

Development of High-Density 3D Dynamic Suspension Culture for
Human Induced Pluripotent Stem Cells Expansion and Hepatic
Differentiation

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Chapter 1 - General Introduction: Physiological and Microenvironmental Conditions in Scalable Culture Systems for Pluripotent Stem Cells Expansion and Differentiation

1.1. Pluripotent Stem Cell Applications

Pluripotent stem cells (PSCs) are undifferentiated cells which exhibit high proliferation rate and are able to differentiate into many types of cell consisted in the human body. They show remarkable promise for various applications and novel strategies in regenerative medicine, drug discovery, and in vitro toxicology for clinical and industrial purposes. The target organs of these technologies vary widely from small organs such as retinal tissue¹ to large organs such as the heart², pancreas³, and liver⁴.

For all of these applications, a sufficient quality and quantity of PSCs are needed, which can be generated in scalable 3D culture systems that support a higher density of cell culture and growth rate and are good manufacturing practice, compatible, simple, and easy to automate⁵⁻⁷. Although numerous research groups have attempted to develop optimum culture systems for PSCs, currently available culture systems show some limitations, such as difficulties in scaling up, automation, and standardization. Therefore, the effectiveness of scalable culture technology for PSC expansion and differentiation must be improved.

1.2. Pluripotent Stem Cell Types

Generally, PSCs can be derived from embryonic or reprogrammed from adult tissue. They are classified as embryonic stem cells (ESCs) or induced pluripotent stem cells (iPSCs), respectively.

ESCs were first isolated from the inner cell mass of blastocysts by Thomson et al. in 1998⁸. ESCs have some disadvantages, such as the requirement to extract and manipulate the embryo, which is limited by ethical considerations. In clinical use, because ESCs are derived from embryonic stage cells, it is more difficult to obtain immunocompatible cells for transplantation. This increases the risk of immune rejection in allogeneic transplantation. To overcome this limitation, several alternative sources were investigated.

iPSCs were first generated by Yamanaka et al. in 2006⁹⁻¹¹. Adult somatic cells were reprogrammed into pluripotent cells by overexpressing transcription factors in somatic cells via transfection of 4 genes into human fibroblasts⁹⁻¹¹. This achievement is very promising for both regenerative medicine and industrial-related applications such as drug screening.

iPSCs not only showing similar characteristics such as remarkable pluripotency to ESCs but also present minimum ethical issues such as those involving embryo destruction for ESCs^{5,12,13}. Moreover, this technology provides opportunities to generate autologous iPSCs from patients via tissue biopsy, which may minimize immune rejection when transplanted back into the patient.

1.3. The Development of Stem Cell Culture

The first method for large-scale production of animal cells was developed for baby hamster kidney cells by Capstick et al. in 1962^{14,15}. This technology has been applied in a broad range of biological products, such as monoclonal antibodies, hormones, vaccines, and other pharmaceutical products¹⁶. In PSC cultivation, the purpose of mass production is to prepare large amounts of cells for industrial and clinical use. For example, pancreatic islet transplantation requires at least 6×10^8 beta cells in an approximately equivalent to cells covered 0.6 m² of culture area; this corresponds to 600 6-well plates¹⁷. To regenerate 30% of liver tissue, 6×10^{10} liver cells are required, covering 60 m² of culture area or 60,000 6-well plates¹⁸. To obtain high-quality cells for these applications, it is important to sustain the self-renewal properties, maintain the pluripotency, and provide cryopreservation to maintain established cultures by using proper microenvironments for cellular growth.

Selection of the culture system for PSCs largely depends on cellular characteristics during cultivation (Table 1). In all culture methods, a single hPSC is known to undergo apoptosis without Rho-associated protein kinase (ROCK) inhibition. These conditions are thought to be caused by anoikis, the apoptosis mechanism induced by a lack or alteration of

cell-cell or cell-matrix interactions¹⁹. When hPSCs dissociated into single cells, the loss of E-cadherin, a key molecule in intercellular adhesion, can activate the ROCK-dependent signaling cascade to cause myosin hyperactivation. These cascades can increase myosin contraction, impacting cellular vulnerability and causing apoptosis^{20–22}. Thus, hPSCs are typically seeded as clusters or single cells with ROCK inhibitors such as Y-27632 and HA-1077^{21,22}. For other common mammalian cells, adherent culture is the most widely used method for culturing undifferentiated PSCs. However, these cells also can be cultured in suspension as single cells or aggregates²³.

1.3.1. Adherent Culture Methods

Adherent culture is the most widely used cell culture method for biological studies. For adherent PSCs, the interactions between PSCs and feeder cells are essentially required to provide support, such as producing secreted growth factor, expressing specific ligands, releasing cytokine, and provide the cell-cell interaction for stable attachment which is important for the cell growth and pluripotency^{24,25}. Several proteins which required for maintaining the pluripotency, such as transforming growth factor beta 1 (TGF- β 1)²⁶, activin A²⁶, bone morphogenetic protein (BMP)-4²⁶, Fibroblast growth factor 2 (FGF2)²⁶, and Wnt-3²⁷ were secreted by the feeder cells.

Inactivated mouse embryonic fibroblasts are traditionally used as a supportive feeder layer to sustain hPSC propagation^{28–30} (Figure 1A). However, co-culturing PSCs with animal feeder cells present the transmission of unwanted genes, viral contaminations, or immunogenic nonhuman saccharides such as sialic acid³¹, as well as variability in

experimental results³². To overcome these limitations, many studies have developed xeno-free human feeder cells, such as human foreskin fibroblasts^{26,33,34} human adipose-derived stromal cells^{35,36}, amniotic epithelial cells^{37,38}, fetal skin cells^{39,40}, and amniotic mesenchymal stem cells (MSCs)⁴¹. Autologous adult human fibroblasts are preferable for use as feeder cells in clinical applications because they minimize xenogeneic substances from animals and are easy to obtain. Moreover, human PSCs can be generated from isogenic parental cells as feeder cells, thus the compatibility of cell-cell interactions can be better maintained⁴². However, maintenance in the culture systems using feeder cells is costly and time-consuming.

Therefore, in large-scale culture, the application of feeder layer can be eliminated to obtain a higher yield and reduce production costs⁴³. Alternatively, feeder cells can be replaced with a conditioned medium and extracellular matrices (ECM), such as Matrigel⁴⁴, fibronectin⁴⁴, laminin^{45–47}, and vitronectin⁴⁸, which are normally produced by feeder cells to maintain the PSCs in culture. The medium consists of non-xenogeneic compounds that are also essentially required for the propagation and differentiation of hPSCs used in clinical applications. In addition, the growth factor such as such as activin A, TGF- β 1, FGF2, Insulin, Transferrin, and Nodal also added in the medium formulation to support the proliferation and reconstitute its production from the feeder cells⁴⁸. Currently, feeder cell-conditioned medium or commercially available synthetic medium for feeder-free culture have been used for PSCs culture. Media selection varies depending on the cell type and it is necessary to optimize the media conditions for different culture methods^{7,49,50}.

The other challenge in adherent culture is the method to harvest the PSCs. The conventional adherent method requires cell removal by mechanical or enzymatic separation, which may damage the cells and affect the quality and quantity of mass-produced PSCs⁵¹. These conditions make the use of human PSCs for clinical applications were more difficult to achieve.

1.3.2. Suspension Culture Methods

Current development in cell culture technology has enabled adherent PSCs to be adapted for suspension culture (Figure 1). This method can provide better mass transfer of nutrition and does not require enzymatic or physical removal, which can disrupt the cell membrane. The cell interaction between PSCs and feeder cells or ECM were not required since their native interaction by direct contact between the PSCs, self-auto/paracrine, and other factors can be achieved. Elimination of the feeder layer and synthetic matrices simplified the procedure and minimized contamination. In industrial applications, this system can also increase the scalability by enabling high-density cell culture ($> 1 \times 10^7$ cells/mL) and significantly reducing the costs involved¹⁶. A recent experimental study revealed that global gene expression, mainly the pluripotency gene and functional characteristics, were comparable to those maintained under adherence conditions⁵². Differentially expressed genes between suspension and adherent PSC cultures were mainly associated with cell adhesion and extracellular matrix interactions⁵².

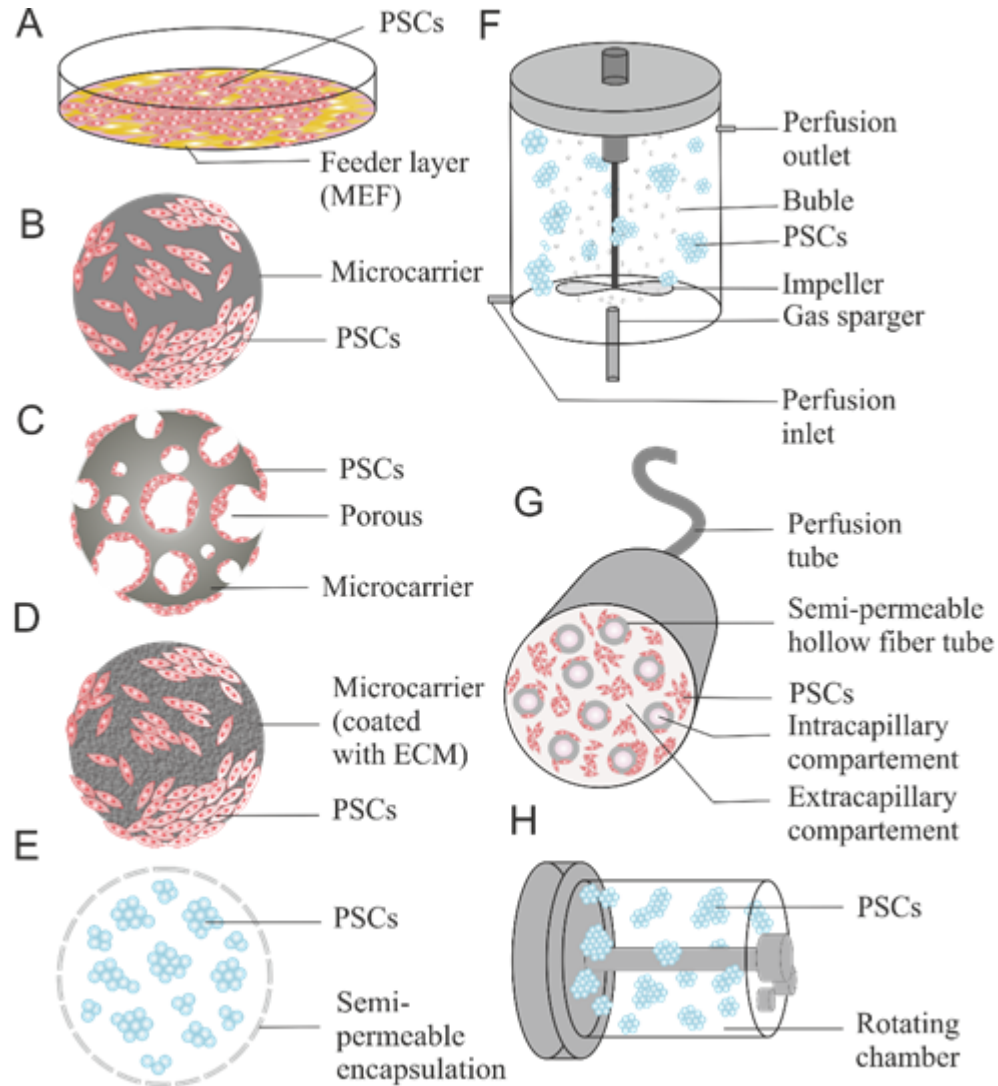


Figure 1. Different culture systems in PSC cultivation : 2D static culture (A), solid filled-microcarrier (B), porous microcarrier (C), coated microcarrier (D), cell encapsulation (E), stirred bioreactor (modified by oxygen sparger and perfusion systems) (F), hollow fiber systems (G), and rotary culture systems (H)

