et al., 2016), while such effect was not observed with fibroblasts that was used to generate hiPSCs tested, nor with neurons generated from the tested hiPSCs (Hossini et al., 2016). Another study that decreased activity of CDK1, which would indirectly inhibit PI3K/AKT signaling, resulted in loss

of pluripotency (Wang et al., 2017). There studies all suggested the important role of PI3K/AKT in survival and maintenance of hPSCs.

Transforming growth factor β (TGFβ) superfamily is a well conserved family of related polypeptides, including TGFβs, activins, Nodal, bone morphogenetic proteins (BMPs), and growth and differentiation factors (GDFs). These members need transmembrane serine/threonine kinase receptors for signaling. Two types of receptors, type I and type II, would form heterotetramers (two type I and two type II) upon ligand binding. This would subsequently lead to activation of Smad signaling or non-Smad signaling (Wrana, 2013). There is a total of eight Smads that respond to activation by different TGFβ family members. When activated, the Smads would form trimers with Smad4, then relocate into the nucleus to bind directly to DNA. The most important Smad 2 and 3, which are activated by TGFβ/activin/nodal, are known to target Nanog, leading to maintenance of hPSC pluripotency. Other non-Smad pathways are activated independently through adaptor proteins or signaling mediators with the two types of receptors (Sakaki-Yumoto et al., 2013).

Cell signaling is complicated with cross-talk between different pathways happening at various levels. It has been reported that both high and low levels of fibroblast growth factor 2 (FGF2) activate MAPK/ERK signaling and maintain ERK activity at a basal level that leads to self-renewal of hPSCs. However, high levels of FGF2 also activate PI3K/AKT that would suppress MAPK/ERK so that its activity is restrained at a low level. Besides FGF2, PI3K/AKT can also be activated by insulin, insulin growth factor (IGF), as well as epithelial growth factor (EGF). When activated, PI3K has cross-talks with TGFβ signaling to maintain Smad 2 and 3 activity for pluripotency maintenance (Dalton, 2013). Another step taken towards pluripotency maintenance, is suppression of signaling pathways that lead towards differentiation, notably Wnt

signaling. PI3K/AKT pathway does this by targeting ERK 1, 2, which would subsequently suppress GSK3, an important member of Wnt pathway (Singh et al., 2012; Dalton, 2013; Van Camp et al., 2014).

1.2.2 Characteristics of Substrate

Finding the right substrate is an important part of a successful *in vitro* culture system for human pluripotent stem cells. When mitotically inactivated mouse embryonic fibroblasts (MEF) (Conner, 2001b) and Matrigel were used for 2D *in vitro* culture of human pluripotent stem cells, properties of the substrates did not have much significance because they are either live cells or from live tissues and consist of various extracellular matrix (ECM) proteins as well as signaling molecules which all provide support for adhesion and maintenance of human pluripotent stem cells. During the past decade, researchers are putting more and more effort in finding well-defined xeno-free substrates for hPSCs. This is when characteristics of the substrates themselves become important. When trying to create synthetic substrates for human pluripotent stem cell culture, several research groups (Mei et al., 2010; Saha et al., 2011; Celiz et al., 2014; Chang et al., 2013; Chen et al., 2012b; Reimer et al., 2016) discovered that human pluripotent stem cells prefer certain substrates on which they were able to grow normally. Though the substrates found by each group were different, there existed some characteristics that was shared among these "hit" polymers.

Mei and colleagues (2010) examined cell adhesion on different synthetic materials using microarrays coated with fetal bovine serum (FBS). Their results showed that surface roughness did not have a strong correlation with cell survival. However, water contact angle (WCA) was a major factor with around 70° associated with optimal colony formation. Surface analysis using

time-of -flight secondary-ion mass spectrometry (ToF-SIMS) revealed that polymers that positively supported colony formation often contains hydrocarbon ions like $C_2H_3^+$, oxygen-containing ions like CHO_2^- from esters, and fragments from cyclic structures. Later study by Saha et al (2011) applied short-wavelength UV light to virgin polystyrene for a short period of time, followed by coating with FBS and cell seeding. They also examined the treated surface with ToF-SIMS and reached similar conclusion as Mei et al (2010) that surfaces containing hydrocarbon ions and oxygen-containing ions from esters had positive impact on cell adhesion and colony formation.

Celiz et al (2014) created an array that contained 141 synthetic polymers and examined their ability to support hESC line HUES7. Though WCA was previously proposed as a predictor of hPSC interaction (Mei et al., 2010), this study could not find correlations between WCA and cell adhesion. However, Examination of surface chemistry by ToF-SIMS showed that the "hit" polymers identified contained monomers that had cyclic structures or thiol group and were rich in esters.

When conjugating poly-acrylamide (PAm) with poly (sodium-4-styrenesulfonate) (PSS) to form a polymer substrate for hPSC, Chang et al (2013) found a correlation between sulfonate content and cell adhesion. This is similar to previous study that identified "hit" polymers containing thiol group (Celiz et al., 2014). Besides, they also discovered that cells attached better to substrates with high rigidity (elastic modulus around 344 kPa), but WCA for optimal cell attachment was about 23.0^{o}which was not in accordance with Mei et al (2010), suggesting WCA may not be a good indicator for cell attachment.

Compared with the studies above, Reimer et al (2016) chose to investigate the effect of topographies instead of examination of different polymers. Their topography library was

constructed on tissue culture polystyrene by differing density, feature size and roundness of each unit. After short-term culture of hiPSC with E8 medium and ROCK inhibitor, small feature size, high wavenumber and high feature density was found to be the most important factors for hiPSC maintenance.

Another study used reactive ion etching (RIE) to produce glass surfaces with different nanoroughness and patterns, followed by vitronectin coating and hESC seeding. The results indicated that hESC attached and maintained better on surfaces with low nanoroughness (Chen et al., 2012b).

Among all studies mentioned above, Reimer et al (2016) were the only group who did not include a pretreatment of the culture surface with serum or ECM proteins or serum-containing culture media like MEF-conditioned medium (MEF-CM). However, cells seeded onto their culture surface were only maintained for a short period of time. According to results reported by these researchers, the observed relationship between substrate properties and cell survival was more likely due to difference in protein adsorption between different substrates. It was known that surface with some degree of hydrophobicity led to better adsorption of proteins like fibronectin (Smetana, 1993). This is likely one reason Mei et al (2010) observed optimal colony formation on substrates that had a WCA around 70°. Besides, protein adsorption experiment carried out by Chang et al (2013) showed that the substrate that best supported maintenance of several hPSC lines (PAm_6-co-PSS_2) adsorbed higher amount of bovine serum albumin (BSA) and vitronectin (VN). It was also reported that cells cultured on this substrate express higher levels of multiple ECM proteins as well as integrins.

There has been various reports on synthetic substrates that supported adhesion and growth of hPSC (Villa-Diaz et al., 2010; Irwin et al., 2011; Brafman et al., 2010; Kolhar et al.,

2010; Mahlstedt et al., 2010), but studies on how substrate characteristics affect protein adsorption did not emerge until recently. A study compared protein adsorption from MEF-CM on to plasma etched tissue culture polystyrene (PE-TCPS) (Mahlstedt et al., 2010) and normal tissue culture polystyrene (TCPS) because the former was reported to support long term hESC maintenance with MEF-CM but the latter wasn't. Proteomics analysis showed that some proteins are uniquely adsorbed to PE-TCPS. These identified proteins were then mixed pairwise and deposited onto another synthetic polymer polyHPhMA (Celiz et al., 2014; Celiz et al., 2015) to test their ability to support growth of a hESC and an hiPSC line. Since polyHPhMA worked in combination with StemPro media, which has defined composition and is much simpler than MEF-CM, this screening method was able to show difference in cell adhesion abilities between various proteins. The results, interestingly, showed that the combination of heat shock protein-1 (HSP) and heat shock protein 90 (HSP90) was as effective as fibronectin coating in supporting cell adhesion (Hammad et al., 2016). HSP belongs to the HSP70 family, both HSP70 and HSP90 are parts of the heat shock protein family. Though it was not clear how exactly the combination of the proteins support hPSC adhesion and maintenance, some studies suggested that HSP was involved in Nanog upregulation in mouse and HSP90 played critical role in maintaining mouse ESC pluripotency (Prinsloo et al., 2009; Bradley et al., 2012).

Besides the types of proteins adsorbed, conformation of proteins was also an important factor. Different conformations have different binding affinities to regulate the various interactions and signaling pathways. These conformations can be affected by concentration of certain cations, as well as mechanical tension (Hytönen and Wehrle-Haller, 2014), which are all environmental changes that can be controlled through altering the substrates or culture media.

This is probably part of the reason topographic properties like feature density and size had an impact on cell adhesion.

1.2.3 Extracellular Matrix Proteins and Cell Surface Receptors

Adhesion of human pluripotent stem cells on to culture surfaces largely depends on interactions between extracellular matrix (ECM) proteins and cell surface receptors and cell-cell interactions. These interactions can affect signaling pathways in order to maintain viability and pluripotency of hPSCs.

Integrins are transmembrane proteins that link the cells to ECM proteins, they are heterodimers consist of α and β subunits. 18 α subunits and 8 β subunits make up a total of 24 integrins. On the extracellular side, they bind to different ECM proteins, on the intracellular side, most integrins are linked to actin filaments. Based on differences in ligands, integrins can be categorized into three groups: collagen receptors, laminin receptors, and RGD receptors. Each of the integrins has appeared to have its unique and specific functions (Hynes, 2002).

It has been reported that collagen I can act as a reservoir for basic fibroblast growth factor (bFGF) and heparin sulfate proteoglycans (HSPGs) to provide protection from proteolytic environment, as well as regulation through controlled release (Kanematsu et al., 2004). Collagen I-bound α2β1 integrin has shown to support maintenance of mouse ESCs through activation of PI3K/AKT pathway (Suh and Han, 2011). On the other hand, collagen IV was reported to enhance growth of transplanted hPSCs through pretreatment, and cell migration on collagen IV coated surface was improved. These was shown to be mediated by α2β1 integrin (Li et al., 2011).

Laminin is another important component of ECM proteins, and is also a major component of Matrigel (Hughes et al., 2010). Laminins are large heterotrimer glycoproteins that

contain α, β, and γ chains. Various laminin isoforms have been identified that bind to different receptors including many β1 and β4 integrins (Colognato and Yurchenco, 2000b). Laminin-511 and -521 was shown to reduce cancer cell apoptosis by binding preferably to α3β1 integrins to upregulate PI3K/AKT signaling (Gu et al., 2002). It was also demonstrated that human fibroblasts are able to support hESC growth express laminin-511 and α3β1, α6β1, α7β1 integrins that binds laminin (Hongisto et al., 2012). Similarly, laminin-511 were also produced by undifferentiated hESCs, and use of laminin-511 with nidogen-1 on single-cell hESC suspension drove the cells to resemble into aggregates through binding with α6β1 integrins (Evseenko et al., 2009). Other researchers not only confirmed expression of laminin-511 by ESCs but also proved that laminin-511 can serve as a substrate to support long-term culture of both mouse and human pluripotent stem cells (Domogatskaya et al., 2008; Rodin et al., 2010). Binding of laminin-511 to α6β1 integrin would upregulate the PI3K/AKT pathway for self-renewal, and can partially replace ROCK inhibitor Y-27632. This effect is likely due to the association between α6β1 integrin and Fyn, which is part of the Fyn-RhoA-ROCK signaling. Interaction between laminin-511 and α6β1 integrin would upregulate Fyn-RhoA-ROCK signaling to improve cell survival. A recent research confirmed high expression level of α6 and β1 integrins in embryonic stem cells, but only α6 integrin was downregulated during differentiation (Villa-Diaz et al., 2016). It was shown that focal adhesion kinase (FAK) was kept in an inactive state and its N-terminal was relocated into nucleus to react with two pluripotent transcription factors Oct4 and Sox2. This regulation of FAK activity and pluripotency was related to the expression of laminin α5 and α6 integrin in pluripotent stem cells (Villa-Diaz et al., 2016). On the other hand, while not able to support hPSC maintenance alone, binding of laminin-332 to α6β4 integrin is involved in hemidesmosomes organization, which is important for adhesion and migration of various

somatic and cancer cells (Nakashima and Omasa, 2016). There is no data on how α6β4 integrin interacts with hPSC signaling pathways, but it is no doubt that additional laminin binding with integrin would improve cell adhesion, which is essential for hPSC survival.

Besides the two ECM proteins, integrins can recognize specific peptide sequence, the most important is arginine-glycine-aspartic acid (RGD). This adhesion sequence was first discovered in fibronectin, and was later found to exist in various adhesive proteins including vitronectin, laminin, bone sialoprotein, tenascin, entactin, and even some collagens. Though there are a lot of proteins that bear the RGD sequence, not all of them can mediate cell adhesion because of inadequate position and conformation (Ruoslahti, 1996).

One known RGD receptor, α5β1 integrin, binds to fibronectin (Nakashima and Omasa, 2016). It has been shown that this integrin can bind efficiently to soluble fibronectin to induce redistribution of focal adhesion and Rho activation, affecting fibroblast-matrix adhesion and morphology (Huveneers et al., 2008). Another research showed that α5β1 integrin assist epithelial growth factor (EGF) signaling by forming complex with activated EGF receptor and leads to effective activation of MAPK/ERK and PI3K/AKT pathways (Lee and Juliano, 2002). Another fibronectin receptor, α8β1 integrin, was shown to promote tumor cell survival when serum was deprived through activation of PI3K/AKT pathway (Farias et al., 2005).

Except for these two β1 integrins, there is also αVβ1 which binds to RGD sequence in vitronectin in addition to fibronectin, laminin and collagen IV (Bodary and McLean, 1990; Dedhar and Gray, 1990). Binding to vitronectin is tightly related to αVβ1 for certain human melanoma cells, and αVβ1 was detected to cooperate with α5β1 for efficient cell attachment on fibronectin (Marshall et al., 1995). Similar to αVβ1, αVβ3 integrin is another vitronectin RGD receptor that can cooperate with α5β1 for melanoma cell attachment on fibronectin. Besides,

αVβ3 can bind to fibronectin with high affinity when vitronectin or other RGD-containing adhesion proteins are absent (Charo et al., 1990).

With advances in the search for defined substrates for *in vitro* culture of human pluripotent stem cells, vitronectin was reported to support long term culture in combination with various culture media (Braam et al., 2008; Chen et al., 2011b; Prowse et al., 2010). Adhesion of hPSCs to vitronectin was mediated by αVβ5 integrin (Braam et al., 2008). Adhesion efficiency was not affected even when N- and C-terminal of full length vitronectin was truncated (Chen et al., 2011b).