Table 1. Comparison and example of various culture systems application for PSCs expansion and differentiation

Culture Systems	Ease of scale up	Ease of monitoring	Ease of harvesting	Mass transfer	Shear stress	References of application in PSCs expansion	References of application in PSCs differentiation types
General culture systems	Conventional static culture	low	low	high	low	low	hiPSCs ^{29,53,54} , hESC ²⁹ , miPSCs ⁵⁵ , mESC ⁵⁶ , Hepatocyte ⁵⁷ , chondrocytes ⁵⁸ , muscle fiber cells ⁵⁹ , lung and thyroid progenitor cells ⁶⁰ , odontoblast ⁶¹ , cardiomyocytes ⁶²
	Automation	medium	high	high	low	low	hiPSCs ⁶³⁻⁶⁷ , hESC ⁶⁸ , mESC ⁶⁶ , miPSCs ⁶⁹ , Cardiomyocyte ⁶⁴ , neural cells ⁶⁶
	Stirred bioreactor	high	high	high	high	high	hiPSCs ^{53,70-72} , hESC ^{70,71} , miPSCs ⁷³ , Hepatocyte ⁷⁴ , cardiomyocytes ⁷⁵⁻⁷⁷ , pancreatic β cells ⁷⁸
	Rotary bottle	medium	medium	high	high	medium	mESC ⁷⁹ , hESC ⁸⁰ , Cardiomyocyte ^{81,76} , osteogenic cells ⁷⁹
	Hollow fiber bioreactor	medium	low	low	medium	low	mESC ⁸² , hESC ⁸³ , Hepatocyte ^{84,85}
Others culture techniques	Microcarrier	high	low	low	medium	high	mESC ⁸⁶ , hESC ⁸⁷ , hiPSCs ⁸⁷ , Neural progenitor ⁸⁸ , endoderm progeny ⁸⁹ , hematopoietic cells ⁹⁰ , hepatocytes ⁹¹ , cardiomyocytes ^{78,92,93}
	Cell encapsulation	medium	low	low	medium	low	miPSCs ⁹⁴ , mESC ^{95,96} , hESC ^{97,98} , Cardiomyocyte ⁹⁹ , pancreatic cells ¹⁰⁰ , definitive endoderm ⁹⁸ , osteogenic cells ⁷⁹

1.4. Mechanical and Physiological Environment in Suspension Culture Systems for PSC Expansion and Differentiation

Suspension culture system provides expandable culture volume and dynamic physiological environment compared to conventional dish culture¹⁰¹. Ideally, suspension culture systems for PSC cultivation should meet some essential requirements, such as efficient mass transfer of oxygen and nutrients, waste transfer, and minimum shear stress¹⁰². These microenvironmental factors may affect cell pluripotency and fate during differentiation. Depending on the cell type, optimal PSC expansion and differentiation can be conducted under various conditions (Table 2). Generally, in the culture systems, PSCs are incubated at 37°C and pH 7.4. A rapid decrease in temperature and increase in pH can inhibit cell proliferation, reduce pluripotency, and decrease cell viability^{103,104}.

Viscosity is correlated with fluid movement in culture systems. Appropriate medium viscosity properties are required to maintain the proper PSC culture. Medium with lower viscosity exhibits more rapid mass transfer and uniform conditions in mass production. In contrast, a previous experiment showed that hPSC culture in less viscous media may cause cell agglomeration and shear-induced apoptosis, leading to lower yields [8]. In addition, a recent study showed that culture medium in which continuous viscoelasticity was optimized by using a non-toxic polymer, such as low acyl gellan gum which can form large numbers of uniformly sized aggregates and maintain cells in a suspension without agitation is one promising culture strategy¹⁰⁵.

Oxygen is one of the most important components in cell culture systems and is involved in nearly all cellular aerobic metabolic cycles. In PSC propagation, the

concentration of oxygen in the culture media is a crucial factor in pluripotency and proliferation that maintains the cellular characteristics of stem cells¹⁰⁶. An in vitro study clearly demonstrated that differentiation was significantly reduced in hESCs under hypoxic culture conditions (1–5% O₂) compared to under normoxic culture conditions (21% O₂) by morphologically distinct growth area examination and expression of pluripotency markers according to biochemical and immunohistology assays¹⁰⁷. Another study showed that hypoxia of iPSCs cultivated under 5% O₂ improved the efficiency of iPSC proliferation and enhanced the expression levels of transcription factors for iPSC reprogramming (Oct3/4 and Nanog), which are required to maintain pluripotency¹⁰⁸. Repeated passaging of hESCs under normoxic culture conditions resulted in an increased number of differentiated cells and reduced self-renewal¹⁰⁹. ESC growth under hypoxia conditions promotes the formation of EBs during repeated passaging¹⁰⁷. Forsyth et al. reported that physiologic oxygen (2%) may reduce chromosomal damage and induce recovery after oxidative damage post room oxygen (20%) treatment¹¹⁰. These studies clearly showed that oxygen concentration affects PSC growth, differentiation, and EB formation.

Cell aggregation control is very important for maintaining PSCs in an undifferentiated state and preserving cellular viability. In suspension hPSC propagation in vitro, cell-cell interactions are essentially required. Therefore, PSCs must grow in appropriate-sized aggregates, known as embryoid bodies (EBs). EBs are three-dimensional aggregates that are morphologically similar to the early stages of the developing embryo and can differentiate into all three germ layer cell types. A recent study demonstrated that excess aggregation can direct PSCs into spontaneous differentiation, which occurred as the size of

EB aggregates increased¹¹¹⁻¹¹³. Moreover, insufficient oxygen and nutrition exposure can result in necrotic cells in the center of the aggregates. Some approaches for controlling aggregation in suspension culture have been reported. One of recently developed method for controlling aggregation is the addition of lipid-rich albumins, such as Albumax. This compound can reduce aggregation by inhibiting E-cadherin-mediated cell-cell attachment. A previous study showed that administration of 0.2-0.5% Albumax is optimal for obtaining aggregates of appropriate size and higher hiPSC yields in 12-well plates while maintaining pluripotency¹¹⁴.

In suspension culture, the agitation mechanisms are significantly important to increase the homogenous environment by the medium mixing, enhancing the O₂ transfer, and also controlling the aggregates size and avoid the excess aggregation. This agitation can be achieved by using the dynamic culture systems, such as stirred bioreactor or the rotary culture. However, in consequence, the hydrodynamic stress caused by agitation can induce the negative effect for the suspended cells itself. This hydrodynamic shear stress is a particularly important biomechanical factor in mammalian cell culture. Most cell types respond to shear stress by physiological or gene expression alterations¹¹⁵. Generally, excess hydrodynamic stress through rigorous agitation or bubble bursting dynamics can decrease the viability of mammalian cells. In addition, excess agitation during cultivation may affect the phenotype and characteristics of PSCs. For example, an excessive centrifugal force of 1000×g or more may induce phenotypical shifting and decrease the proliferation rate during expansion¹⁰³. A study conducted by Sargent et al. revealed that exposure to hydrodynamic stress conditions not only significantly affected EB formation and structure, but also induced downregulation

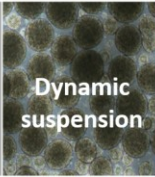
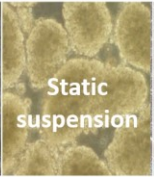

of pluripotency genes, resulting in spontaneous differentiation into three germ lineages^{116,117}. Moreover, in the suspension culture systems, cells in aggregates are exposed to higher shear stress than individual cells because of their large particle diameter¹¹⁷. As an alternative, the dynamic rotary suspension culture can be utilized to enhance the mixing of the medium by mild agitation. This systems also has been demonstrated its potential to enhances PSC expansion and differentiation into the mesoderm and endoderm¹¹⁸.

In optimizing the scalable culture systems for expansion and differentiation of PSCs, traditional adherent cell culture can be also utilized on polymer- or hydrogel-based microcarrier beads which provide a high surface area for cell attachment and proliferation. This technique enables adherent-type cells to be treated in a manner similar to that in suspension culture. Compared to conventional static adherent culture, the main advantage of microcarriers is that they can accommodate a larger number of cells and occupy less space in the bioreactor^{89,119}. In general, microcarriers are divided into several types: nonporous microcarriers, coated microcarrier and porous microcarriers (Fig. 1B and C). In PSCs expansion and differentiation, the coated microcarrier with ECM such as collagen or fibronectin to enhance cell attachment and adhesion^{119,120} (Fig. 1D). These coatings are completely dissolvable in trypsin solution, which allows for sampling, counting, and harvesting of the cells without interference from the carriers. Moreover, the poor growth and gradual loss of pluripotency which occurred because of a lack of ECM interactions can be avoided¹²¹. The porous microcarrier can create a microenvironment which allows auto/paracrine interaction inside the beads, protecting the cells from shear and frictional stresses caused by aeration and agitation in a dynamic bioreactor. However, the cell

collection was more difficult and the variability among cultured PSCs undergoing expansion or differentiation which caused by the gradients of oxygen, nutrition, and the waste product inside the pores and the surface.

The other alternative to improve the PSCs suspension culture systems is by enveloping the viable cells or aggregates within layers of semi-permeable and high biocompatibility membranes to protect the cells from shear stress and to accumulate products secreted from cells while exchanging nutrients and wastes¹²². Enclosing cells in hydrogel can create a microenvironment with cell-hydrogel contact and auto/paracrine effects. In contrast to these advantages, the application involves an encapsulation and decapsulation process, which is costly and challenging when using hydrogel with non-reversible gelation⁹⁴. In addition, cells showing high proliferation such as PSCs can escape from hydrogel capsules and grow outside of the capsules.

Table 2. Comparison between general PSCs culture systems

Pros / Cons		 Dynamic suspension	 Static suspension	 Monolayer Culture
	Scaleability	High	High	Low
	Cell-cell interaction	High	High	Low
	Nutrition + oxygen transfer	High	Moderate	Low
POTENTIAL	Cost (Growth factor usage)	Low	Low	High
	Size control	Good	Poor	N/A
DRAWBACKS	Shear stress	High	Low	Low

1.5. Development of Dialysis Culture Systems in PSC Expansion and Differentiation

The current technology of culture systems device also provides the feasibility to adapt the physiological condition of the cell in a tissue compartment with nutrition supply by using dialysis culture system. One of the most developed dialysis culture systems for cellular mass production is hollow fiber bioreactor. This culture system was based on perfusion methods that use small semi-permeable membrane hollowed fiber tubes. By circulating the culture medium, this system can provide nutrients to cells and eliminate wastes from the cell culture compartment (Fig. 1G). Principally, this system mimics the microenvironment of blood vessels *in vivo* by using two different compartments. The first compartment is an intercapillary space within the hollow fibers, while the second is extra capillary space surrounding the hollow fibers¹⁰². The cells are cultured in the extra-capillary space, and oxygenated medium at an appropriate pH is circulated through hundreds to thousands of capillaries within the hollow-fiber cartridge using perfusion systems. Oxygenation and pH control is achieved by a gas-permeable surface in a CO₂ incubator. The exchange of nutrients and waste products is achieved by diffusion through the semi-permeable membrane fibers. A separate culture compartment and perfusion compartment produces the low-stress environment required for maintaining PSC pluripotency and viability. In this system, the culture compartment can be filled with up to 10⁸ cells/mL (near the tissue density *in vivo*)¹²³. Therefore, this system requires less medium and fewer growth factors than traditional culture methods. This high-density culture also can facilitate the three-dimensional growth resembling the *in vivo* tissue structure to support PSC differentiation. Recent studies showed

that the functional activity and formation of several tissue-like structures were increased in hepatocyte-derived hiPSC aggregates after hepatic differentiation using this system⁸⁵.

However, hollow fiber systems have some disadvantages, such as difficulties in monitoring and controls. At high cell densities, spatial concentration gradients in the extra-capillary compartment can form, based on the distance of the PSCs to the intercapillary compartment. Previously, a study conducted by Roberts et al. evaluated the potential and feasibility of using hollow fiber systems for hESC mass production. The results indicated that hESCs can be scaled up in a hollow fiber bioreactor without significantly decreasing pluripotency⁸³.

1.6. Thesis composition and Objective

The final objective of this thesis is to develop the efficient culture system for expansion and hepatic differentiation through medium refinement and utilization of native biological interaction which achieved by high-density suspension culture. This objective was achieved by several investigation and improvement (Figure 2) which is described in thesis composition as follows:

- Chapter 1 Introduce the various culture systems for pluripotent stem cells culture. The effect of the various microenvironmental condition of currently available culture systems was also explained. Some problems regarding culture condition and alternative methods to overcome and improving the available culture systems were also mentioned in this chapter.

- Chapter 2 consist of initial small scale multicompartment dialysis culture system design and it's a performance in actual operation for high-density hiPSCs expansion with different initial cell inoculation density. The role of dynamic culture environment in cellular proliferation inside the spheroid also investigated in cell density-dependent manner. This chapter also clarifies about the effect of basal medium refinement and growth factor accumulation in the culture compartment to the hiPSCs pluripotency and differentiation capability into three lineages which recapitulated organ development.
- Chapter 3 consist of the investigation of the effect of the spheroid size which formed from different cell density in hepatic differentiation. This chapter also discussed the effect of WNT/ β -catenin in stepwise hepatic differentiation between the spheroid size. In addition, the optimal initial spheroid size will be practically applied for high-density hepatic differentiation.
- Chapter 4 consist of the expansion of the high-density hiPSCs culture utilization for hepatic differentiation. In this chapter, the effect of differentiation medium in combination with gellan gum during hepatic differentiation is observed. High-density hepatic differentiation was initiated by using optimized initial spheroid size and performed by stepwise differentiation by addition of the differentiation cocktail consisted of stage-specific growth factor and WNT/ β -catenin inhibitor. Afterward, the initial definitive endodermal differentiation was performed by using similar gellan gum combination in a mini dialysis culture system.
- Chapter 5 summarizes the overall discussion of the previous chapter. This chapter also emphasizes the feasibility of the high-density suspension culture into various type of

stem cells. Further investigation and improvement are suggested to realize both clinical and industrial application of pluripotent stem cells.

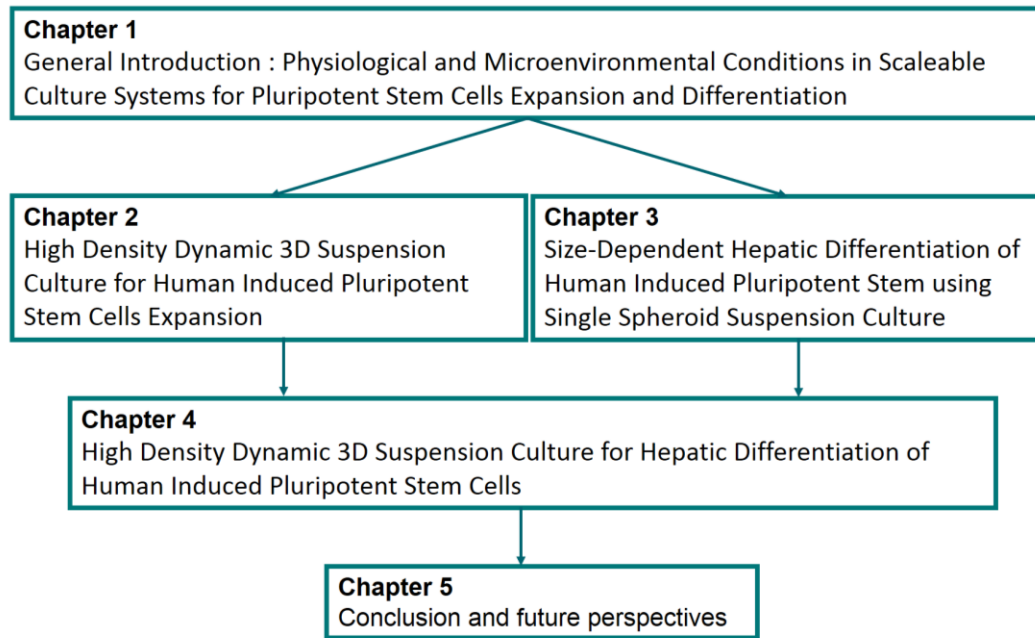


Figure 2. The composition of this thesis

Chapter 2 - High Density Dynamic 3D Suspension Culture for Human Induced Pluripotent Stem Cells Expansion

2.1. Introduction

Human induced pluripotent stem cells (hiPSCs) is a potential source to generate many cell types consisted of the human body. Because of these characteristics, the hiPSCs can be utilized for various application, such as transplantation or cell based assays. However, to realized this application, the large number of hiPSCs were essentially required. Currently, the hiPSCs expansion and maintenance are still remained difficult, regarding the culture condition problems such as the inadequate nutrition supply limitation and waste product

removal, mechanical stress, and biological interaction between the hiPSCs. This condition can rise impact in cell quality (pluripotency and viability) and requirements of high-cost production.

The control of spheroid size was also important in suspension culture since the larger spheroid can potentially induce the spontaneous differentiation. In addition, the necrotic area often occurred in the central region of the spheroid caused by the mass transfer limitation of the oxygen supply, nutrition transfer, and waste product removal inside the spheroid altogether with increasing of the spheroid size.

Rotational culture systems can be applied to achieve the homogenous culture environment and enhance the oxygen transfer and avoid the excess aggregation. Moreover, this dynamic condition also can improve the mass transfer capability between the dialysis membrane. However, the mechanical stress caused by this culture system also can increase the risk of damaging the cells at the same time. Therefore, we applied the gellan gum biopolymer to avoid the excess spheroid aggregation and reducing the mechanical stress by increasing the medium viscosity. Gellan gum is an extracellular polysaccharide, secreted by the bacteria *Sphingomonas paucimobilis* during anaerobic fermentation¹²⁴.

In suspension culture, the low acyl gellan gum forms a microfiber network structure which keep the cell floating and well dispersed and blocking the interaction between spheroid by Ca^{+} mediated-interaction.

The high-density suspension culture systems provide the advantage to expand the cells with high volumetric production rates. However, in terms of its application in

pluripotent stem cell, several factors need to be considered, such as cell density variable, metabolic demand, and mechanical stress. Moreover, the extensive requirement of small molecular weight molecule nutrition supply and waste product removal cannot be handled by conventional culture systems.

In this study, we utilized the mini dialysis culture system for very high-density suspension culture by continuous refinement of the micromolecules only-basal medium while preserve and recycled the accumulated macromolecules which were required for proliferation and maintenance of hiPSCs pluripotency.

2.2. Methods

2.2.1. Design of Dialysis Device for High-Density Suspension Culture

The dialysis culture systems consist of two compartment, the culture compartment and a lower compartment. This culture insert was modified by cutting and removing the bottom mesh layer. To selectively permeate the nutrition or cellular waste product, the dialysis membrane was carefully affixed into the bottom side of the strainer using alkyl- α -cyanoacrylate-based surgical grade tissue adhesive Aron Alpha A (Daiichi Sankyo). The upper compartment then placed in 6 deep well plates as dialysate compartment (Corning).

2.2.2. Penetration Test of Dialysis Culture Device in Cell-free Condition

To evaluate the device performance in low molecular mass nutrition and waste product exchange, 2 ml of DMEM low glucose (Sigma Aldrich) containing 0.2% gellan gum and 0.8 g/L lactic acid (Sigma Aldrich) were placed in culture compartment and 15 ml

DMEM high glucose (Sigma Aldrich) was placed in dialysate compartment. The dialysis culture systems then placed in 120 rpm rotary shaker. In order to measure the change of glucose and lactic acid concentration, 50 μ m of the medium sample were collected every 2 hours during 12 hours.