Integrin αVβ6 and αVβ8 are also RGD receptors. Different from other integrins that interact mostly with ECM proteins, αVβ6 and αVβ8 play important roles in TGF-β signaling. TGF-β1, when secreted, was in an inactive form that carries a latency-associated peptide (LAP). This LAP contains RGD sequence that is recognized by αVβ6 and αVβ8 integrins. Binding with these two integrins is an important step in TGF-β1 activation and subsequent signaling (Munger et al., 1999; Yang et al., 2007; Aluwihare et al., 2009). Considering the importance of TGF-β signaling pathway, interaction with TGF-β in hPSC culture media is essential in maintenance of human pluripotent stem cells.

Integrins are responsible for most cell-ECM protein interactions, there are also cadherins that mainly mediate cell-cell junctions. Cell-cell adhesion through cadherins is dependent on Ca^{2+} ions, the intracellular portion of cadherins links to the cytoskeleton, while the extracellular domain binds to another cadherin of the same type. Cadherin superfamily contains a large variety of members, including three well studied classical cadherins: E-cadherin (epithelial), N-cadherin (neural), and P-cadherin (placental), that are named after the tissues they were found (Alberts et al., 2014).

E-cadherin is essential for cell-cell adhesion during embryoid body formation, and is downregulated during the differentiation process (Dang et al., 2004). Studies on several somatic cells showed that E-cadherin mediated cell-cell junctions can regulate activities of Rac1, RhoA, and Cdc42, which are Rho family GTPases affecting actin cytoskeleton (Noren et al., 2001). It was later confirmed that similar phenomenon exists in hESCs. Improved cell-cell junctions through E-cadherin after single cell dissociation can suppress Rho-ROCK signaling, and thus promote hESC survival (Xu et al., 2010). E-cadherin is anchored to actin cytoskeleton in cells through interactions with p120-, β- and α-catenins. Catenins are also important signaling molecules. It was found in cancer cells that the E-cadherin and catenin complex can recruit members of PI3K/AKT pathway to activate it for improved cell survival and proliferation (De Santis et al., 2009). In the case of hESCs, a small GTPase Rap1 was found to interact with E-cadherin to promote formation of E-cadherin mediated cell-cell junctions and indirectly assist in the maintenance of human pluripotent stem cells (Li et al., 2010a). Studies on mouse ES cells demonstrated that E-cadherin is important for LIF/BMP mediated pluripotency through their binding with β-catenin. Also, forced expression of E-cadherin in feeder cells, instead of E-cadherin supplemented media, resulted in enhanced pluripotency marker expression in mouse ESCs, suggesting that E-cadherin functions through direct cell-cell contact (Soncin and Ward, 2011). Based on these results, researchers have developed substrates for hPSCs using E-cadherin alone (Nagaoka et al., 2010) or in combination with other ECM proteins (Rodin et al., 2014).

Compared to E-cadherin expressed in most stem cells, epithelium and carcinomas, N-cadherin and P-cadherin are less common. Their roles in cell signaling are studied mostly using cancer and somatic cells. N-cadherin was found to upregulate PI3K/AKT in PC3 cells that also express E-cadherin (Tran et al., 2002). Another research reported that N-cadherin enhanced AKT

activation to regulate β-catenin Wnt signaling in cortical precursors without mentioning E-cadherin (Zhang et al., 2013). However, N-cadherin was able to support mESC derivation, maintenance and even iPSC generation from fibroblasts without E-cadherin (Bedzhov et al., 2013). These observations suggest that cell-cell adhesion mediated by these cadherins is likely more important than the specific type of cadherin in signaling events. But P-cadherin seems more different from E- and N-cadherin. It can also activate Rho GTPases Rac1 and Cdc42 like E-cadherin, this function of P-cadherin is essential for migration and invasion of ovarian cancer regardless of E-cadherin and was mediated by accumulation of p120 catenin (Cheung et al., 2010). On the other hand, P-cadherin was reported to regulate expression and activation of α6β4 integrin, a laminin receptor. By studying a breast cancer cell model, α6β4 appeared to be a downstream target of P-cadherin. High level of P-cadherin lead to increased α6β4 integrin expression and cell adhesion to laminin. This effect, in turn, activate AKT through focal adhesion kinases (FAK) and results in tumor growth and invasion (Vieira et al., 2014).

1.2.4 Soluble Growth Supplements

Though interactions with substrates is crucial for maintenance of human pluripotent stem cells, roles of soluble growth supplement provided by medium cannot be ignored. Basic fibroblast growth factor (bFGF), also known as FGF2 or FGF-β, is a common supplement in many hPSC media formulas. There have been various reports on the ability of bFGF to help maintain hPSC cultures (Levenstein et al., 2006; Xu et al., 2005; Saxena et al., 2008), but the exact mechanism of bFGF signaling in hPSCs is not clear. Basic FGF can activate major signaling pathways in hPSCs including MAPK/ERK and all three branches of PI3K/Akt (Nakashima and Omasa, 2016), and can act both exogenously and endogenously. It was found

that exogenous bFGF has slightly different effect compared to bFGF produced by hESC themselves, while inhibition of the latter resulted in rapid differentiation (Dvorak et al., 2005). Some researchers discovered that bFGF signaling may lead to inhibition of bone morphogenetic protein signaling to help maintain pluripotency (Diecke et al., 2008). More interestingly, another group reported that exogenous FGF interact with FGFR1 on differentiated hESC fibroblast-like cells, leading to secretion of insulin growth factor-II (IGF-II), that in turn interact with human ESCs to support self-renewal and pluripotency (Bendall et al., 2007).

As mentioned earlier, TGFβ signaling is a major pathway in human pluripotent stem cells. Activin, Nodal and TGF-β are the most studied, while Activin A and TGF-β are commonly used as hPSC media supplement. Activin/Nodal interact with transmembrane type II Activin receptors (ActRII/IIB) to recruit and activate ALK4, or ActRIB, which is a member of type I Activin receptors (ALKs). Similarly, TGF-β binds to different receptors TGFBRI and TGFBRII, also known as ALK5. ALK activation triggers formation of Smad2/3 complex, which interact with Smad4 and relocate into the nucleus to maintain expression of Oct4 and Nanog (Pauklin and Vallier, 2015). TGFβ signaling is important in maintenance of pluripotency (Xiao et al., 2006; Yamasaki et al., 2014; Singh et al., 2012). However, evidences suggested complicate cross-talk between TGFβ signaling and FGF signaling. It was shown that without bFGF, Activin A alone was able to support long-term hESC culture (Xiao et al., 2006), and FGF inhibition can be rescued by extra Activin addition (Pauklin and Vallier, 2015). In addition, it was reported that FGF signaling can be inhibited through addition of an Alk4/5/7 receptor inhibitor SB431542. This suggested that FGF signaling was at a certain level depending on TGFβ signaling (Vallier et al., 2005).

Except for these two most important ones, insulin/insulin growth factor (IGF) is also important, as the insulin-like growth factor-1 receptor (IGF-1R) signaling is crucial for glucose metabolism, consequently affect cell survival (Wang et al., 2007). Investigation of gene expression pattern in hESCs also shown that IGF1R was among the 20 most positively significant receptor genes (Sperger et al., 2003). Unlike bFGF and TGFβ, IGF appeared to be functionally independent and might be dispensable in hPSC maintenance. One group reported successful long-term hESC culture in medium lacking IGF-II (Montes et al., 2009).

Bovine serum albumin (BSA) is another supplement found in many media formulas. It acts as lipid carrier (Garcia-Gonzalo and Izpisúa Belmonte, 2008), and provide nutrients for MEF, as well as protection against β-mercaptoethanol in feeder-dependent culture system (Nakashima and Omasa, 2016). Some researchers also find that BSA adsorption on to synthetic surfaces can enhance cell adhesion, but this effect can be weakened by other media components (Irwin et al., 2011). Other common supplements usually act to provide functions similar to those found under *in vivo* conditions. For example, transferrin functions as iron transporter to ease oxygen utilization and promote cellular enzyme activities; Selenium, on the other hand, is part of cellular antioxidant defense system and is effective in protecting cells from oxidative damages (Nakashima and Omasa, 2016).

Besides these essential ones, there is also an optional supplement, Rho kinase inhibitor (ROCKi) Y-27632. Rho is a small GTP-binding protein that functions through Rho kinases and affects various cellular processes like cell shape, motility, proliferation, and even gene expression (Liao et al., 2007). Y-27632, (R)-(+)-trans-4-(1-aminoethyl)-N-(4-pyridyl)cyclohexanecarboxamide dihydrochloride monohydrate ($C_{14}H_{21}N_3O \cdot 2HC1 \cdot H2O$), is a synthetic compound. It inhibits ROCK by competing with ATP for the catalytic site. But the

inhibition mechanism of Y-27632 is more complex and potent that the reaction cannot be reversed by elevated ATP concentration (Narumiya et al., 2000). Y-27632 was chosen as a solution to the apoptosis problem of hPSC culture after complete dissociation and was proven to promote cell survival in both feeder-dependent and feeder-free conditions (Watanabe et al., 2007). It was also demonstrated that addition of Y-27632 during the thawing process can largely improve recovery of cryopreserved hPSCs (Claassen et al., 2009). The exact mechanism behind the function of Y-27632 is not yet clear. It has been suggested that loss of E-cadherin mediated cell-cell contact upon complete dissociation would lead to myosin hyperactivation mediated by ROCK, resulting in apoptosis and Y-27632 can block this process. Another theory proposed actin-myosin contraction as the major reason of apoptosis and Y-27632 could disrupt this contraction to promote cell survival (Kurosawa, 2012).

1.3 Summary

A defined 3D culture system for long term maintenance of hiPSCs is needed to advance researches on hiPSC and its potential clinical application. But currently available options cannot replace 2D culture methods in maintenance of hiPSCs as they are hard to operate, has limited testing on long term hiPSC maintenance, or do not allow some common testing methods like fixation and immuno-staining. Besides maintenance of hiPSC in 3D culture systems, it is also meaningful to explore the differences in hiPSCs cultured in 2D and 3D, as the great differences in culture conditions would possibly cause changes in cell reaction, including gene expression levels, regulations of signaling pathways, requirements of some micro-nutrients.

Objectives

The objective of this research was using a new peptide based hydrogel – hiPSC-qualified PGmatrix (PGmatrix-hiPSC) to develop a 3D culture system that is defined, easy to operate and can support long term culture of human induced pluripotent cells while maintaining their pluripotency. By comparing gene expression of hiPSCs cultured continuously in 2D and 3D, we plan to explore the effect on different growth environment on gene expression that may indicate the importance of culture method for more accurate results in developmental and clinical researches.

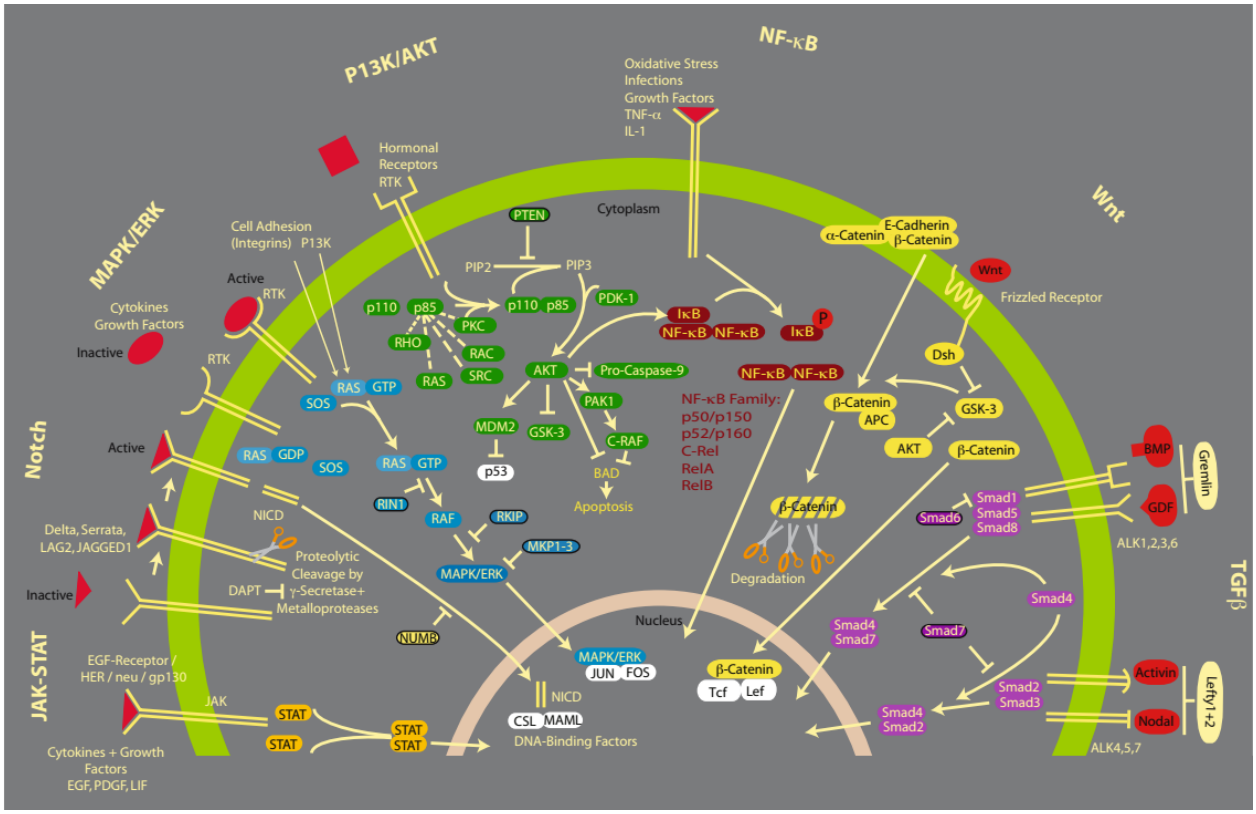

Figure 1.1 Major signaling pathways in cancer and embryonic stem cells.

MAPK/ERK, PI3K/AKT, and TGFβ are the most important pathways in human pluripotent stem cells as they tightly regulate cell growth as well as maintenance of pluripotency. MAPK/ERK pathway is activated by growth factors, integrins as well as through crosstalk with PI3K/AKT. When activated, signal is relayed from receptor tyrosine kinase (RTK) to MAPK/ERK through a series of phosphorylation and eventually affect gene expression in the nucleus. PI3K/AKT also receives signals from RTKs and controls cell survival and growth through a network centered on AKT protein. TGFβ pathway is activated by growth factors belonging to TGFβ family and affect gene expression and protein production through a complex Smad network. Wnt signaling has been found related to hPSC differentiation and is centered on β-catenin. The role of NF-κB is not clear in hPSC, but both Wnt and NF-κB have crosstalks with PI3K/AKT.

Note: from Dreesen, O., and A.H. Brivanlou. 2007. Signaling pathways in cancer and embryonic stem cells. Stem Cell Rev. 3:7-17. Copyright was permitted by Springer.

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Chapter 2 - Materials and Methods

2.1 2D Cell Culture

One of the human iPSC lines was obtained from Applied Stemcell, Inc., Milpitas, CA (ASC-hiPSC), the other hiPSC line was bought from Thermofisher, Waltham, MA (TF-hiPSC). The hiPSCs from ASC were cultured on six-well plates coated with Matrigel (Corning Life Sciences, Tewksbury, MA) in mTeSR1 medium (Stemcell Technologies, Vancouver, Canada) with 1% penicillin/streptomycin (Quality Biological Inc, Gaithersburg, MD) added, while the hiPSCs from Thermofisher were cultured on six-well plates coated with Vitronectin-XF (Stemcell Technologies) using the same culture medium. Passage time was determined according to cell confluency by microscope visual assessment, and the cells were usually grown for 4 to 6 days after seeding. To passage hiPSC from 2D culture, hiPSCs were treated with 1 mL ReLeSR™ (Stemcell Technologies) and incubated for 5 minutes, then ReLeSR™ was removed and 700 µL DMEM/F12 was added. The plates were gently tapped to detach cells from 2D surface, cell suspension was collected and gently pipetted to be dissociated into small clusters. After 5 minutes of centrifugation at 200×g, cell pellet was collected and resuspended in mTeSR1 medium supplemented with 1% penicillin/streptomycin and 10 µM ROCK inhibitor Y-27632 (Stemcell Technologies). Cell suspension was re-plated on to Matrigel-coated (for ASC-hiPSC) or Vitronectin-XF coated (for TF-hiPSC) six-well plates at a seeding density of around 5×10^4 cells/cm^2 in mTeSR1 medium with 1% penicillin/streptomycin and 10 µM ROCK inhibitor Y-27632. Cells were fed by changing medium on day 2 with mTeSR1 with 1% penicillin/streptomycin and every day after that.

2.2 Surface Plating of hiPSC on PGmatrix

100 µl 0.5% hiPSC-qualified PGmatrix (PGmatrix-hiPSC) was prepared by mixing 45 µl mTeSR1 medium with 5 µl PGwork and 50 µl PGmatrix-hiPSC. The mixture, after thorough mixing, was placed in one well of a 24-well plate to cover the entire bottom. The plate was incubated for 30 minutes at 37°C for gelation. Then 1 ml of hiPSCs suspension in mTeSR1 was added gently on top of the formed gel. Culture medium was changed daily starting from day 2. PGmatrix-hiPSC were also supplemented with small amount of ECM proteins vitronectin (Sigma-Aldrich, St. Louis, MO) and laminin (Sigma-Aldrich, St. Louis, MO) to promote surface adhesion as for comparison purposes: vitronectin-PGmatrix-hiPSC (PGV) and vitronectin-laminin-PGmatrix-hiPSC (PGVL).

2.3 Optimization of 3D culture conditions

According to information obtained from literature review, survival and growth of hiPSC depends on cell-cell interactions, growth supplement (growth factors), as well as elasticity and stiffness of the substrate. The method of encapsulating hiPSCs into PGmatrix-hiPSC was described in detail in Section 2.3. Optimization of 3D culture condition for hiPSC was centered on four factors: cell seeding density, level of growth supplement, gel strength and level of ROCK inhibitor Y-27632 supplementation. Three seeding densities were used: low (1×10^5 cells/ml), medium (5×10^5 cells/ml), and high (1×10^6 cells/ml). Culture medium used in this project is mTeSR1, which is prepared by combining basal medium with solution of growth supplement. By adjusting the ratio of basal medium and growth supplement, three levels of growth supplement in mTeSR1 basal medium were tested: 1× (normal level), 1.5×, and 2×. Gel strength was changed by using different amount of PGmatrix-hiPSC stock solution during the encapsulation step,

yielding three final gel concentrations of 0.2%, 0.3%, and 0.5%, corresponding to gel strength of about 380 Pa, 500 Pa, and 800 Pa, respectively. Supplementation of ROCK inhibitor Y-27632 was conducted by adding different amount of prepared stock solution into culture medium to obtain three different final concentrations: 0 μM, 10 μM, and 30 μM.

2.4 3D Cell Culture

To transfer hiPSCs from 2D to 3D, hiPSCs were suspended in mTeSR1 medium with 1% penicillin/streptomycin and 10 μM ROCK inhibitor Y-27632, and counted with a cellometer Auto2000 (Nexcelom Bioscience, Lawrence, MA). Cells were encapsulated in 3D hydrogel using PGmatrix-hiPSC kit (PepGel LLC., Manhattan, KS). The seeding density of hiPSCs was 1.8×10^5 cells/mL for ASC-hiPSC, and 2 - 3×10^5 cells/mL for TF-hiPSC. For 3D cell culture in 24-well plate with 0.5% gel strength, 240 μL of cell suspension were mixed with 10 μL PGwork in a 1.7 mL microtube, then 250 μL PGmatrix-hiPSC were added and mixed thoroughly without introducing air bubbles. The mixture was plated into one well of a 24-well plate. The process was repeated to produce more wells of 3D culture. The plate was then incubated at 37℃ for 30 minutes to allow gel formation. 1 mL of mTeSR1 medium with 1% penicillin/streptomycin and 10 μM ROCK inhibitor Y-27632 was added gently on top of the formed hydrogel after incubation. Medium was changed daily started from day 2.

To thaw hiPSCs stored in liquid nitrogen, one vial of stored cells was taken out from liquid nitrogen storage and warmed in 37°C until half thawed. The vial was sprayed and wiped with 70% ethanol and took into a biosafety hood. Pre-warmed mTeSR1 medium was added and pipetted, the mixture was collected into a 15 mL conical tube. More medium was added to the same tube and centrifuged at 200×g for 5 minutes and the cell pellet was resuspended in

mTeSR1 medium supplemented with10 μM Y-27632. The cell suspension was counted, and encapsulated in PGmatrix-hiPSC at three seeding number: 1×10^5 cells/well, 3×10^5 cells/well, and 5×10^5 cells/well using a 24-well culture plate following the method described above. The cells were grown for 5 days.

To release cells from 3D PGmatrix-hiPSC culture system, medium on top of the gel was removed and 500 μL DPBS without Ca^{2+}/Mg^{2+} was added. The gel was disrupted by pipetting thoroughly and the mixture was collected into a 50-mL conical tube. Another 1 mL DPBS was added to rinse the well and collect any remaining materials. DPBS without Ca^{2+}/Mg^{2+} was then added to the conical tube to dilute the mixture more than 20 folds. The tube was centrifuged at 200×g for 6 minutes and the supernatant was removed. 1 mL 0.5 mM EDTA solution was added and gently pipetted to resuspend the cells and incubated at 37℃ for 9 minutes to dissociate cell colonies. Then 0.5 mL mTeSR1 medium was added to stop the dissociation. The cell suspension was centrifuged at 200×g for 5 minutes and the cell pellet was resuspended in 1 mL of mTeSR1 medium with 1% penicillin/streptomycin and 10 μM ROCK inhibitor Y-27632 for re-encapsulation in 3D PGmatrix-hiPSC.

2.5 Immunochemistry in 3D

To stain cells directly in 3D PGmatrix-hiPSC culture system, medium on top of the gel was gently removed, the culture was washed twice with DPBS. 1 mL 10% formalin (Fisher Scientific, Waltham, MA) was added to each well and incubated at room temperature for 40 minutes for fixation. The fixed samples were rinsed with DPBS and then with washing buffer (DPBS with Ca^{2+}/Mg^{2+} + 0.2% Triton X-100 (Sigma-Aldrich, St. Louis, MO) + 0.1% gelatin (Sigma-Aldrich, St. Louis, MO)). The samples were then blocked with10% normal rabbit serum

(Thermo Fisher Scientific, Waltham, MA), followed by incubation with Goat Oct 3/4 antibody (Santa Cruz, Dallas, TX) overnight. The samples were then rinsed with washing buffer and incubated with rabbit anti-goat antibody alexa fluor 488 (Thermo Fisher Scientific, Waltham, MA). After incubation with secondary antibody, the samples were rinsed with washing buffer, followed by addition of glycerin for imaging.

2.6 Flow Cytometry

Cell spheroids grown in 3D PGmatrix-hiPSC system was released following the 3D cell harvesting procedure in section 2.4, then incubated with 0.5 mM EDTA solution for 15 minutes to obtain single cell suspension. Cell samples were stained first with two surface marker antibodies anti-SSEA4 and anti-TRA-1-81 (BD Biosciences, San Jose, CA), then fixed and stained for transcription factors Nanog, Oct3/4 and Sox2 using human pluripotent stem cell transcription factor analysis kit (BD Biosciences, San Jose, CA). Three stained cell samples, one unstained sample, an isotype control together with six single color controls were analyzed with BD LSRfortessa X-20 flow cytometer (BD Biosciences, San Jose, CA). The data acquired was analyzed using BD FACSDiva v8.0.1 software.

2.7 RT-qPCR Analysis

RT-qPCR analysis was performed to characterize expression levels of pluripotency-related gene in the two hiPSC lines by Kansas State Veterinary Diagnostic Laboratory. Total RNA samples were extracted from each hiPSC sample using Direct-zol RNA MiniPrep kit (ZYMO Research Corp., Irvine, CA, USA) according to the manufacturer's description. The concentration of the total RNA samples was diluted to 20 ng/µl for real-time RT-PCR. RT-qPCR

reactions were conducted with Bio-Rad CFX96™ Touch™ Real-time PCR Detection System using iTaq Universal SYBR Green One-Step Kit (Bio-Rad, Hercules, CA). A volume of 20 µL RT-qPCR reaction contains 10 µL of 2×iTaq universal SYBR Green reaction mix, 0.25 µL iScript reverse transcriptase, 1 µL of 10 µM forward and reverse primer mix solution (final concentration 500 nM), 5 µL RNA sample, and 3.75 µL nuclease-free water. The first reaction was reverse transcription reaction at 50°C for 10 min, then polymerase activation and cDNA denaturation at 95°C for 1 min, followed by 45 cycles of denaturation at 95°C for 10s and annealing and extension at 60°C for 40s for hTERT, ESG-1, DNMT3B, and 3 House Keeping genes (EID2, ZNF324B, and CAPN10) (Holmgren et al., 2015), or annealing at 52°C for 30s, and extension at 72°C for 20s for the rest genes (UTF1, NANOG, OCT4, REX1, SOX2, Myc, DPPA4, FGF4, and GDF3). Three replicates were conducted for each gene in all samples. The Ct values were analyzed using Bio-Rad CFX Manager 3.0 software. The expression fold change of a target gene in a tested sample was compared with corresponding control samples and normalized with the average expression levels of the 3 housekeeping genes.

2.8 Karyotype Analysis

ASC-hiPSCs cultured in 3D PGmatrix-hiPSC continuously for 37 passages were harvested and fixed. Karyotype analysis of the fixed sample was conducted by Cell Guidance System LLC.

2.9 Statistical Analysis

Statistical analyses were done using the statistical software SAS university edition. For comparisons, the means were compared using t-test with α level set as 0.05.

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Chapter 3 - Results and Discussion

3.1 Optimization of 3D culture conditions

As mentioned in Chapter 1, previous studies have shown that survival and growth of hiPSC depends on many factors. Among them, some factors can be controlled through the culture environment, for example, nutrient supply, cell-cell interactions, and substrate properties. Nutrient supply is controlled through culture medium formulation. Cell-cell interaction activities are significantly influenced by cell seeding density: as the farther cells are from each other, the harder for them to transmit signaling molecules. For 3D culture based on hydrogel, gel strength is an important substrate property that is adjusted by changing gel concentrations.

We first tested three cell seeding densities and increasing growth supplement levels in culture medium to evaluate how hiPSC survival and colony formation would be affected without addition of ROCK inhibitor Y-27632. Changes in growth supplement level did not have significant influence on hiPSC survival when comparing morphology of hiPSC with the same cell seeding density cultured using mTeSR1 that had different growth supplement levels (Figure 3.1A-C). Through hiPSCs with growth supplement level increased to 1.5× seemed to form slightly more small colonies. By comparing hiPSC with the same 1.5× supplement at different cell seeding densities, it was clear that higher cell seeding density had largely improved hiPSC survival and colony formation (Figure 3.1B, D, E), probably because the shorter distance between cells facilitated cell-cell interactions. When cell seeding density is high enough, the effect of increased growth supplement level is almost not observable (Figure 3.1E, F). These results suggested that without ROCK inhibitor, higher cell seeding density is more important for hiPSC survival and colony formation than additional growth supplement.

Furthermore, we examined the effect of gel strength without the addition of ROCK inhibitor. When hiPSCs were encapsulated in 0.2% or 0.3% PGmatrix-hiPSC, they behaved similar to hiPSCs in suspension. Since gel strength is relatively low, hiPSCs can approach each other slowly and eventually form giant clusters (Figure 3.2A, B). When increased gel concentration to 0.5%, gel strength was largely improved, cells were restrained and the colonies were more uniform in sizes (Figure 3.2C).

Without addition of ROCK inhibitor, cell survival and colony formation were generally non-efficient, the formed colonies are usually large with dense and dark centers that resemble the morphology of embryoid bodies. When ROCK inhibitor Y-27632 was added, hiPSCs form small, clear spheres in a short time, both population expansion and viability were greatly improved. But, when final concentration of Y-27632 was higher than 10 μM, population expansion and viability decreased (Figure 3.2D-F). This indicated that though ROCK inhibitor Y-27632 can greatly improve hiPSC survival and colony formation, it does not function in a dose-dependent manner, there is an optimum concentration of ROCK inhibitor. According to the results, 10 μM ROCK inhibitor Y-27632 was used in the following studies.

From these results, we found that hiPSC 3D culture system requires a gel strength around 800 Pa to form uniform colonies, and 10 μM ROCK inhibitor Y-27632 is needed with mTeSR1 medium at normal growth supplement level. Cell seeding number per well for 3D culture here was similar to that used for 2D cultures. But when recovering from cryopreservation, seeding density should be 1~2 times higher than regular 3D cell culture for better cell-cell interactions that improve hiPSC survival.

3.2 Differences in hiPSC Performance between 2D and 3D cultures

3.2.1 HiPSC Morphology in 2D and 3D

As shown in Figure 3.3, hiPSCs grown in 2D and 3D possess distinctively different morphologies. When grown in 2D on Matigel (Figure 3.3A) or Vitronectin-XF (Figure 3.3C) coating, cell adhesion depends solely on the one side that get in contact with the coated surface. Therefore, hiPSCs grown in 2D assume a flattened shape and form compact, single-layered colonies. When hiPSCs started to grow rapidly, expansion of these formed colonies is limited on the coated surface, and is thus restricted by the surface area available.