In order to check the device capability to localize the macromolecule in culture compartment and the differences of permeation in different size of molecules, 2 μ g/ml of each FITC with the different molecular weight (4kDa, 10kDa, and 20kDa) was added in culture compartment. This experiment was performed by using DMEM basal medium in both compartments similar to penetration test for micromolecule. The medium sample was isolated from culture medium during 12 hours and the FITC concentration was measured by Wallac Arvo SX 1420 multilabel counter.

2.2.3. Monolayer hiPSCs Culture

Human induced pluripotent stem cells (TkDN-4M cell line) were cultured and maintained in vitronectin coated-tissue culture dish using complete supplemented Essential 8 (E8) culture medium and maintained following manufacturer protocols.

2.2.4. hiPSCs Spheroid forming in Suspension Culture

HiPSCs spheroid was generated by seeding 2×10^6 single cell suspension per well of 6 well plates for 24 hours in E8 media supplemented with 0.2% free fatty acid-bovine serum albumin (BSA) and placed in the 90 rpm rotary shaker.

2.2.5. Lactate Dehydrogenase Assay in Culture Medium

To evaluate the cellular injury caused by hydrodynamic stress, the lactate dehydrogenase (LDH) leakage from the cells were performed. The spheroid was previously formed was moved into 12 well plates consisted of +GG or -GG medium under 120 rpm rotational culture. The cell was harvested by sedimentation in 10 ml tubes and the medium supernatant was isolated for LDH analysis. The supernatant was analyzed by LDH cytotoxic assay kit (Wako Chemical) following the manufacturer's instruction.

2.2.6. High-Density hiPSCs Suspension Culture

The dialysis membrane of the culture insert was activated by pre-wetted the dialysis layer using 2ml of sterile H₂O 15-30 minutes before the dialysis culture was started. The dialysis culture insert was placed in 6 deep well plates. After the H₂O was completely removed, 15 ml of DMEM were inserted into dialysate compartments (6 deep well plates) and 2 ml of E8 consisting FcEM using the formulation from manufacturer protocols and 0.2 % BSA were inserted into culture compartments. The hiPSCs spheroids which previously formed were harvested and transferred into the culture compartment. The spheroids maintenance performed by 120 rpm rotary shaker for 4 days. As a control group, the suspension culture using the same condition also performed without any dialysis support and with or without manual 24-hour medium replacement.

2.2.7. Morphological Analysis

Each of the spheroids group was moved into 12 well plates and the macroscopic and microscopic image were taken. The image of the spheroid colony was observed and the spheroids diameter analyzed by FIJI/ImageJ software.

2.2.8. Cell Number Determination

After the morphology was observed directly by light microscope, the spheroids were collected, centrifuged at 1000 rpm for 3 minutes, and the supernatant removed from the tube. To obtain the single cells, 1 ml of TrypLE dissociation reagent were added into the spheroids and incubated for 10 to 15 minutes at 37°C following homogenization by gentle pipetting. Afterward, the cells were diluted 100 times in PBS and counted by using Haemocytometer.

2.2.9. Glucose and Lactate Concentration Measurements

To measure the glucose and lactate during 4 days culture in different high-density culture configuration, 50µl of culture media sample were isolated every 24 hours and measured by YSI 2950 multipurpose bioanalyzer.

2.2.10. Haematoxylin-Eosin Staining of Thin Cross Sectioned-Spheroid

The spheroid was collected and fixed by 4% paraformaldehyde solution (Wako) in PBS for 30 minutes at room temperature, washed in PBS and replaced with 30% PBS buffered sucrose solution at 4°C overnight. The sucrose solution was discharged, and the

Spheroid placed in the cryomold following embedding with OCT at -20 OC until hardening. 10 µm thin sections were taken by cryostat and mounted onto glass slides for 2.8 hematoxylin-eosin staining.

2.2.11. bFGF and Nodal Concentration Measurement by ELISA

The 100 µl solution containing 1 g/L bFGF primary antibodies (R&D Biosystems) were immobilized in each well of 96 well plates by incubation at 37°C for 3 hours. Afterward, the plates were washed with 200µl/well PBS-Tween, then the 100 µl/well of blocking buffer was added and incubated for 1 hour at room temperature. The 100µl of a serial dilution of the medium sample and standard solution were plated in each well and incubated 2 hours at room temperature. The plates were washed with PBS Tween and 100 µl/well of 1g/L diluted bFGF secondary antibodies (R&D Biosystems) added and incubated for 2 hours at 37°C. The plates were washed by PBS Tween solution and 50µl Streptavidin-HRP (R&D Biosystems) were added and incubated at room temperature for 30 minutes-1 hour. To obtain the color reaction, the plates washed by PBS tween and 100µl of color solution consist of 2.5 mg 0-phenylene diammonium chloride and 0.5 µl H₂O₂ per ml citrate buffer were added to each well following incubation for 30 minutes in room temperature. The color reaction was stopped afterward by adding 50µl/well H₂SO₄ (4N) without removing the previous solution. The fluorescence intensity was measured by Wallace Arvo SX 1420 multilabel counter.

The Nodal detection was performed by human Nodal ELISA kit (LSBio) by using manufacturers instruction. The fluorescence intensity was measured by Wallac Arvo SX 1420 multilabel counter.

2.2.12. Random Differentiation Assay

The random differentiation assay was performed by dissociating the spheroid population into a single cell and seeded in U-shape ultra-low attachment 96 well plates to formed embryoid bodies for 24 hours following additional 2 days culture using E8 medium. Afterward, the embryoid bodies population were moved into DMEM basal medium containing 10% Foetal calf serum for 7-day differentiation using low attachment 60mm tissue culture dish in static suspension condition. The gene expression of the trilineage marker of the differentiated spheroid then analyzed by qPCR.

2.2.13. Gene Expression Analysis by qRT-PCR

The mRNA was isolated from 10^6 cells using Trizol reagents (Life Technologies). The purified RNA solution was reverse transcribed by using ReverTra Ace qPCR RT Master Mix (Toyobo) followed by qPCR analysis using Thunderbird SYBR qPCR Mix (Toyobo) using manufacturers instruction. The gene amplification and expression analysis performed by using Pikoreal Real-Time PCR System (Thermofisher)

2.3. Results

2.3.1. Mass Transfer Performance of Dialysis Device in Cell-free Condition

The glucose and lactic acid measurements in 12 hours showed that the device was able to maintain the equilibrium of glucose concentration after 12 hours of culture, and also able to maintain the equilibrium of the lactic acid in 8 hours. Based on the measurements, there is no significant difference of mass transfer performance between the +GG group and -GG group.

The penetration test of FITC was confirmed the device capability to accumulate the macromolecule selectively based on their size. During 24 hours of the penetration test, the only macromolecule which can pass through the 12kDa MWCO dialysis membrane is only 4kDa FITC, while the 10 kDa FITC and 20 kDa FITC was still remained at the culture compartment without detected in dialysate compartment.

Evaluation of Gellan Gum Effect in Hydrodynamic Shear Stress Induced-cell Injury

In order to minimize the cell injury by the other factor, such as change of pH by elevation induced by intolerable elevation of lactate accumulation in medium, the hiPSCs spheroids were cultured at 6 hours under rotational culture. The results showed that the addition of gellan gum was significantly reduced the LDH leakage caused by membrane rupture of the damaged cells. In contrast, the control group without gellan gum addition apparently experienced injury in relatively similar percentage among the different density population.

2.3.2. Cellular Proliferation and Spheroids Morphology in High-Density Suspension

Culture

At the end of the hiPSCs expansion in spheroids form, 500 spheroids were measured from each culture population and compared with initial spheroid size at day 0. The population in negative control were excluded regarding the limiting number of spheroid below 500 at the end of the expansion. In general, the spheroid size was relatively increased in all of the spheroids population cultured in dialysis fed-batch system. In the positive control, the number of spheroids was largely depended on initial spheroid seeding density. The higher initial cell seeding density mostly tended to reduce its size after 5 days of culture, while the lower initial cell seeding density can keep increasing its diameters. Interestingly, the smaller spheroids with higher uniformity also increase in higher initial spheroid density group population in all culture systems. The spheroid was increasing below 680 μm in diameters during 4-day expansion and the histological morphology showing an absence of necrotic area at the center of spheroid were usually caused by mass transfer limitation of nutrition and secreted toxic metabolite.

The cellular proliferation was significantly increased in HD culture groups compared to conventional suspension culture and a negative control without medium change. In conventional suspension culture, the proliferation rate of all initial cell seeding density population at day 4 was limited up to approximately 5×10^6 cells/ml.

2.3.3. Glucose Consumption and Lactate Secretion During 5 Days Culture

Based on glucose measurements, the high-density dialysis systems show better performance in glucose delivery compared to positive and negative control group. Compared to the two control, the glucose concentration in dialysis systems can be maintained in a density-dependent manner at around 2.2-3.0 g/L which is nearly the original concentration of culture medium. This results indicated that in terms of glucose delivery, the dialysis systems have been able to support feeding requisite for high cell density culture.

The lactate concentration in dialysis culture systems has successfully maintained the equilibrium of concentration between 0.1 and 0.5 g/L. This concentration was much lower than the critical lactate accumulation determined by the previous study. These conditions also confirmed by higher lactate accumulation in all initial cell density in both positive and negative control which rise above the lethal limit of lactate concentration.

2.3.4. Accumulation of bFGF and Nodal in Culture Medium

By using 12 kDa MWCO dialysis membrane, the bFGF concentration can be better maintained during 4 days expansion in the HD culture system. This condition was occurred because of the bFGF were continuously retained in high density during the expansion period. In CS culture system, the bFGF concentration was limited by 24-hour medium removal which contain the accumulated circulating bFGF and replaced with new E8 medium containing approximately 80-100 ng/ml bFGF. Meanwhile, the bFGF in NC were decreased without any medium replacement during expansion.

Nodal is an important macromolecule which not externally supplemented in E8 medium. In our experiment, this growth factor act as a native cytokine which natively secreted by the hiPSCs itself. The result shows that at the end of the expansion, the Nodal were circulated and accumulated in higher concentration at HD culture medium in comparison with both CS or NC. It's concentration was relatively increased in larger initial cell density in HD culture. In contrast, the nodal concentration was decreased in larger initial cell density.

2.3.5. Gene Expression Level of Pluripotency Transcription Factor

The gene expression analysis was performed to measure the capability to maintain the pluripotent capability after 5 days of culture. In general, the upregulation of most of the pluripotency marker SOX2, OCT4, and Nanog was expressed in HD culture with dialysis support. Although the OCT4 and SOX2 were expressed in an almost similar level, the expression level of Nanog was significantly higher altogether with increasing cell density. In

contrast, general pluripotency marker was expressed in a relatively lower level in conventional suspension culture.