On the other hand, when cultured in 3D through encapsulation with PGmatrix-hiPSC, hiPSCs are surrounded by soft hydrogel that consists of over 99% liquid. The hydrogel mimics the *in vivo* environment and allows cells to populate in a three-dimensional manner. As a result, colonies form small spheres like those shown in Figure 3.3B and 3.3D. This 3D culture method make good use of the space along Z axis.

3.2.2 Adhesion to PGmatrix-hiPSC in 2D

Since hiPSCs encapsulated in PGmatrix-hiPSC formed spheres and expand rapidly, we initially explored hiPSC adhesion by plating cells on a thin layer of formed gel. Without supplementation of ROCK inhibitor, ASC-hiPSCs did not survival nor attach to the gel (Figure 3.4A). When 10 μM ROCK inhibitor was added, ASC-hiPSCs form spheres that did not attach to the gel very well, cells were easily lost during media changes (Figure 3.4C). Many researches have shown that ECM proteins like vitronectin and laminin can serve as substrates for 2D culture of hiPSCs (Braam et al., 2008; Chen et al., 2011; Miyazaki et al., 2008; Miyazaki et al., 2012), therefore, we incorporated some ECM proteins to PGmatrix-hiPSC to improve cell adhesion.

With 0.2% vitronectin in PGmatrix-hiPSC, some ASC-hiPSCs formed colonies without ROCK inhibitor supplement, morphology of the colonies was similar to those formed in Matrigel 2D cultures (Figure 3.4B), but colony forming efficiency was low. When both vitronectin and laminin were added, colony formation was significantly improved without ROCK inhibitor, but severe differentiation can be observed (Figure 3.4D).

These results from surface plating revealed that mechanism of hiPSC adhesion and survival is very different in 2D from 3D environment. When surrounded by hydrogel in a 3-dimensional way, hiPSCs did not seem to require binding with specific ECM proteins. However, in 2D environment, cells require strong binding in order to survive and form colonies. In addition to vitronectin, laminin improved colony formation, but severe differentiation was found in hiPSCs cultured on vitronectin-laminin-PGmatrix-hiPSC (PGVL), which could be a result of insufficient binding, as only small amount of vitronectin (0.5%) and laminin (0.2%) was added. Comparison between Figure 3.4A and C indicated that ROCK inhibitor promote cell survival through enhanced cell-cell interaction, instead of improving cell-matrix adhesion.

3.2.3 HiPSC Growth and Viability in 2D and 3D

In 3D PGmatrix-hiPSC culture system, hiPSCs go through distinctive growth phases. At the beginning day 0 and day 1, cells recover from passaging treatments and adapt themselves to the environment, thus show basically no growth. At around day 2, cells started to expand their population and grow rapidly. Increase in sphere size is clear when compared day 3 photo with day 2 in Figure 3.5. During continuous 3D culture, cells were usually harvested on or around day 5, when the spheres were large and cell growth become slower. To illustrate a clear growth curve, 7 wells of a 96-well plate were set up by encapsulating 0.9×10^5 cells per well in 0.3%

PGmatrix-hiPSC. One well of cells were harvested every day to observe cell growth during a 7-day period. The population fold expansion and viability were shown in Figure 3.6, presenting that cell growth remained stagnant for the first two days, then started rapid expansion, which confirmed the observation as shown in Figure 3.5. Around day 6, cells start to compete for the limited space and nutrients, therefore, cell growth decreased on day 7. Based on these results, the passage time of hiPSCs in 3D PGmatrix-hiPSC system should be around day 5 for sufficient population expansion, as well as to avoid nutrient depletion.

ASC-hiPSC and TF-hiPSC were grown continuously in both 3D and 2D environment for multiple passages for comparison purposes. The continuous 3D culture of the two cell lines were maintained using PGmatrix-hiPSC, while 2D culture of ASC-hiPSC was maintained in Matrigel-coated plates and TF-hiPSC culture was maintained in Vitronectin-XF-coated plates. At the end of each passage, cells were harvested and counted with a cellometer Auto2000 using AO/PI staining method, the number was recorded and used to determine the seeding number for next passage. The total population fold expansion was calculated through dividing the total number of cells harvested by the number of cells seeded. Population fold expansion and viability of two hiPSC lines in 3D and 2D for multiple passages were shown in Figure 3.7 (ASC-hiPSC) and 3.8 (TF-hiPSC). Statistical analysis of these data showed that for both ASC-hiPSC and TF-hiPSC, the total population fold expansion and viability in 3D PGmatrix-hiPSC system was significantly ($P < 0.05$) higher than in 2D culture. The distribution of population fold expansion and viability for both cell lines in 3D was also significantly smaller than those in 2D systems (Figure 3.7C, 3.7D, 3.8C, and 3.8D). The results indicated that hiPSCs maintained more consistent growth and viability in 3D PGmatrix-hiPSC system than on Matrigel and Vitronectin-XF.

These results demonstrated that 3D PGmatrix-hiPSC system, comparing with 2D culture systems, is more robust for maintenance of hiPSC culture. When cultured in 2D Matrigel or Vitronectin-XF-coated plates, cells adhere to a thin layer of ECM proteins, and are exposed directly to culture media and can sense changes in the environment spontaneously. This sensitivity may be advantageous for genetic engineering, but for expansion and maintenance of cell integrity, this sensitivity made hiPSCs prone to changes in media nutrients and pH, as well as atmosphere oxygen content which is much higher than that *in vivo*. These factors would negatively affect cell growth and integrity. On the contrary, hiPSCs cultured in 3D PGmatrix-hiPSC were surrounded by hydrogel, where the hydrogel acts as a protecting cushion that slows down changes happened to the environment. For example, when new culture medium was added on top of the gel, the nutrients had to diffuse into the gel to reach the cells, instead of add new medium directly on cells, allowing cells to adapt to changes such as pH and temperature. In addition, the hydrogel also acts as an oxygen buffer to maintain oxygen content desirable for cells. Inside human body, the physiological oxygen level is about 2% - 9%. It has been proven that many human stem cells, like embryonic stem cell and mesenchymal stem cell were maintained better under low oxygen condition (hypoxia). There is also proof that hypoxia can improve generation of hiPSC. This effect was explained by the discovery of hypoxia inducible factors (HIFs). This family of transcription factors are only activated under hypoxic conditions to regulate a wide range of genes critical to the survival and maintenance of human stem cells (Mohyeldin et al., 2010). Lower oxygen content is beneficial in maintenance of hiPSC, which is one of the factors that contributes to the consistent population expansion and better viability of hiPSCs in 3D PGmatrix-hiPSC system.

3.3 Cryopreservation and Thawing of hiPSCs Grown in 3D

It has been proven that hiPSCs can be transferred easily from conventional 2D culture into 3D PGmatrix-hiPSC system. To escape from the 2D culture steps, we cryopreserved hiPSCs that have been cultured in 3D PGmatrix-hiPSC and thawed them directly into the same 3D PGmatrix-hiPSC system using a 24-well plate at three different seeding densities: 1×10^5 cells/well, 3×10^5 cells/well, and 5×10^5 cells/well. As shown in Figure 3.9A-C, hiPSCs survived very well at all three cell seeding densities. When harvested on day 5, the total population fold expansion and viability for each cell seeding density were shown in Figure 3.9D, demonstrating that hiPSC can be thawed directly into 3D PGmatrix-hiPSC culture system and still maintain rapid growth rate with satisfied expansion and viability with seeding density ranging from 1 ~ 3 times of that used for routine 3D hiPSC culture in PGmatrix-hiPSC.

3.4 Expression of Pluripotency-related Markers and Genes

3.4.1 Expression of Pluripotency Markers

To date, none of the reported 3D culture systems for hiPSCs have yet conducted immune-staining directly in 3D. We successfully fixed a 5-day 3D culture of ASC-hiPSC, and conducted staining for Oct 4 (Figure 3.10). The background fluorescence was influenced by cells in other plenary locations, and can be minimized by adjustment of microscope settings and image processing software. To better evaluate pluripotency of hiPSCs culture in 3D PGmatrix-hiPSC system, we conducted flow-cytometry test on both ASC-hiPSC and TF-hiPSC cultured continuously in 3D PGmatrix-hiPSC system for more than 25 passages. The expression of two surface pluripotency markers SSEA4 and TRA-1-81, and three transcription factors Oct 3/4, Sox2, and Nanog were evaluated. Results showed that Sox2, Oct 3/4, Nanog and SSEA4 were

detected on most of ASC-hiPSC cells (Figure 3.11). For TF-hiPSC, Sox2, Oct 3/4, and SSEA4 were detected on most cells, but Nanog expression was detected in about 50% of tested cells, though the PCR results discussed in the following section indicated that the expression level of Nanog was actually slightly higher for TF-hiPSCs cultured in 3D PGmatrix-hiPSC than those cultured in 2D system. TF-hiPSCs were cultured for more than 30 passages before we started to culture them in our lab, the length of *in vitro* culture of TF-hiPSC may induce certain level of cell degradation, making this cell line more prone to differentiation, causing decrease in the detected expression of Nanog by flow-cytometry.

Compared with the expression of SSEA4 and TRA-1-81 data obtained from various hPSC lines cultured in 2D around the world (Adewumi et al., 2007), SSEA4 was detected at a higher level (> 99%) and TRA-1-81 was significantly lower (~ 7%) for the two hiPSCs lines used in 3D culture of this research. This difference is likely related to the different culture environment provided by 2D and 3D culture systems. Both SSEA4 and TRA-1-81 are recognized as surface antigens. SSEA4 antigens are glycosphingolipids, and TRA-1-81 is an oligosaccharide structure on podocalyxin, which is a cell adhesion protein (Natunen et al., 2011; Schopperle and DeWolf, 2007). Though both SSEA4 and TRA-1-81 are often used as a pluripotency marker, their expression is not crucial for maintenance of pluripotency. It has been reported that depletion of SSEA3 and SSEA4 in hESCs did not affect their pluripotency as confirmed by *in vitro* differentiation and teratoma formation (Brimble et al., 2007). Other researchers have reported the generation of naïve state human pluripotent stem cells that did not have SSEA4 expression and consisted of both TRA-1-81 negative and positive populations (Pastor et al., 2016). The exact cellular function of SSEA4 and TRA-1-81 antigens are not yet clear, but TRA-1-81 antigen keratan sulfate is structurally related to SSEA1 epitopes expressed

in murine embryos (Henderson et al., 2002). However, both SSEA4 and TRA-1-81 antigens are surface glycolipids or proteoglycans and may involve in cell adhesion. Cell adhesion mechanism in 3D PGmatrix-hiPSC is different from that in conventional 2D culture system. This difference may cause hiPSCs to modify expression of surface antigens to adapt to this new environment, and thus resulted in high expression of SSEA4 antigens and low expression of TRA-1-81 antigens.

3.4.2 Gene Expression Profile of hiPSCs Grown in 2D and 3D

Expression levels of 12 pluripotency related genes in ASC-hiPSCs and TF-hiPSCs cultured continuously in 2D Matrigel (ASC-hiPSC, passages 10 and 15) /Vitronectin-XF (TF-hiPSC, passages 8 and 20) and 3D PGmatrix-hiPSC (passages 2, 5, 10, 20 of ASC-hiPSC, and passages 5, 15, 20, 24 of TF-hiPSC) were determined by RT-qPCR and the data were analyzed with SAS university edition software. Fold change results were normalized using results of passage 10 (2D Matrigel) and passage 8 (2D Vitronectin-XF) as references for ASC-hiPSC and TF-hiPSC, respectively. Initial comparison between different passages did not reveal any distinctive trend in gene expression changes. Therefore, average fold change results from hiPSC cultured in 2D and 3D were compared to identify any possible changes or trends in gene expression as a result of different culture conditions.

Results from the two hiPSC lines had some differences, but the expression levels of Nanog, Oct4, Sox2, DNMT3B, DPPA4, Myc, and ESG1 were not significantly different between cells cultured in 2D and 3D environments in general. For both cell lines culture in 3D system, expression levels of UTF1 and hTERT were significantly higher, and expression level of REX1 was lower than those cultured in 2D systems (Figure 3.12).

As a pluripotency related gene, expression of undifferentiated transcription factor 1 (UTF1) is closely connected with stem cell pluripotency and is more sensitive to pluripotency changes than Oct4 and Nanog. More recent evidence has suggested that UTF may have a role in specific ESC chromatin formation (Morshedi et al., 2013). Another critical factor contributing to proliferation capability of pluripotent stem cells is the integrity of telomeres. Telomeres are DNA caps at the ends of human chromosomes that provide protection against degradation. Well maintained telomere would improve proliferation ability of hiPSCs. It is reported that telomerase is the enzyme responsible for telomere synthesis, which is regulated by transcription of human Telomerase Reverse Transcriptase (hTERT) (Ramlee et al., 2016). Another study found that overexpression of hTERT in human mesenchymal stem cells (hMSCs) shifted their gene expression profile towards an ESC-like state that had better differentiation potential, and have decreased spontaneous differentiation of hMSCs in culture (Tsai et al., 2010). Therefore, higher expression of UTF1 and hTERT in hiPSCs cultured in 3D PGmatrix-hiPSC suggested that these cells can maintain chromosomal integrity and pluripotency better as well as proliferate better than those cultured in 2D systems.

REX1 or Zfp42 is a zinc-finger encoding gene expressed exclusively in early embryos, and have been widely used as pluripotency markers (Kai Chuen Lee et al., 2013). However, there have been studies that found out REX1-/- ESCs can still proliferate and maintain their pluripotency (Masui et al., 2008; Scotland et al., 2009). A recent study discovered that REX1 regulate human stem cell pluripotency by promoting mitochondrial fission to keep mitochondria in immature state and to keep stem cells in a highly glycolytic state (Son et al., 2013). This function of REX1 is critical for hiPSCs culture in 2D environment because of the high oxygen content that would trigger mitochondrial oxidative phosphorylation, which is more important for

energy supply in differentiated cells. However, in 3D PGmatrix-hiPSC system, oxygen level has been "regulated" by the hydrogel, therefore it's likely less necessary to promote REX1 expression to protect cells from oxidative stress caused by atmosphere oxygen content.

It was also observed in Figure 3.12 that the expression of FGF4 and GDF3 was different for hiPSCs cultured in 2D and 3D, and the trends were also different between the two hiPSC lines: expression levels of FGF4 and GDF3 were lower for ASC-hiPSCs in 3D, but higher for TF-hiPSCs cultured in 3D. Fibroblast growth factor 4 (FGF4) was initially thought to be fibroblast-specific. Some studies found that FGF4 depletion in mouse ESC showed defects during differentiation though no effect on ESC growth was observed (Wilder et al., 1997). It has been reported that FGF4 can activate ERK1/2 signaling pathway, which later contributes to neural and mesodermal differentiation. ERK signaling, as mentioned in Chapter 1, is tightly controlled in hPSC for proper pluripotency maintenance (Dalton, 2013). Growth differentiation factor 3 (GDF3) encodes a secreted ligand that belongs to the TGF-β superfamily. This ligand maintains hiPSC pluripotency by inhibiting BMP signaling, which pathway is known to cause differentiation. When hiPSCs differentiated, GDF3 expression reduce to allow differentiation (Ariel J. Levine and Ali H. Brivanlou, 2006).

In 3D PGmatrix-hiPSC system, hiPSCs express high levels of UTF1 and hTERT that all contributes to pluripotency maintenance and connect at certain degree to the core Oct 3/4, Nanog, Sox2 pluripotency regulators (Kosaka et al., 2009). Therefore, FGF4 and GDF3 expression may be less important for regulating ERK signaling and suppress differentiation. However, the differences in expression of FGF4 and GDF3 for the two hiPSC lines tested may relate to the cell source. It has been reported that hiPSCs generated using the same method but from different cell sources would have slightly different gene expression and differentiation

potential (Hu et al., 2016; Veronica Sanchez-Freire et al., 2014). Compared to gene expression in ASC-hiPSC, the results for TF-hiPSC had a generally larger standard deviation for both 2D and 3D cultured cells. Therefore, this large variation in gene expression observed is more likely related to the cell line itself, not culture conditions nor culture length or PCR procedures.

3.5 Karyotype Analysis

Chromosome is a compact structure resulted from a single long DNA molecule tightly packed together with proteins. Human has a total of 46 chromosomes, including 22 pairs of homologs and two sex chromosomes. These chromosomes are numbered 1 – 22 according to their approximate order of size, and can be isolated from cells during mitosis, when they are most compact for better visualization. To detect changes and abnormalities, isolated chromosomes are stained with Giemsa stain to observe the resulted banding patterns. Because banding pattern for each chromosome is unique, any changes in this pattern can be used to determine chromosomal abnormalities in addition to aberrant copy numbers (Alberts et al., 2014). It has been reported that some hESCs would show aberrant karyotype after prolonged culture, these abnormalities are commonly found on chromosome 1, 12, 17 and 20, and includes extra copies of the chromosomes as well as translocations (Amps et al., 2011). It is thus important to perform analysis examining whether hiPSCs cultured in 3D PGmatrix-hiPSC are karyotypically normal.

Karyotype analysis was conducted on ASC-hiPSC that has been cultured in 3D PGmatrix-hiPSC continuously for 37 passages. Results from 20 cells examined showed an apparently normal male karyotype (Figure 3.13). This result demonstrated that long term culture of hiPSCs in 3D PGmatrix-hiPSC would not cause any chromosomal abnormalities.

Maintenance of chromosomal integrity is likely related to upregulated expression of UTF1 and hTERT observed in PCR analysis results. The two genes encode proteins that involved in chromatin organization and DNA protection, their upregulation would enhances these mechanism to protect genetic materials from mistakes in organization and replications.

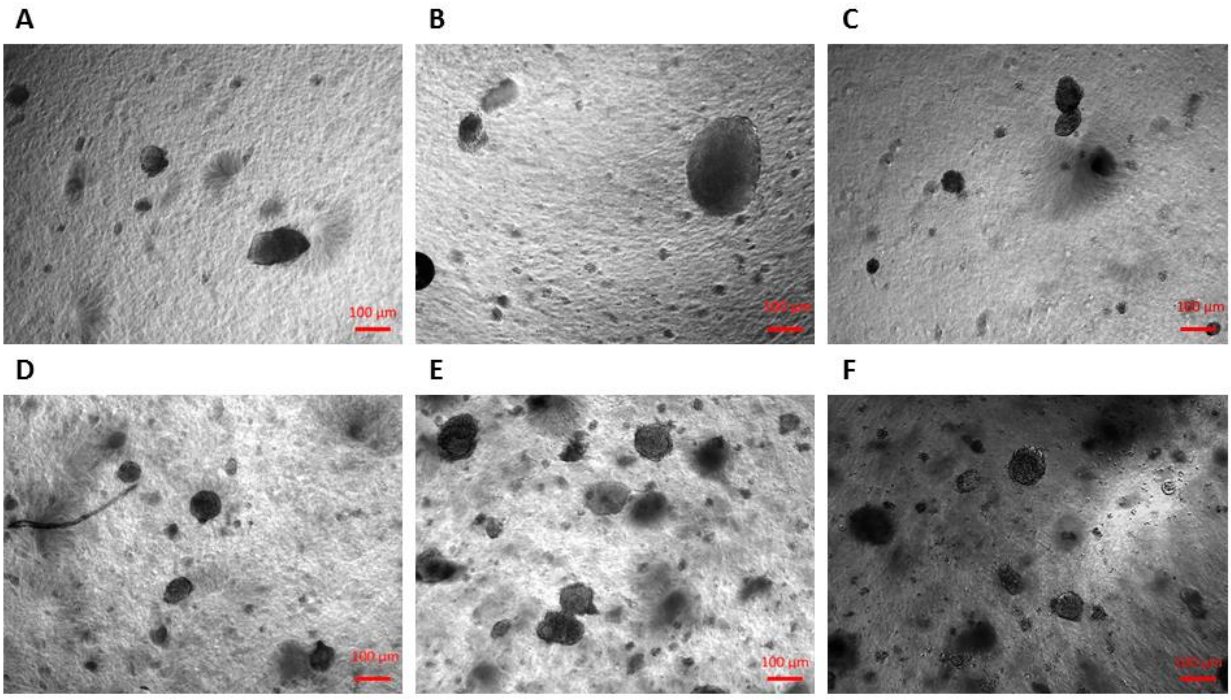

Figure 3.1 Optimization of Seeding Density and Growth Supplement Level.
A. hiPSC in 0.5% PGmatrix-hiPSC at low seeding density (1×10^5 cells/ml) with 1× normal level mTeSR1 growth supplement, day 7; B. hiPSC in 0.5% PGmatrix-hiPSC at low seeding density with 1.5× mTeSR1 growth supplement, day 7; C. hiPSC in 0.5% PGmatrix-hiPSC at low seeding density with 2× mTeSR1 growth supplement, day 7; D. hiPSC in 0.5% PGmatrix-hiPSC at medium seeding density (5×10^5 cells/ml) with 1.5× mTeSR1 growth supplement, day 7; E. hiPSC in 0.5% PGmatrix-hiPSC at high seeding density (1×10^6 cells/ml) with 1.5× mTeSR1 growth supplement, day 7; F. hiPSC in 0.5% PGmatrix-hiPSC at high seeding density with 1× mTeSR1 growth supplement, day 7;

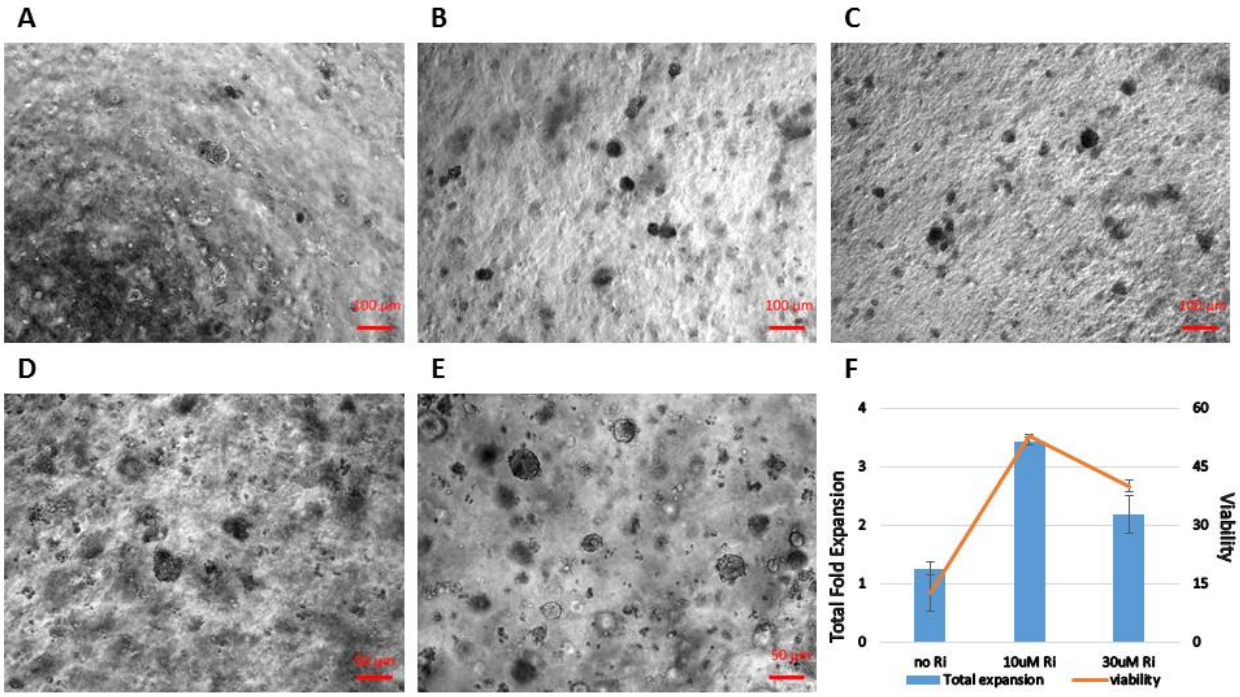

Figure 3.2 Optimization of Gel Strength and Concentration of ROCK inhibitor Y-27632. A. hiPSC in 0.2% PGmatrix-hiPSC with 1.5× mTeSR1 growth supplement, day 5; B. hiPSC in 0.3% PGmatrix-hiPSC with 1.5× mTeSR1 growth supplement, day 5; C. hiPSC in 0.5% PGmatrix-hiPSC with 1.5× mTeSR1 growth supplement, day 3; D. hiPSC in 0.3% PGmatrix-hiPSC with normal mTeSR1 complete medium, day 3; E. hiPSC in 0.3% PGmatrix-hiPSC with normal mTeSR1 complete medium supplemented with 10 µM ROCK inhibitor Y-27632, day 3; F. Comparison of total population fold expansion and viability between hiPSCs cultured in 0.3% PGmatrix-hiPSC using mTeSR1 complete medium supplement with different levels of ROCK inhibitor Y-27632.

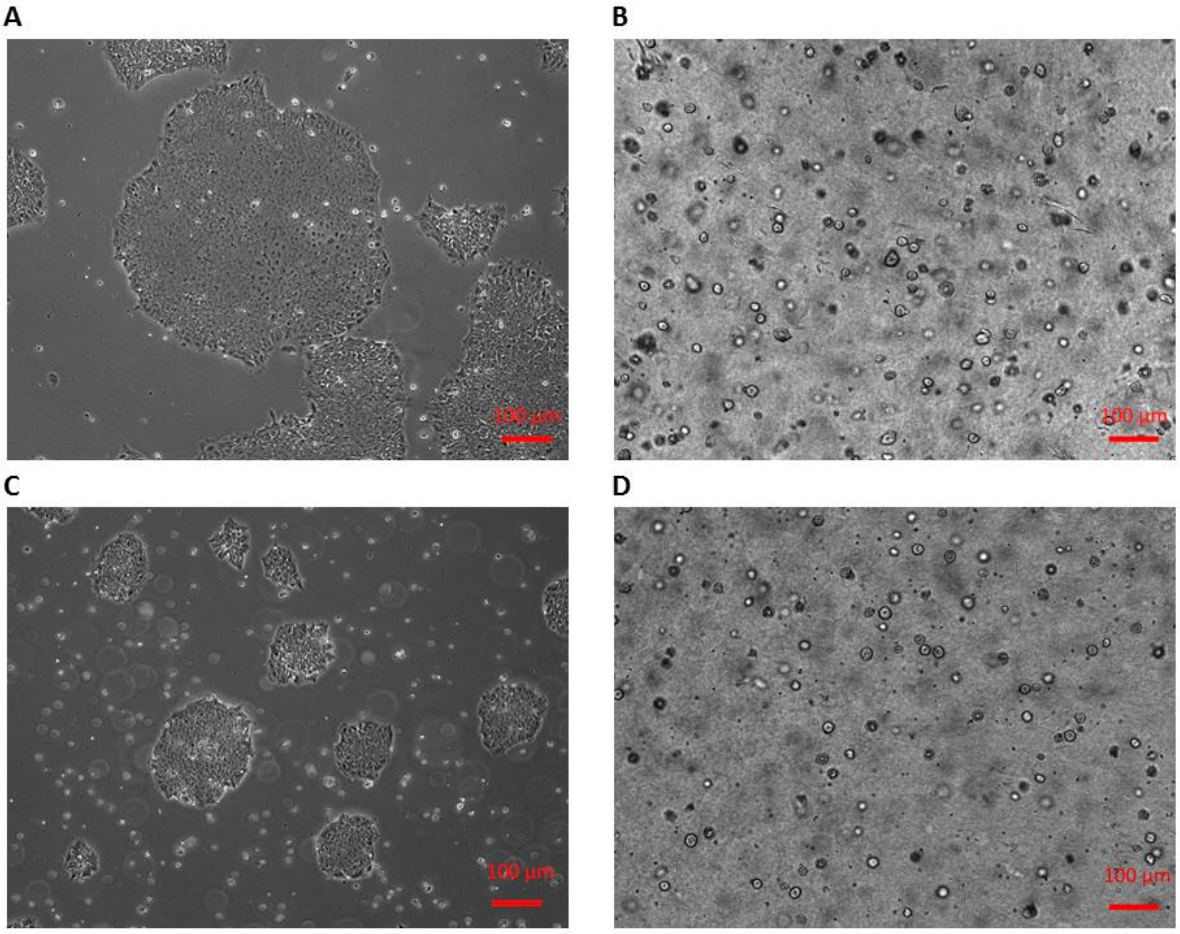

Figure 3.3 Typical hiPSC morphologies in 2D and 3D culture systems.

A. ASC-hiPSCs cultured on 2D Matrigel-coated plate (day 3); B. ASC-hiPSCs cultured in 3D PGmatrix-hiPSC system (day 3); C. TF-hiPSCs cultured on 2D Vitronectin XF-Coated plate (day 2); D. TF-hiPSCs cultured in 3D PGmatrix-hiPSC system (day 3).