2.3.6. Differentiation Potential into Three Germ Layer Lineage

The distinguished morphological shifting was observed after the hiPSCs spheroid were differentiated by DMEM basal medium consisted of 10% fetal calf serum. The spherical hiPSCs spheroid was altered into the swelling embryoid bodies which are found to be less at higher density population of HD culture.

In order to observe the differentiation potential into a specific cell type which reconstituted from three germ layer lineage, the analysis of gene expression level was analyzed by using specific marker OTX2 (ectoderm), Brachyury (mesoderm), and GATA4 (endoderm). The results showed that compared to conventional suspension culture, the hiPSCs spheroid cultured in dialysis culture insert showed higher differentiation capability into three germ layer. In addition, this potential significantly increased altogether with increasing final cell density in HD expansion.

2.4. Discussion

Our primary objective in high-density hiPSCs culture systems is to achieve the very high-density cell yield in a well maintained biological environment. Generally, the high-density mammalian cell culture is referring to 10^7 cells/ml¹²⁵. Although the current conventional culture can be utilized to expand the hiPSCs at higher cell density, several

factors can confine the limitation. The mechanical stress, medium formulation, nutrient transfer, and accumulation of toxic metabolite is the main factor which can determine the maximum cell amount which can be maintained in culture systems. The main concept in the utilization of this systems is to localized the very high density of hiPSCs and high molecular weight growth factor in a certain area to maintain self autocrine/paracrine while continuously exchange the low molecular weight nutrition and removal of lactic acid as a waste product⁸⁰. The mass transfer capability of our device was currently confirmed by cell-free penetration test and showing its capability to perform medium refinement while selectively localized the macromolecule based on their molecular weight.

At day 5 of expansion, the lower proliferation rate was noticed in positive control. Moreover, the decreasing number of spheroids occurred in a more intense manner in higher initial cell density of negative control. This two phenomenon were increased altogether with higher initial cell density. Interestingly, the glucose concentration both in positive control and even negative control still remained at sufficient amount at the end of the expansion. This evidence was clearly shown that the caused of the decreasing number of viable cells in most of the higher density cells in the control population were not caused by glucose starvation. Apparently, the accumulation of lactate produced by very high initial spheroids seeding number severely damage the cells and prevented the spheroid growth from the early days of expansion. In conventional suspension culture with regular 24 hours of medium replacement, the proliferation of the spheroid in all of initial cell density group was stopped at a certain amount of density threshold in around 5×10^6 cells/ml. This two results indicated the certain limitation mainly in terms of lactate accumulation as toxic metabolite which

decrease the pH and inhibit the cellular growth related with volume limitation. This evidence were confirmed by elevated lactate concentration which reaching or exceeding the lethal lactate accumulation of concentration which previously studied, while the glucose were remaining in significant amount in culture medium. These results showed that the main limiting factor in high density cell culture is the lactate accumulation.

Compared to the positive- and negative control population, the results of hiPSCs expansion were significantly enhanced in dialysis culture systems. The hiPSCs were rapidly expanding regardless its high populations density. As expected, with the continuous lactate removal by using the dialysis fed batch support, the concentration of lactate in culture compartement can be maintained below the lethal lactate concentration, and the growth limiting condition were caused by lactate accumulation can be eliminated. Moreover, the dialysis systems also showed its capability to steadily keeping the high glucose concentration in culture compartment at the same time.

The spheroids density influences cellular behavior and properties due to cell-cell communications, localized/concentrated of auto- / paracrine factors, spheroid shape, and mechanical interactions. In rotational suspension culture, the dynamic condition were utilized to significantly prevent the excess spheroid agglomeration. On the other hand, the mechanical stress caused by this dynamic condition also induce the unwanted spontaneous differentiation and affecting the cellular viability. In order to reduced these agitational effect and prevented the excess agglomeration, we added the gellan gum biopolymer in culture medium. The previous study was revealed the potential of gellan gum biopolymer to prevent the agglomeration in hiPSCs suspension culture¹⁰⁵. Our comparation results in rotary

suspension culture were also showed the decreasing of cellular injury in significant amount. The reduction of cell injury were possibly occurred by increased the medium viscosity with rheological properties modification of culture medium by GG addition. In addition the excess agglomeration also did not observed during expansion, which showed its potential to be used in hiPSCs based-tissue engineering for regenerative medicine. As well as live-cell contamination, bacteria-derived materials such as GG are susceptible to contamination with endotoxins, which posses the possibility to trigger immunogenic reactions and thereby compromise implanted cells or organ¹²⁶. For this reason, the GG intended for cell culture, particularly in vivo testing, should be purified of endotoxins. Several methods exist for endotoxin removal, including phase separation, chromatographic and ultrafiltration techniques, and the levels of remnant endotoxins can be quantitatively determined using limulus amoebocyte lysate (LAL) tests¹²⁷. However, low endotoxin or partially synthesized GG products are commercially available, and will likely can be potentially used as an appropriate feedstock material for most GG tissue engineering purposes¹²⁸.

During rotational culture, the different mixing zone were distinct between the distance from the central area of the culture compartment. The previous study showed that the higher and fluctuated shear stress were mostly occurred in pheriphery region compared to the central area¹²⁹ (Appendix). These non-uniform mixing in dynamic culture systems can promote the spheroid heterogeneity within different mixing zone¹³⁰. In lower density population, the spheroid apppearently experienced different hydrodynamic exposure. Consequently, the non uniform spheroid were produced and distributed in certain area. The larger spheroids were mostly located at the periphery region of the spheroid colony while the

smaller spheroid were concentrated at the center of the colony. In contrast, the higher density spheroid were mostly spread homogeneously through the dialysis insert while the hydrodynamic force were distributed equally between the hiPSCs spheroids, produced heterogenous smaller spheroids. Apparently this uniform and well controlled size condition were also influence the pluripotency by reducing the mass transfer limitation of nutrition, toxic metabolite, and macromolecule substance (growth factors/ cytokine) which usually occurred in very large spheroid.

The main challenges in high density culture system is the accumulation of lactic acid as toxic metabolite which can increase the culture medium pH which inhibit the cellular proliferation. Moreover, hiPSCs exhibit higher anaerobic respiration and metabolism compared to adult cell types. In consequence, the lactate secretion were much more higher and the glucose consumption were lower than adult cell types. To compensate this situation in conventional suspension culture, the medium need to replaced frequently to manually remove this toxic metabolites.

The hiPSCs natively maintain their homeostasis by continuously secrete several growth factor, such as bFGF, TGF β 1, LIF, Nodal, Insulin, and other unknown factor which required to maintain the pluripotency and cellular proliferation (Table 3). However, this important secreted cytokine were frequently removed by medium replacement, meanwhile the costly additional macromolecules such as bFGF, TGF β 1, and insulin were required to be supplemented continuously in culture medium.

Basically, by using our proposed HD culture system, we expect the retainment of these macromolecule on culture compartment. bFGF is one of the growth factor which

regularly supplemented in culture medium at high concentration (100 ng/ml). This growth factor were accumulated altogether with the secreted bFGF. On the other side, this macromolecule were also used by the hiPSCs to maintain its homeostasis, which indicated by the existence of the circulating bFGF. These two phenomenon were depend on the increasing of the cell number. In the HD culture, the availability of the bFGF can be maintained while the hiPSCs were proliferate at very high number.

The other growth factor which also have a role as important cytokine to maintain the pluripotency is Nodal^{131–133}. This macromolecule were not externally supplemented or consisted in E8 culture medium formulation. Despite of very high number of the resulted HD expansion, the nodal were detected at large amount and its increase altogether with increasing of density, while the nodal concentration of the medium from the resulted small cell density in conventional culture were significantly lower than HD culture. Interestingly, the nodal were still detected in higher amount from death cells in negative control. This indicated that the nodal usage of the viable cells is one of the important factor which affecting the amount of the circulating nodal. The nodal signaling was previously identified as one of the main mechanisms to maintain the PSCs pluripotency by enhanced the upregulation of Nanog¹³¹. Our results showing the result which correspond with this mechanisms. The nodal concentration were consistently showing similar pattern with Nanog gene expression at the end of expansion period.

Table 3. Medium component used in this experiment

No.	Molecule component in culture system	Molecular Weight	Conventional suspension	High density suspension
1.	DMEM/F-12	< 1 KDa	2 ml	refined
2.	Ascorbic acid-2-phosphate magnesium	278.4 Da	64 µg/mL	omitted
3.	Sodium selenium	172.94 Da	14 ng/mL	omitted
4.	FGF2	18 kDa	100 ng/mL	100
5.	Insulin	5.74 kDa	19.4 µg/mL	19.4 µg/mL
6.	NaHCO ₃	84 Da	543 ng/ml	omitted
7.	Transferrin	80 kDa	10.7 µg/mL	10.7 µg/mL
8.	TGFβ-1	25 kDa	2 ng/mL	2 ng/mL
9.	Low acyl gellan gum	500 kDa	2%	2%
10.	Other native cytokine		-	Accumulated

In suspension culture, the hiPSCs were normally tend to form spheroids due to strong cell-cell interaction. The activation of some membrane receptor, such as E-cadherin was essentially required to maintain the pluripotency by direct interaction between the cells. This condition can be achieved in direct cellular contact between them in three-dimensional spheroid form. By using dialysis support, the supported macromolecule components which externally administrated and released from the hiPSCs into the medium were expected to be accumulated and recycled in culture compartment (Table 2.1). The accumulation of this macromolecule also has been identified as the important factor of auto- / paracrine signaling complex which is essentially required to maintain the state of pluripotency and proliferation. This phenomenon was observed in our experiment by gene expression measurements. In general, the higher spheroid density in dialysis culture resulted in better pluripotency gene expression and trilineage differentiation potential. Meanwhile, the pluripotency was significantly decreased in the higher density of the control population. In this phenomenon, we hypothesized that the accumulation of this secreted auto-/paracrine in upper compartment

enhanced the self-maintenance of pluripotency regulatory between spheroids when supported by the continuous small molecule nutrient transfer and lactate removal.

2.5. Conclusion

Based on these findings, we suggest that the dialysis culture system support was successfully maintained the culture condition in the hiPSCs proliferation up to 7 fold expansion rate in well preserved-pluripotency. This experimental results showed the potential for cost-effective hiPSCs expansion methods with efficient growth factor usage in combination with low shear stress culture environment by GG administration.