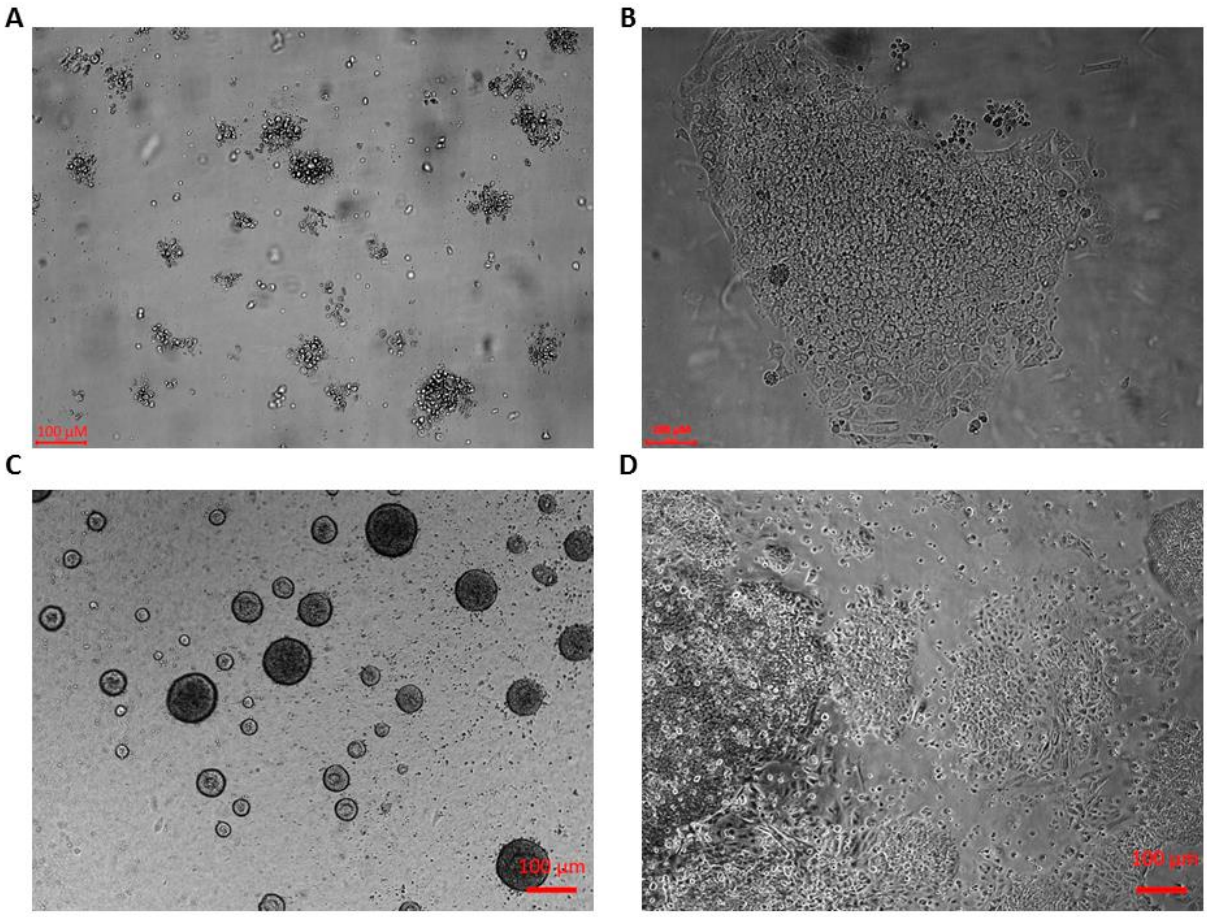

Figure 3.4 Surface plating of ASC-hiPSC on PGmatrix-hiPSC with mTeSR1 medium.
A. ASC-hiPSC plated on PGmatrix-hiPSC without ROCK inhibitor Y-27632; B. ASC-hiPSC plated on vitronectin-PGmatrix-hiPSC (PGV) without ROCK inhibitor Y-27632 formed some colonies; C. ASC-hiPSC plated on PGmatrix-hiPSC supplemented with 10 µM ROCK inhibitor Y-27632; D. ASC-hiPSC plated on vitronectin-laminin-PGmatrix-hiPSC (PGVL) without ROCK inhibitor Y-27632 showed better colony formation efficiency but with severe differentiation.

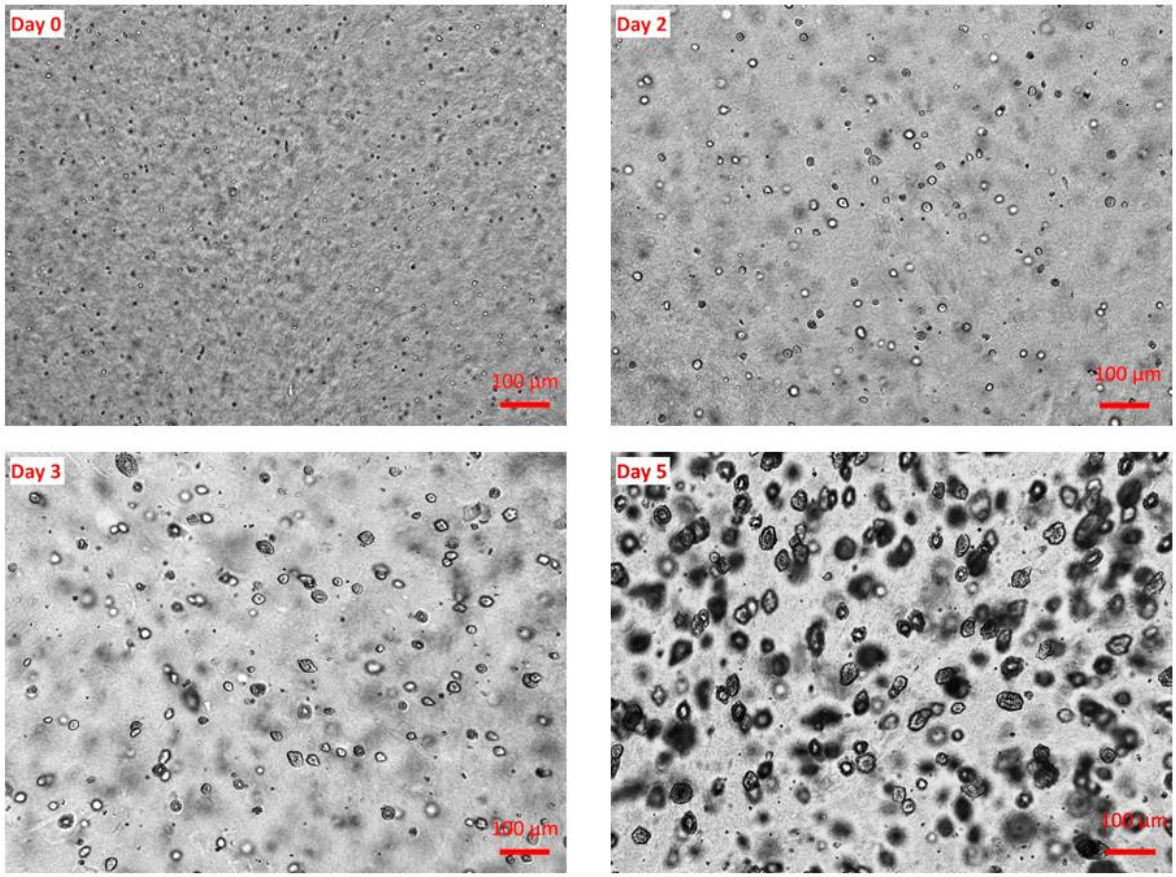

Figure 3.5 ASC-hiPSC growth during a 5-day period.

ASC-hiPSC 3D passage 34 was encapsulated in 0.5% PGmatrix-hiPSC, photos were taken on day 0 (day of encapsulation), day 2, day 3, and day 5 (day of harvesting).

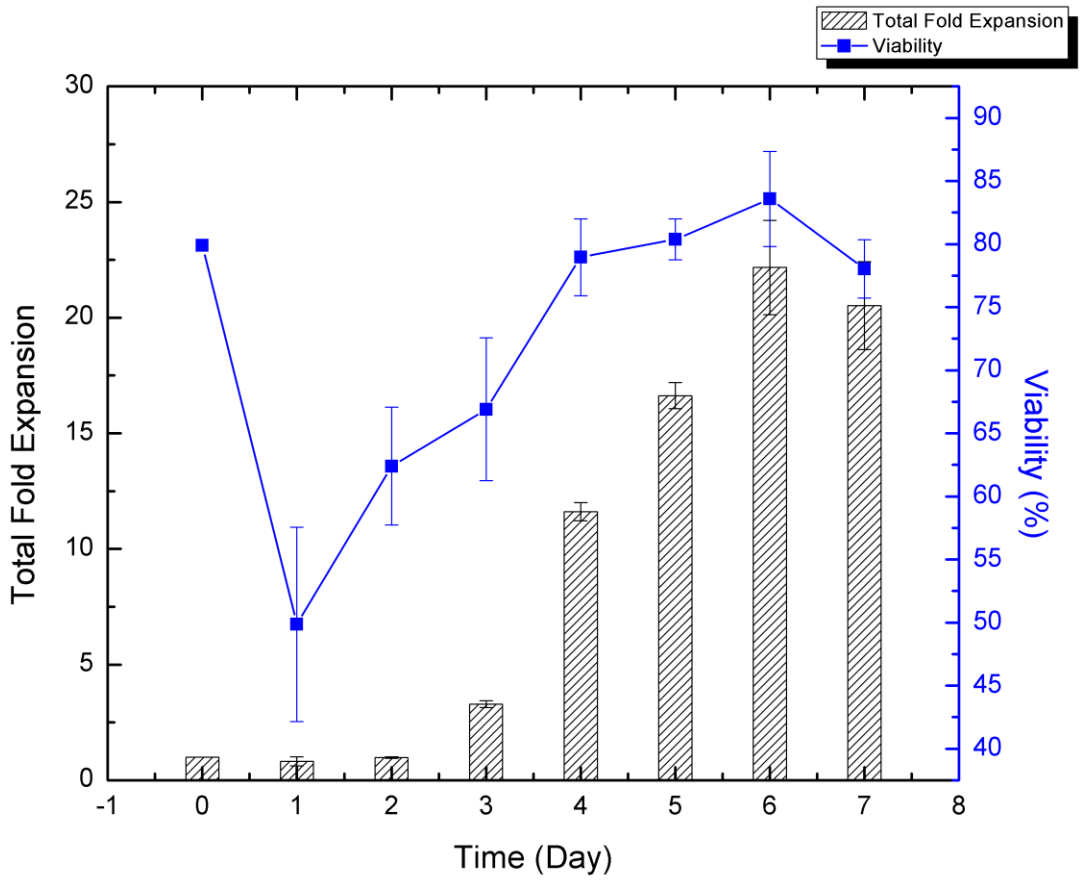

Figure 3.6 Growth and viability of ASC-hiPSC during a 7-day period.

ASC-hiPSCs encapsulated in 0.3% PGmatrix-hiPSC in 96-well plate. Total fold expansion = total cell number harvested / cell number seeded. Viability was obtained from a cellometer Auto2000 using AO/PI staining.

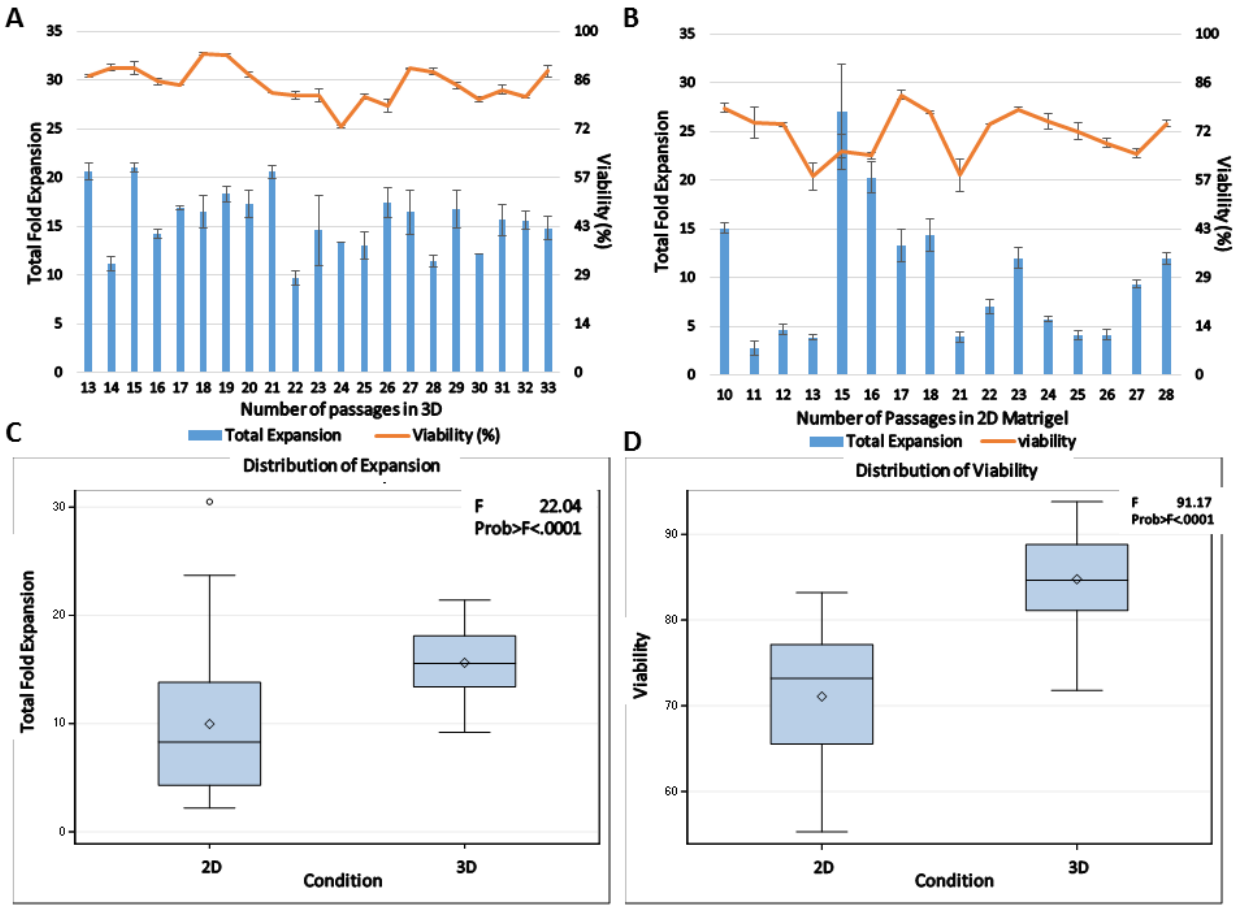

Figure 3.7 Growth and viability of ASC-hiPSCs across 20 passages in 3D and 15 passages in 2D Matrigel.