Chapter 3 - Size-Dependent Hepatic Differentiation of Human Induced Pluripotent Stem using Single Spheroid Suspension Culture

3.1. Introduction

The *in vivo* propagation of fresh hepatocyte isolated from adult liver was provided the opportunity for many applications⁵⁷. However, to obtain the hepatocytes the limitation in the shortage of the donor organ still be a problem⁸⁴. The human induced pluripotent stem cells (hiPSCs) derived-hepatocyte (iHeps) represent a potentially novel source for various applications. For these practical applications, it is necessary to generate a higher number of iHeps in similar functional properties with primary hepatocyte as ideally original liver counterparts^{134,135}.

One of the potential methods to increase the yield production of iHeps is by cultured and performed the differentiation in three-dimensional (3D) culture¹³⁶. Despite the development of differentiation methods in 3D spheroid form, the production of functional iHeps from hiPSC cells still technically challenging. This problem arose by insufficient maturation and the heterogeneous population of different lineage resulted from hepatic differentiation^{137–139}. Moreover, the emergence of the resulted 3D cyst-like structure population in hepatic differentiation was often reported to decrease the hepatic differentiation capability by shifting its characteristics into other lineage¹⁴⁰.

During 3D spheroid culture, the size-related factor can be affecting both quality and quantity of the resulted cell yield. This phenomenon was indicated the importance of controlling the spheroid size in various application such as hepatic differentiation, mainly when using larger scale culture system. Currently, there are several studies which performing 3D hepatic differentiation with several types of the bioreactor, such as stirred tank bioreactor^{140,141}, perfusion culture¹⁴², or rotary culture¹⁴³. However, the optimization study of initial spheroid size for long term hepatic differentiation were still few.

To overcome this problem, we evaluate the size-dependent hepatic differentiation of hiPSCs spheroid to identify the effect of initial spheroid size and obtain the optimal initial spheroid size specified for long term hepatic differentiation. Therefore, we perform the single spheroid forming and hepatic differentiation in suspension culture using each well of 96 well U shape low attachment well plates to analyze the independent spheroid in a size-specific manner.

3.2. Methods

3.2.1. *hiPSCs Maintenance on Matrigel Coated Tissue Culture Dish*

The TkDN4-M human hiPSCs cell line (provided by Stem Cell Bank, Center for Stem Cell Biology and Regenerative Medicine, The University of Tokyo)¹⁴⁴ cultured by mTeSR1 medium (Sigma) in Matrigel (Corning) coated-6 well plates tissue culture dish (Iwaki). The medium refreshment was carried out every 24 hours.

3.2.2. *Hepatic Differentiation in 2D Monolayer Culture*

10⁵ hiPSCs were seeded in Matrigel-coated-6 well plates tissue culture dish and maintain under regular monolayer hiPSCs culture conditions until it reaches around 80% confluency. Afterward, the culture medium was replaced by differentiation medium every 24 hours following previous monolayer hepatic differentiation protocol by Si-Tayeb *et al.*, 2010⁵⁷.

3.2.3. *Hepatic Differentiation in Suspension Culture of Single 3D Spheroid*

The hiPSCs were harvested from monolayer culture and dissociated using Trypsin-EDTA solution (Life Technologies). The single cells obtained by passing the hiPSCs clumps through the cell strainer. To generate the various size of the spheroid, 200 µl of mTeSR1 medium solution consisted a different number of cells were seeded into each well of ultra-low attachment round bottom-96 well plates for 24 hours at the 37°C incubator in the static condition. Afterward, the hepatic differentiation was initiated by replacing the medium with

200 μ l of differentiation medium with the same formulation as described in monolayer hepatic differentiation. The medium replacement was carried out in every 24 hours. In order to slightly mixing the medium, the plates were placed under 105 rpm rotary shaker during the spheroid forming and differentiation period.

3.2.4. Morphological Analyses of hiPSCs Spheroid during Differentiation

The morphological analyses conducted by direct observation using the light microscope (Olympus inc.). Afterward, the pictures were taken and the spheroid size was analyzed by using Fiji (NIH) image analysis.

3.2.5. Glucose Measurement in Culture Medium

To analyze the glucose consumption during hepatic differentiation, 200 μ l of the culture medium were isolated every day and measured using multipurpose analyzer (YSI).

3.2.6. Gene Expression Analyses by qRT PCR

The Spheroid was dissociated using Trypsin-EDTA solution (Life Technologies). The RNA isolation was performed using Trizol reagents (Life Technologies). The purified RNA solution was reverse transcribed by using ReverTra Ace qPCR RT Master Mix (Toyobo) following qPCR analysis using Thunderbird SYBR qPCR Mix (Toyobo). The gene

expression analysis performed by using Pikoreal Real-Time PCR System (Thermofisher). All of the procedure was performed by using the manufacturer protocols.

3.2.7. Albumin Concentration Measurements by ELISA

The 100 μ l solution containing 1 g/L albumin primary antibodies (Bethyl) were immobilized in each well of 96 well plates by incubation at 37°C for 3 hours. Afterward, the plates were washed with 200 μ l/well PBS-Tween, then the 100 μ l/well of blocking buffer was added and incubated for 1 hour at room temperature. The 100 μ l of a serial dilution of the medium sample and standard solution were plated in each well and incubated 2 hours at room temperature. The plates were washed with PBS Tween and 100 μ l/well of 1g/L diluted HRP labeled-secondary antibodies (Bethyl) added and incubated for 2 hours at 37°C. to obtain the color reaction, the plates washed by PBS tween and 100 μ l of color solution consist of 2.5 mg 0-phenylene diammonium chloride and 0.5 μ l H₂O₂ per ml citrate buffer were added to each well following incubation for 30 minutes in room temperature. The color reaction was stopped afterward by adding 50 μ l/well H₂SO₄ (4N) without removing the previous solution. The fluorescence intensity was measured by Wallac Arvo SX 1420 multilabel counter.

3.2.8. Histological Analyses of Thin Cross Sectioned-iHeps Spheroid

The spheroid was collected and fixed by 4% paraformaldehyde solution (Wako) in PBS for 30 minutes at room temperature, washed in PBS and replaced with 30% PBS buffered sucrose solution at 4°C overnight. The sucrose solution was discharged, and the

Spheroid placed in the cryomold following embedding with OCT at -20 °C until hardening. 10 µm thin sections were taken by cryostat and mounted onto glass slides for 2.8 hematoxylin-eosin staining.

3.2.9. Statistical Analyses

The statistical analysis was performed by using GraphPad Prisms 7.04. Statistical significance between each dataset was determined by ordinary one way ANOVA methods, and values presented as means ± standard deviation.

3.3. Results

3.3.1. Morphological Pattern and Proliferation Rate of hiPSCs Spheroids During Hepatic Differentiation

During definitive endoderm differentiation, several amounts of the hiPSCs were detached from the outer layer of the spheroid which caused by activin A-induced apoptosis¹⁴⁵. However, most of the spheroid maintained their size by keep proliferating up to day 5. During day 10-15 of differentiation, most of the larger spheroid size (6250-25000 initial cell number) was increased in size, and gradually forming the smaller dense compact spheroid at hepatic maturation stage. The partial swelling was started to occur at day 10-15 in several amounts of all size of the spheroid, which gradually reduced in hepatic maturation stage. However, in smaller spheroid (3125 initial cell number), the swelling area becomes increase from day 10 and altered into the cystic-like structure which mostly tends to keep swelling with the various

size. This smaller spheroid also persistently maintain their structure until the end of differentiation. The higher error bar in small Spheroid showed the variability between the cyst-like structure.

Based on histological examination of cross-sectioned Spheroid, it is was revealed that the dense spheroid showing higher cell viability which exhibits the small central necrotic area was appeared in the dense Spheroid. Apparently, the increase of swelling area was affecting the cell viability inside the spheroid. This phenomenon can be observed in partially swelled spheroid. Moreover, the larger amount of the dead cells were observed in the cystic spheroid.

To evaluate the growth kinetic during hepatic differentiation of hiPSCs, the glucose consumption was determined by its concentration in the differentiation medium. At definitive endoderm differentiation stage, the consumption of glucose was relatively decreased more in larger spheroid. This condition mostly related to the larger surface area which exposed by activin A priming during endodermal differentiation. In terms of proliferation level, the spheroid formed from 6250 and 12500 cells show higher fold increase compared to the other group of initial cell seeding density.

3.3.2. Role of WNT/ β -catenin signaling in foregut endoderm and Hepatoblast

The expression of endoderm gene marker GATA4 was slightly increased in larger spheroid size, indicated that the progress of endodermal differentiation at this step was not yet affected by mass transfer limitation of nutrition, oxygen, and growth factors. WNT/ β -

catenin signaling pathway plays the important role of hepatic differentiation in stage-specific manner¹⁴⁶. During in vivo liver organogenesis, the WNT/ β -catenin were enhanced in endoderm development. However, suppression of this pathway is required to promote foregut endoderm into hepatoblast^{147,148}. Axin 2 is the negative regulator of WNT/ β -catenin signaling which induced by the feedback mechanisms of WNT/ β -catenin signaling¹⁴⁹. The increasing level of Axin 2 can be used as an adequate representative indicator of WNT/ β -catenin upregulation.

At the end of hepatoblast differentiation, we found that the Axin 2 was significantly downregulated in larger Spheroid size. Increasing activity of WNT/ β -catenin signaling had previously known as an inhibiting factor of HNF4 α ¹⁵⁰. In contrast, the result showed that HNF4 α were significantly increased gradually in larger spheroid size.

3.3.3. *Characterization of Resulted iHeps*

The higher albumin gene expression of iHeps was resulted from larger initial spheroid size which confirmed by enzyme-linked immunoabsorbent assay (ELISA). The albumin was only could be detected after hepatic maturation stage and the average albumin secretion was increased in iHeps obtained from larger spheroid. Importantly, the iHeps produced from larger Spheroid possessed higher drug metabolism capability by expressing CYP3A4 in a significant manner compared to the smaller size spheroid. In addition, the larger spheroid exhibits a lower expression level of immature hepatocyte marker AFP. All of the 3D iHeps spheroids also showing lower expression of cholangiocyte marker CK19 compared to 2D iHeps which indicate higher specification rate of resulted iHeps.

3.4. Discussion

HiPSCs cultured and differentiated in the form of spheroid has the capability to form their own microenvironment similar to original organ development *in vivo*^{151,152}. This advantage can be applied in directing the hiPSCs toward hepatocytes in large scale suspension culture to meet the necessity of a large amount of the cells for several applications. Although previous study which utilized single uniform spheroid inoculation such as by using microscale 3D micropattern plate, but the impact of spheroid size still was not determined¹⁵³. To observe the effect of the spheroid size in the hepatic differentiation, we formed the individual hiPSCs spheroid which consisting identical size to perform hepatic differentiation in suspension culture.