A. Total population fold expansion and viability of ASC-hiPSCs cultured for 20 passages continuously in 3D PGmatrix-hiPSC; B. Total population fold expansion and viability of ASC-hiPSCs cultured for 15 passages continuously in 2D Matrigel-coated plates; C. Mean and distribution of total population fold expansion for ASC-hiPSCs cultured in 2D and 3D ($P < 0.05$); D. Mean and distribution of viability for ASC-hiPSCs cultured in 2D and 3D ($P < 0.05$).

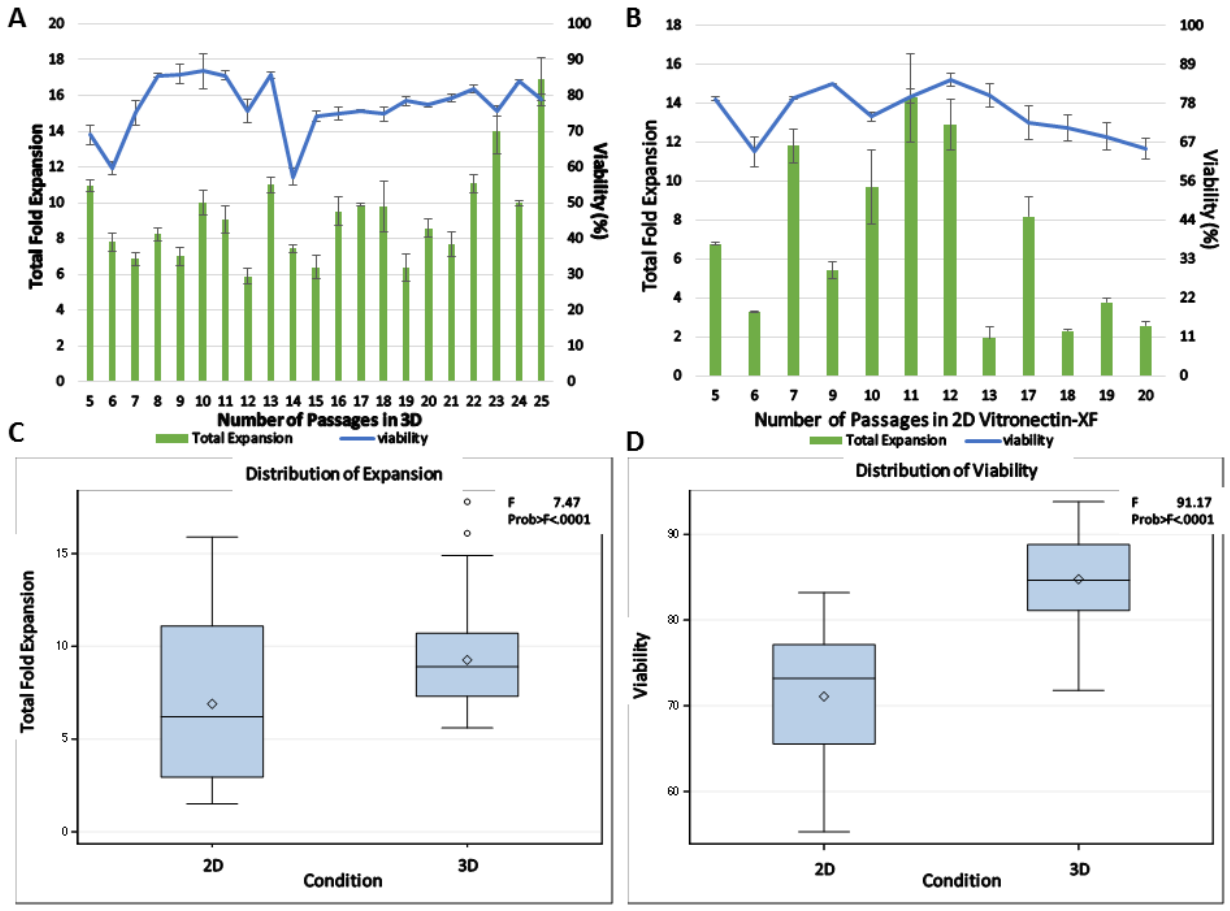

Figure 3.8 Growth and viability of TF-hiPSCs across 20 passages in 3D and 12 passages in 2D Vitronectin-XF.

A. Total population fold expansion and viability of TF-hiPSCs cultured for 20 passages continuously in 3D PGmatrix-hiPSC; B. Total population fold expansion and viability of TF-hiPSCs cultured for 12 passages continuously in 2D Vitronectin-XF-coated plates; C. Mean and distribution of total population fold expansion for TF-hiPSCs cultured in 2D and 3D ($P < 0.05$); D. Mean and distribution of viability for TF-hiPSCs cultured in 2D and 3D ($P < 0.05$).

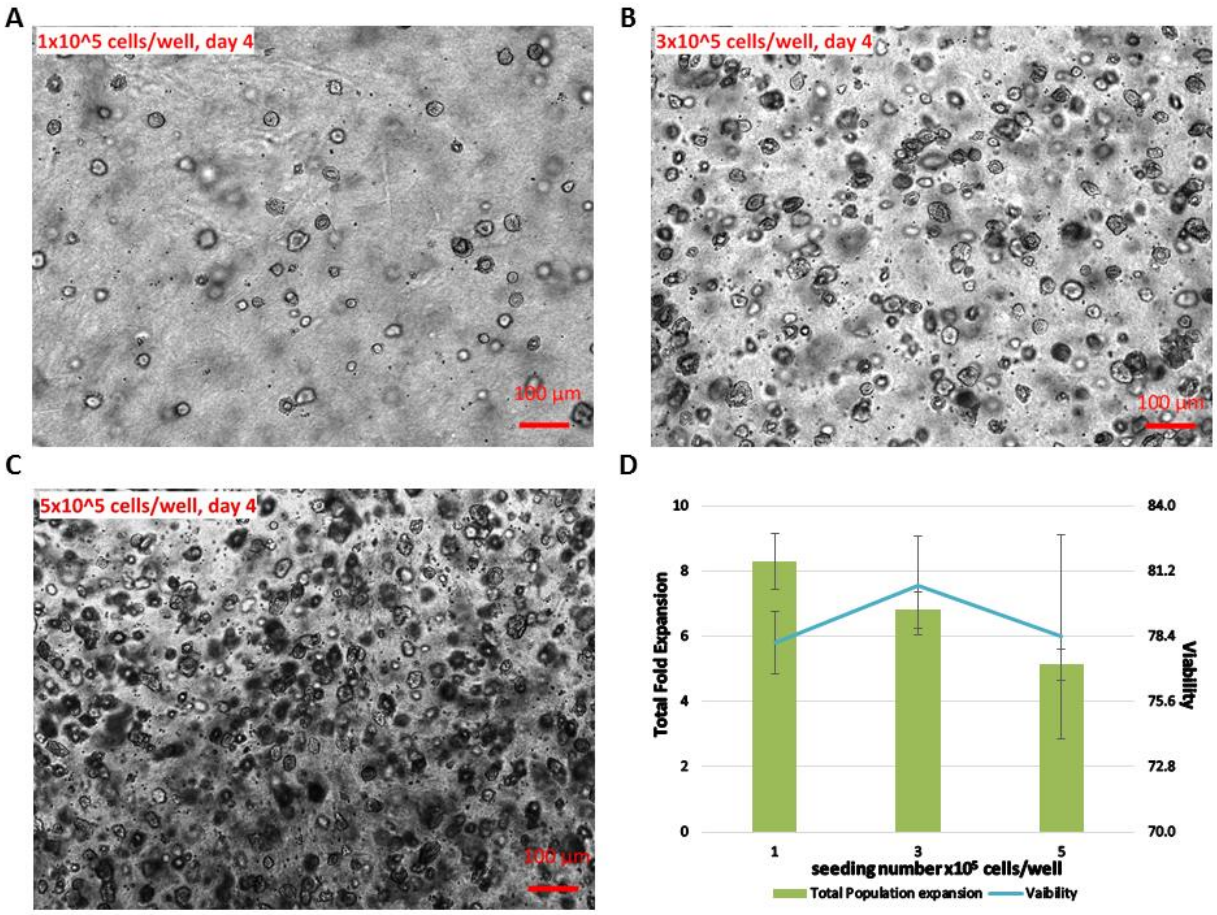

Figure 3.9 Thawing of cryopreserved ASC-hiPSCs directly into 3D PGmatrix-hiPSC system.

A. Day 4 photo of culture with seeding density of 1×10^5 cells/well; B. Day 4 photo of culture with seeding density of 3×10^5 cells/well; C. Day 4 photo of culture with seeding density of 5×10^5 cells/well; D. Total population fold expansion and viability of cultures at three seeding densities (harvested on day 5).

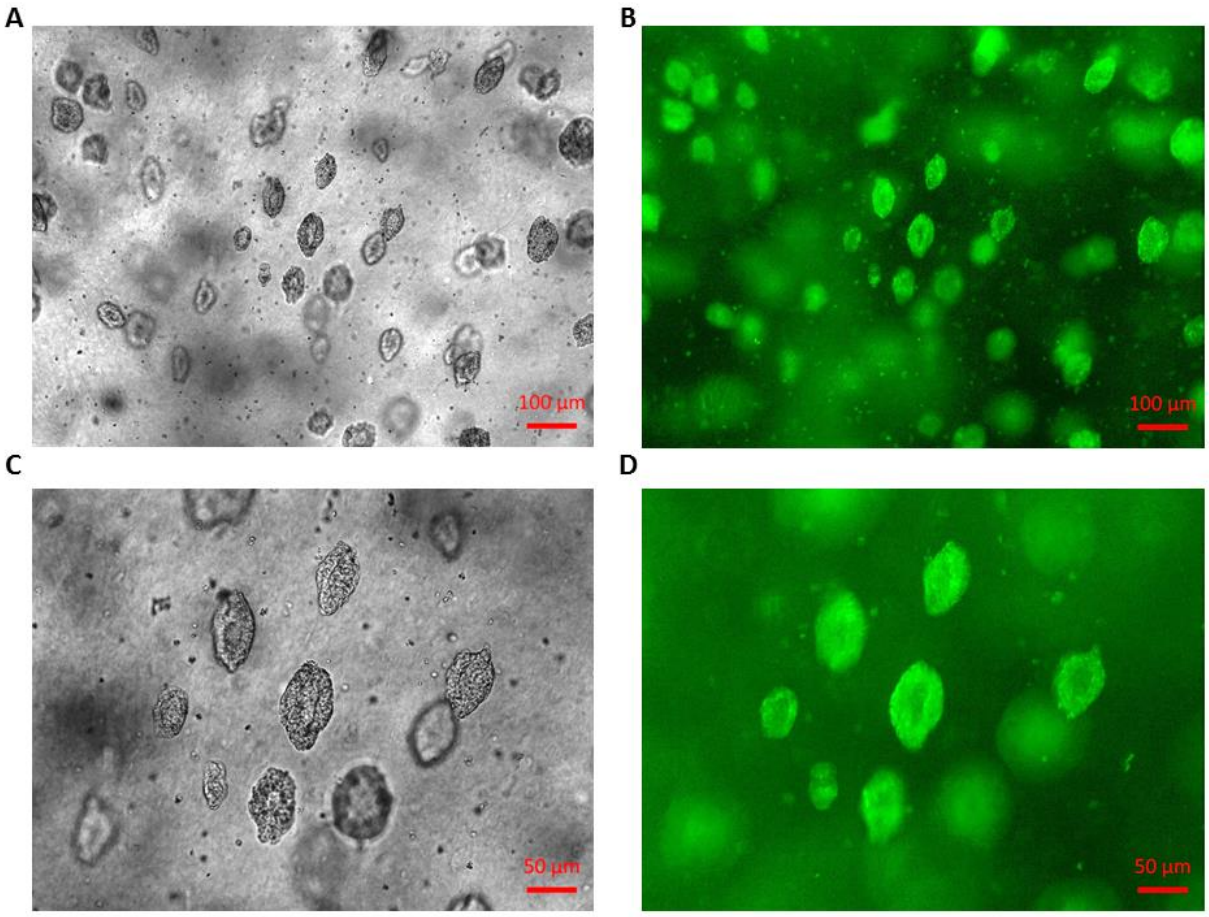

Figure 3.10 Immuno-staining of ASC-hiPSCs in 3D for Oct4 (green).

Bright field image of ASC-hiPSCs fixed in 3D PGmatrix-hiPSC (A. 50× magnification; C. 100× magnification), and fluorescent image of ASC hiPSCs in 3D PGmatrix-hiPSC fixed and stained with anti-Oct4 (green) (B. 50× magnification; D. 100× magnification).

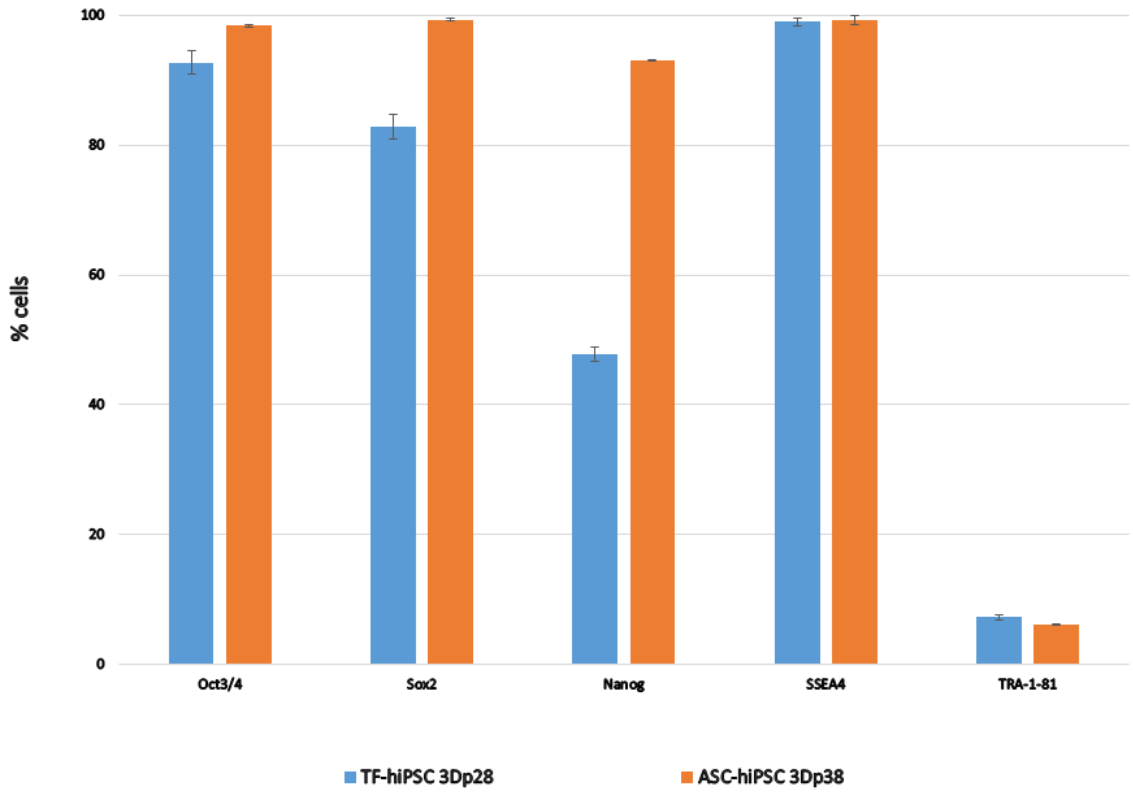

Figure 3.11 Expression of pluripotency markers in TF-hiPSC and ASC-hiPSC lines detected by flow cytometry.