Based on the fold increase of viable cells, the middle range diameter of our experimental set (spheroid formed from 6,250-12,500 cells) resulted in higher expansion rate. We hypothesized that this condition was related to the cystic structure resulted in most of the smallest spheroid population and increasing necrotic area at the center of the dense spheroid. The histological analyses result showing the necrotic area which observed at the center of dense iHeps spheroids. This phenomenon possibly related to the limitation of the mass transfer limitation of oxygen, nutrient, and cellular waste product at the center of spheroid altogether with increased spheroid size¹⁵⁴⁻¹⁵⁷. In contrast, the cystic like-iHeps spheroid showing distinct histological feature indicated an increasing amount of necrotic area in swelling or cystic spheroid which reduced the viable cells in cystic spheroid which mostly form the epithelial like-cells surrounding the cystic wall. Although the partially swelling

structure was found at several numbers of the larger size spheroid, this condition was found more severe in smaller spheroids which mostly formed cystic-like structure at the end of differentiation.

The change of glucose concentration in culture medium was measured to observe the metabolic process during differentiation. In most of the spheroids the glucose consumption rate significantly increased in consistence with increasing of spheroid growth at foregut endoderm and hepatoblast differentiation stage. However, their rate was decreased at approximately early of hepatic maturation stage which related to decreased spheroid diameters or shifting its metabolic signature to oxidative phosphorylation in order to obtain their energy source in late differentiation process¹⁵⁸.

The activity of the stage-specific WNT/ β -catenin signaling taking an important role in hepatic differentiation. Some study was showed the involvement of WNT/ β -catenin signaling both in 2D or 3D hepatic differentiation. However, the involvement of WNT/ β -catenin signaling related to size dependent-spheroid in hepatic differentiation was still few to be reported. Following definitive endoderm differentiation, the hiPSCs differentiated toward hepatoblast by hepatic specification stage. In this stage, repression of WNT/ β -catenin signaling is necessary to direct the definitive endoderm into hepatoblast. This signaling also has previously identified as the inhibitor of early functional hepatic regulatory genes, such as HNF4 α ^{147,159}. In our study, the inhibition of HNF4 α by this signaling activity in the hepatoblast differentiation stage were occurred and increase the reduction of maturation capability, mainly in smaller spheroid. As the result, the downregulation of several hepatic

markers such as ALB and CYP3A4 has occurred altogether with upregulated immature hepatic marker AFP. This condition also confirmed by the decreasing of albumin secretion in lower spheroid size. Other factors which also influence the downregulation of hepatic functionality is increased portion of cystic-like forming which consists of a large number of necrotic cells. Apparently, the loose cell-cell interaction in the cystic structure formed from smaller spheroid reduce the capability to regulate the normal hepatic differentiation mechanism by WNT/ β -catenin signaling inhibition and causing the apoptosis at the same time.

Our present study showed that despite the existence of the necrotic area in a larger dense spheroid, the larger iHeps spheroid showed better physiological maturation. The albumin secretion was only detected at the end of the final stage of differentiation in all experimental group. In addition, the higher albumin gene expression which confirmed by its production in the medium also highly detected in larger spheroids. However, in terms of maturation, the iHeps spheroid resulted from hepatic differentiation still required an improvement since it was showing a significantly lower level of albumin secretion compared to primary hepatocyte cells PXB1. This result has corresponded with the previous study showing the increasing hypoxic area inside the larger spheroids resulting better endodermal differentiation capability which exhibits higher potential of early hepatic progenitor marker AFP¹⁶⁰. Other previous report showed that the low oxygen concentration was able to increase the differentiation capability of stem cells into hepatic progenitor cell in 2D culture system¹⁶¹. Moreover, the higher cell-cell interaction in larger 3D spheroid was necessary for directing the hiPSCs cellular fate into the hepatocyte. The accumulation of their own endogenous

factor apparently contributes to maintaining the microenvironment inside the spheroid by increasing the cell-cell interaction which possibly occurred in larger spheroid size which stimulates the hepatic maturation¹⁵¹.

The hepatic differentiation was often resulting the heterogeneous population consisted of cells expressed hepatic and cholangiocyte marker which related with the bipotential differentiation capability of hepatoblast^{136,162–164}. Moreover, the previous study was shown that the three-dimensional structure hepatoblast promotes the epithelial polarity contributed to the forming of differentiated cholangiocyte which characterized by cystic like structure^{165–167}. Therefore, OSM was administrated at the last stage of hepatic differentiation to blocking the cholangiocyte differentiation and directing differentiation into hepatocyte lineage^{165,168}. To identify the status of the cyst-like spheroid, we analyzed the level of cholangiocyte gene expression marker CK19. Interestingly, the gene expression analyses revealed that the expression level of cholangiocyte marker CK19 was almost similar in all size of the spheroid. In contrast, the differentiated hiPSCs population in 2D culture were demonstrated higher tendencies to differentiate into cholangiocyte by expressing higher CK19. This result demonstrated the importance of three-dimensional structure support to decrease the tendency of the bipotent hepatoblast to differentiated into cholangiocyte.

This study showing the importance to determine the optimum spheroid size for hepatic differentiation. The enhancement of the native paracrine stimulation between spheroids may be maintained in a high-density cell number to increase the differentiation efficiency. However, some challenges such as nutrition transfer, mechanical stress, and

excess aggregation were still remained and need to be addressed in the future. The modulation of WNT/ β -catenin signaling can be administrated in specific differentiation stage to enhanced the hepatic differentiation efficiency^{146,159}.

3.5. Conclusion

In this study, we suggest that the higher size of initial hiPSCs Spheroid at some extent can be able to increase the differentiation efficiency towards hepatocytes. The use of >250 μm diameter initial spheroid size seems to be a potential size to increase the efficiency of hepatic differentiation.

Chapter 4 - High-Density Dynamic 3D Suspension Culture for Hepatic Differentiation of Human Induced Pluripotent Stem Cells

4.1. Introduction

The liver is the largest internal organ which plays important role in metabolism, detoxification, protein synthesis, and other various function. It consisted of various types of cell, such as hepatocyte, stellate cell, Kupffer cell, and endothelial cells. Hepatocytes are the largest portion of cell type in the liver which approximately 70-80% liver cells¹⁶⁹.

This cells can be cultured in vitro and utilized for various application, such as toxicological testing in drug development, in vitro disease modeling, and transplantation for liver failure. However, some remaining problem still needs to be resolved in the practical application which is related to the necessity of a high number of cells. For example, at least $1-3 \times 10^7$ hepatocyte/kg body weight was substantially required for cell based-transplantation for liver treatment^{170,171}. On the other side, culturing primary hepatocyte in vitro remains challenging because of their low proliferation level, prone to apoptosis and a significant decrease in physiological function over time.

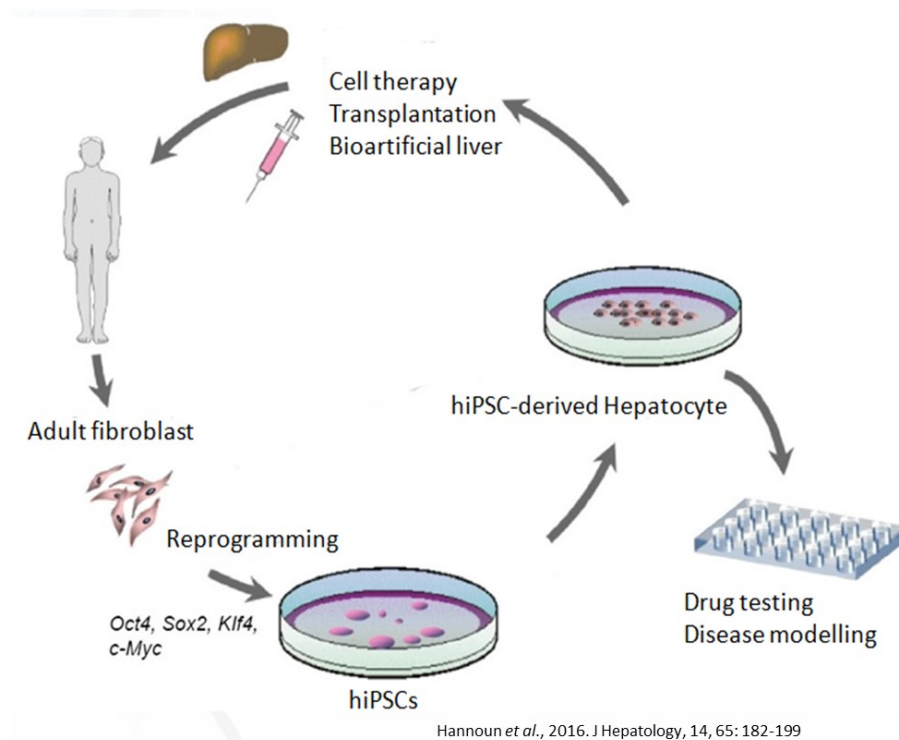


Figure 3. Application of hiPSCs derived-hepatocyte

Human induced pluripotent stem cells (hiPSCs) are considered to be used as an alternative source regarding its high proliferation capability to produce the hiPSCs derived

hepatocyte (iHeps) through stepwise differentiation. However, some remaining problems regarding differentiation efficiency in producing iHeps in a large amount still need to be addressed since the hepatic differentiation itself required an abundance amount of growth factor for directing the differentiation into the hepatic lineage. The three-dimensional suspension culture of hiPSCs has been largely known as cost-effective methods to expand and differentiating the large amount of hiPSCs into various kind of functional cell types. Moreover, the complex three dimensional (3D) structure of PSCs was largely speculated to increase the hepatic differentiation effectivity by providing high self-auto/paracrine interaction inside the 3D microenvironment structure when it is compared to two dimensional (2D) culture⁸⁵.

Our recent study of hiPSCs expansion was resulted in efficient methods to utilize the native secreted cytokine and recycling of growth factor to produce a significant amount of cells. Based on this results, we design the experimental method to extend the application of high-density culture systems for hepatic differentiation to reduce the considerable amount of growth factor during differentiation while increasing the physiological hepatic function by continuous medium refinement.

4.2. Methods

4.2.1. Design of Dialysis Device for High-Density Suspension Culture

The dialysis culture systems consist of two compartment, the upper culture compartment and a lower dialysate compartment. The 40 μm mesh bottom-cell strainer (PluriSelect) was used as culture compartment of dialysis systems. This culture insert was

modified by cutting and removing the bottom mesh layer. To selectively permeate the nutrition or cellular waste product, the 12 kDa molecular weight cutoff Spectra/Por 4 dialysis membrane was carefully affixed into the bottom side of the strainer using alkyl- α -cyanoacrylate-based surgical grade tissue adhesive Aron Alpha A (Daiichi Sankyo). The upper compartment then placed in 6 deep well plates as dialysate compartment (Corning).