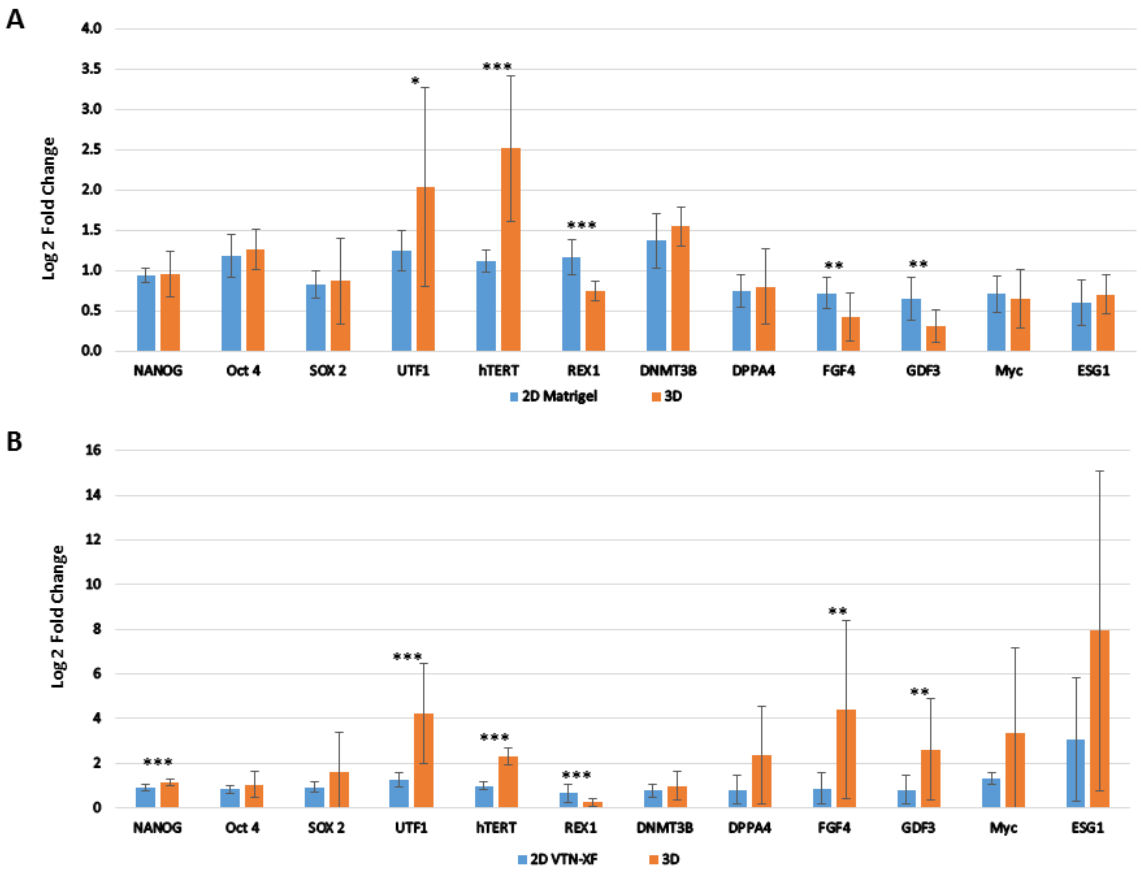

Figure 3.12 Average Expression of 12 pluripotency related genes of ASC-hiPSCs (A) and TF-hiPSCs (B) cultured in 2D and 3D.

Two passages of hiPSCs cultured in 2D (blue) and four passages of hiPSC cultured in 3D (orange) was collected and subjected to RT-qPCR analysis. Fold changes was normalized using 2D passage 10 (for ASC-hiPSC) and 2D passage 8 (for TF-hiPSC) as references. Shown in graph are average fold changes of the 2 passages of 2D-cultured hiPSCs compared with average fold changes of the 4 passages of 3D-cultured hiPSCs. * indicates significant difference ($P < 0.05$), * -- $P > 0.04$; ** -- $0.01 < P < 0.04$; *** -- $P < 0.01$.

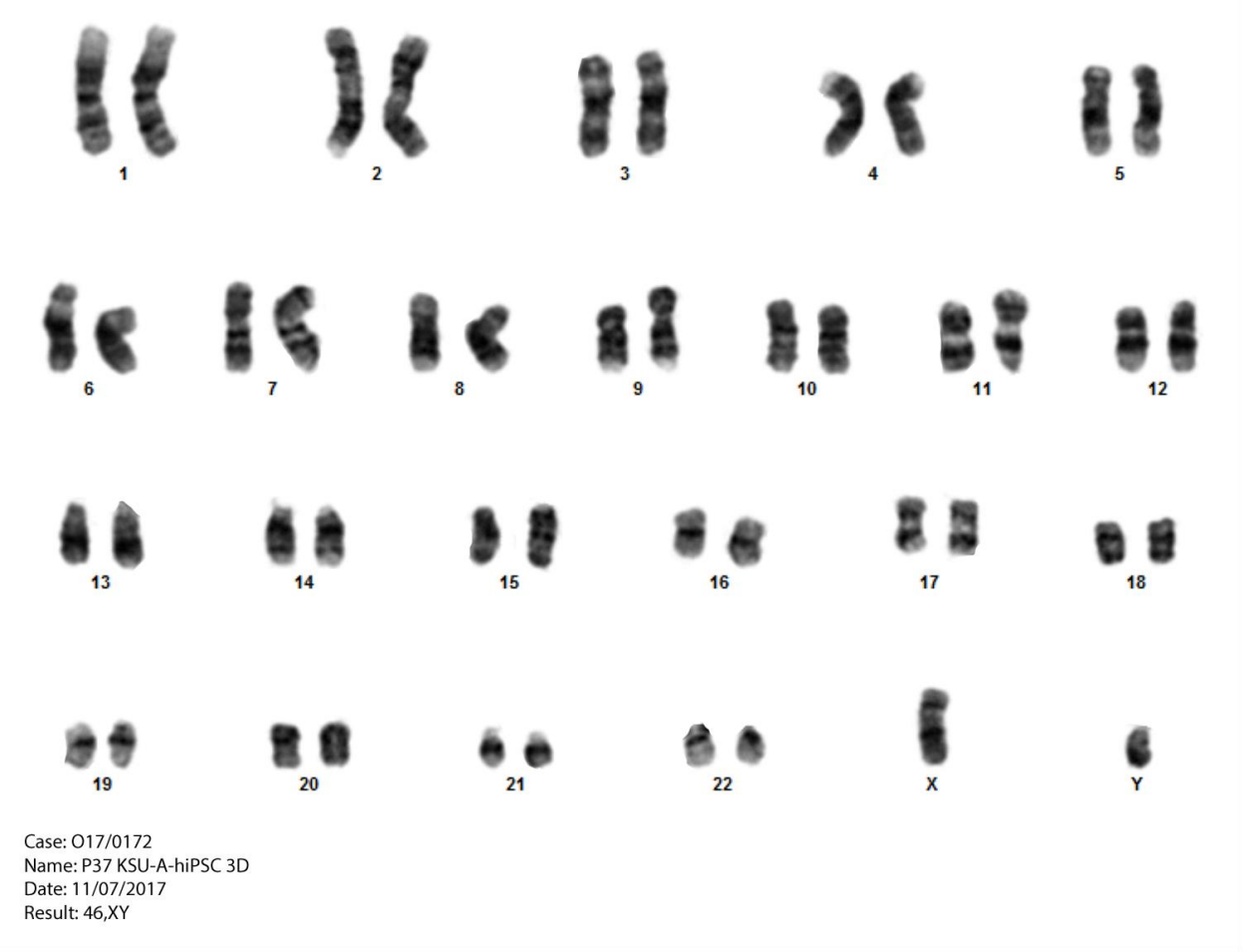

Figure 3.13 ASC-hiPSCs cultured in 3D PGmatrix-hiPSC for 37 passages showed normal male karyotype.

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Chapter 4 - Conclusion and Future Study Recommendations

4.1 Conclusion

We used hiPSC-qualified PGmatrix (PGmatrix-hiPSC) – a hydrogel based on synthetic peptide, to develop a fully defined 3D culture system for human induced pluripotent stem cells (hiPSC). Two hiPSCs lines (ASC-hiPSC and TF-hiPSC) were grown in this new 3D culture system using defined culture medium mTeSR1 supplemented with ROCK inhibitor Y-27632, and compared to the same cells grown in 2D Matrigel or Vitronectin-XF-coated culture plates. HiPSCs formed spheres in 3D culture system, which is different from the flattened and single-layered colonies formed in 2D. Besides differences in morphology, hiPSCs grown in 3D had higher population expansion and viability than 2D with similar passaging schedule. Because of the protective buffer effect of hydrogel surrounding the cells, hiPSCs are protected from environmental impact due to changes like pH, temperature, and negative effect brought by atmosphere oxygen content, resulting in more consistent cell growth and viability. We also developed an applicable protocol that allow t immunochemistry experiment be performed directly on this system with slight modification. In addition, hiPSCs stored in liquid nitrogen can be thawed directly into this 3D PGmatrix-hiPSC system.

Flow cytometry results of the two cell lines cultured for more than 25 passages in 3D PGmatrix-hiPSC showed that Oct4, Sox2, SSEA4 and Nanog are expressed by most of the cells tested. However, we found the surface biomarkers SSEA4 and TRA-1-81 had higher and lower expression levels than those from 2D culture, respectively, which were likely due to the adaptation to 3D culture environment that has less surface adhesion stimulations to the cells. RT-qPCR examination of 12 pluripotency-related genes in ASC-hiPSC and TF-hiPSC cultured in 2D

and 3D showed that the expression of UTF1 and hTERT were upregulated and REX1 expression was downregulated for hiPSCs in 3D PGmatrix-hiPSC system compared to those in 2D. These results are in agreement with our observation that 3D PGmatrix-hiPSC was able to support rapid and consistent population expansion of hiPSCs while maintain relatively high viability. This was also confirmed by Karyotype analysis.

From RT-qPCR analysis, we also found that FGF4 and GDF3 gene expression levels were varied between the two hiPSCs lines cultured in 2D and 3D. This is possibly related to differences between cell lines. But different culture environments can affect hiPSC characteristics, like changes in surface molecules and expression level of some genes. These changes provide clues to better understand functions of some molecules or genes. For example, reduced expression of TRA-1-81 did not affect cell growth and expression level of pluripotency related genes, it is more likely a simple surface molecule that did not involve in major signaling pathways to control cell survival and pluripotency maintenance. On the other hand, differences in gene expression level between hiPSCs cultured in 2D and 3D probably suggested functional redundancy of REX1, and the important role of UTF1 and hTERT.

In summary, hiPSC growth performance are significantly affected by culture conditions, such as cell seeding density, growth factors, gel surface adhesion and gel strength. The fully defined 3D culture system based on PGmatrix-hiPSC was able to maintain long term culture of hiPSCs in undifferentiated state with normal karyotype. In addition, this system can be used directly for immunochemistry as well as thawing of cryopreserved hiPSCs. The differences between hiPSCs cultured in 2D and 3D found in this research suggested the importance of exploring culture environment that is more mimicking *in vivo* conditions as cell characteristics can be affected.

4.2 Recommendations on Future Studies

As demonstrated in this research project, PGmatrix-hiPSC hydrogel provides sufficient support for the fragile hiPSCs to grow in a 3D culture environment that better resembles conditions *in vivo* than traditional 2D culture methods. HiPSCs expanded their population rapidly with high viability, while retaining expression of pluripotency related markers and chromosomal integrity.

With the hiPSC 3D culture system achieved in this study, I have the following further recommendation to further advance hiPSC for clinical research:

1. Scaling up of this hiPSC 3D culture system using PGmatrix-hiPSC would be very important for clinical applications. Usually clinical researches requires a large number of hiPSC cells, to meet the demands, scaling up of hiPSC 3D culture using large culture plates or flasks would be a simple and cost-saving way to produce large number of cells for both experimental and clinical research applications.

On the other hand, there has been suggestions on using hydrogel as microcarrier or encapsulation material for culturing hPSCs in suspension culture. This seems to be a promising solution for industrial large-scale culture. PGmatrix-hiPSC has been proven effective as a material for hiPSC 3D culture under stationary condition, further improvement to strengthen the hydrogel will very likely make it suitable for hiPSC encapsulation in large-scale suspension cultures.

2. The 3D PGmatrix-hiPSC can be used for downstream hiPSC related application studies. Because of its pluripotency, hPSC is more often used as an intermediate in clinical research for disease modeling and regenerative medicine. For example, hiPSCs can be generated

from patient cells that carry certain genetic deficiencies like Parkinson disease. These deficiencies can be preserved in the generated hiPSCs (Singh et al., 2015; Alan Trounson and Natalie D Dewitt, 2016; Nazish Sayed et al., 2016). This method allows researchers to study the process of disease development, which provides information that sample of problematic tissue or animal modeling cannot provide. For some diseases or injuries that cannot be cured by drugs, hiPSCs allow generation of patient specific heathy cells that can be transplanted inside human body to replace damaged cells. This method eliminates the risk of immunorejection resulting from using cells from another source. For drug discovery and testing, hiPSCs provide advantages that animal models cannot compete, due to genetic differences between species.

No matter which application hiPSC is used for, it is best to conduct all these experiments under conditions that mimic *in vivo* environment. This is where 3D culture system is superior than conventional 2D systems, as they are rich in liquid and allow cells to interact with the environment in a 3-dimensional way. To apply 3D system in disease modeling, regenerative medicine and drug testing, there is still a lot of work required to improve it. For example, hiPSC generation and differentiation has not yet been explored in 3D environment because currently available 3D systems do not allow easy access to cells and is hard to control concentration of nutrients and growth supplement.

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