As the control device, the cell strainer was directly affixed into the bottom surface of 6 well non-treated plates (Iwaki) by using alkyl- α -cyanoacrylate-based surgical grade tissue adhesive Aron Alpha A (Daiichi Sankyo). All of the devices were sterilized using EO gas sterilizer prior usage.

4.2.2. Monolayer hiPSCs Culture

Human induced pluripotent stem cells (TkDN-4M cell line) were cultured and maintained in vitronectin coated-tissue culture dish using complete supplemented Essential 8 (E8) culture medium and maintained following manufacturer protocols.

4.2.3. Hepatic Differentiation in Suspension Culture for Gellan Gum Evaluation

HiPSCs spheroid was generated by seeding 1×10^6 single cell suspension per well of 6 well plates for 24 hours in 4 mL of mTeSR1 media supplemented and placed in the 90 rpm rotary shaker. The spheroid was expanded up to 3 days by daily mTeSR1 medium replacement. Afterward, the spheroid were moved into non-treated 12 well plates containing 2 mL of basal differentiation medium consisted of IMDM with F-12 and differentiation

cocktail) Nutrient Mixture (Ham), 5% fetal bovine serum, 1% insulin transferrin selenium-A supplement, 55 μ M monothioglycerol, 100 U/ml penicillin, and 0.1 mg/ml streptomycin (Sigma-Aldrich). The multi-step differentiation was promoted through additional growth factor and WNT/ β -catenin signaling inhibitor during each step of hepatic differentiation. The differentiation towards the definitive endodermal stage was promoted through the administration of 10 ng/ml bFGF, 100 ng/ml Activin-A and 10 ng/ml TGF- β (PeproTech). The second foregut endoderm was promoted by the addition of 10 ng/ml FGF-4 (PeproTech) and 10 ng/ml BMP-4 (R&D Biosystems). The Wnt signaling was required to be absent in hepatobiliary commitment. The 1 μ g/ml WIF-1 (R&D System) and 0.1 μ g/ml DKK-1 (PeproTech) were added to suppress the Wnt signaling. The hepatic maturation was induced by adding 50 ng/ml HGF (PeproTech) and 30 ng/ml Oncostatin A (PeproTech) in the final step. The culture system was placed in 120 rpm rotary shaker. The medium replacement was carried out every 24 hours.

4.2.4. High-Density Hepatic Differentiation of hiPSCs in Suspension Culture

HiPSCs spheroid was generated by seeding 2×10^6 single cell suspension per well of 6 well plates for 24 hours in mTeSR1 media supplemented and placed in the 90 rpm rotary shaker. Afterward, the spheroid was expanded up to 3 days by daily mTeSR1 medium replacement.

The dialysis membrane of the culture insert was activated by pre-wetted the dialysis layer using 2ml of sterile H₂O 15-30 minutes before the dialysis culture was started. The

dialysis culture insert was placed in 6 deep well plates. After the H₂O was completely removed, 15 ml of IMDM/F12 were inserted into dialysate compartments (6 deep well plates) and The hiPSCs spheroids which previously formed were harvested and transferred into the culture compartment using 2 ml of IMDM/F12 basal medium consisting 2% FcEM were inserted into culture compartments. The differentiation cocktail was administrated every 24 hours at the culture compartment only. The hepatic differentiation performed by 120 rpm rotary shaker. As a control group, the conventional suspension culture using the same condition also performed without any dialysis support.

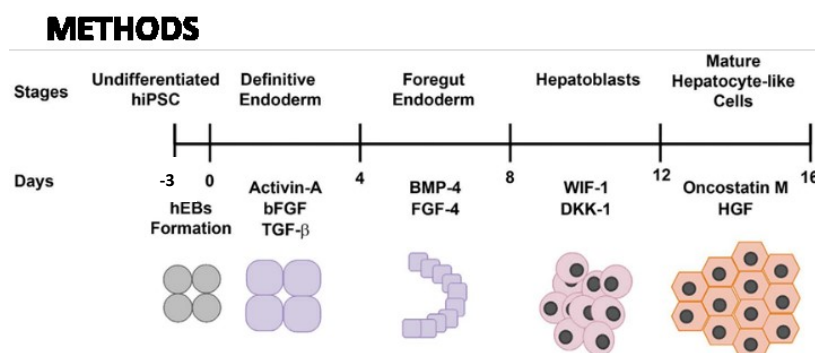


Figure 4. General 3D differentiation protocols in this study

4.2.5. Morphological Analysis

At the end of differentiation, each of the spheroids group was moved into 12 well plates and rotated for 3-5 minutes following visualization of the macroscopic and microscopic image by light microscope (Olympus). The image of the spheroid colony was taken and the spheroids diameter analyzed by FIJI/ImageJ software.

4.2.6. Cell Count Determination

After the morphology was observed directly by light microscope, the spheroids were collected, centrifuged at 1000 rpm for 3 minutes, and the supernatant removed from the tube. To obtain the single cells, 1 ml of TrypLE dissociation reagent were added into the spheroids and incubated for 10 to 15 minutes at 37°C following homogenization by gentle pipetting. Afterward, the cells were counted by using Haemocytometer.

4.2.7. *Gene Expression Analysis by qRT-PCR*

The mRNA was isolated from 10^6 cells using Trizol reagents (Life Technologies). The purified RNA solution was reverse transcribed by using ReverTra Ace qPCR RT Master Mix (Toyobo) followed by qPCR analysis using Thunderbird SYBR qPCR Mix (Toyobo) using manufacturers instruction. The gene amplification and expression analysis performed by using Pikoreal Real-Time PCR System (Thermofisher)

4.3. **Results**

4.3.1. *Evaluation of Gellan Gum Addition in Hepatic Differentiation of hiPSC in*

Conventional Suspension Culture

The two kinds of iHeps spheroid consisted of partially swelling spheroids and dense spheroids resulted after 20 days of hepatic differentiation. After 16 days The partial swelling structure was started to appear at foregut endoderm differentiation stage and increase in size up to the end of hepatoblasts differentiation. Although the protocol was different, these results were consistent with the previous results which previously mentioned in chapter 3. However, the wall of the swelling structure was relatively thicker than the previous study. In terms of morphological appearance, there is no significant difference between the +GG group with -GG group

The gene expression measurement also revealed that although there is no significant difference of CYP3A4 among all group, the ALB was significantly upregulated in +GG

group. In addition, all of the 3D differentiation showed lower expression of cholangiocyte marker

4.3.2. Cellular Proliferation and Spheroids Morphology in High-Density Suspension

Culture (Up to Definitive Endoderm Differentiation)

The cellular proliferation was significantly increased in dialysis systems compared to both positive- and negative control. In positive control, the proliferation rate of all initial cell seeding density population at day 4 were showing maximum expansion threshold up to 5×10^6 cells/ml in conventional culture with 24-hour medium replacement.

At the end of the hiPSCs expansion in spheroids form, 500 spheroids were measured from each culture population and compared with initial spheroid size at day 0. In general, the spheroid size was significantly increased in all of the spheroids population differentiated in HD dialysis fed-batch system. In the conventional suspension differentiation, the number of spheroids was largely depended on initial spheroid seeding density. The higher initial cell seeding density mostly tended to reduce its size after 4 days of endodermal differentiation, while the lower initial cell seeding density can keep increasing in diameters

4.3.3. Glucose and Lactate Concentration during Differentiation

Based on glucose measurements, the high-density dialysis systems show better performance in glucose delivery compared to positive and negative control group. Compared to the two control, the glucose concentration in dialysis systems can be maintained in a density-dependent manner at around 2.5-3.0 g/L which is nearly the original concentration of culture medium. This results indicated that in terms of glucose delivery, the dialysis systems have been able to support feeding requisite for high cell density culture.

The lactate concentration in dialysis culture systems has successfully maintained the equilibrium of concentration between 0.1 and 1.0 g/L. This concentration was much lower than the critical lactate accumulation determined by the previous study. These conditions also confirmed by higher lactate accumulation in all initial cell density in both positive and negative control which rise above the lethal limit of lactate concentration.

4.3.4. Gene Expression Level of Hepatic Endoderm

The gene expression analysis was performed to measure the differentiation efficiency to maintain the pluripotent capability after 5 days of culture. The results showed that, in general, the increase of cell density showed increased expression of endoderm marker GATA4. This result also confirmed by a significant decrease of pluripotency marker OCT4 in higher cell density. The expression level of both genes was comparable in similar initial cell density inoculation group between HD culture and conventional suspension culture.

4.4. Discussion

Based on the study on chapter 3, the Wnt/ β -catenin signaling should be reduced as much as possible in foregut hepatic endoderm stage. Therefore, we adopt the previous suspension differentiation protocol by using Wnt/ β -catenin inhibitor mentioned in the differentiation study by Pettinato et al. (2016)¹⁵⁹. The result showed that in term of hepatic maturation such as albumin expression was significantly enhanced in the +GG group. The reduction of albumin expression might be occurred by higher shear stress in -GG group caused by excess hydrodynamic stress in the rotational culture which is increased in less viscous medium without the gellan gum addition. In addition, the excess shear stress tended to direct the hiPSCs spheroid into mesoderm lineage¹⁴⁰.

During hepatic differentiation, the consistent manner was observed as similar as with our previous result, the resulting iHeps spheroid both from +GG and -GG population significantly exhibited lower expression of cholangiocyte when compared to two-dimensional culture. This results also strengthen hypothesis about the advantage of three-dimensional structure in hepatic commitment which decreasing variability of resulted iHeps which can consist of cholangiocyte remained by bipotential hepatoblast development.

After we ensure the capability of gellan gum to increase the hepatic differentiation by changing the rheological properties of differentiation medium, we apply this biopolymer combination in practical HD differentiation by using differentiation cocktail formulated in Table 4.1. The glucose consumption and lactate secretion were significantly higher metabolisms rate when compared to our previous study in HD hiPSCs expansion which mentioned in chapter 1. This condition was potentially caused by a high dose of activin A

administration which previously identified as a proliferating agent in pluripotent stem cells¹⁷². Moreover, in simultaneous combination with bFGF^{172,173} and TGFβ-1¹⁷⁴, activin A can evade the apoptosis and increasing the effectivity during endodermal differentiation stage in comparison with using Activin A alone. In consequence, this condition resulted in a significant escalation of lactate secretion in culture compartment, mainly in higher cell density. In terms of differentiation efficiency, the increasing density showed better differentiation which can be observed by increasing endodermal marker GATA4 following by reduced pluripotency marker OCT4. Interestingly, although the expression level between HD differentiation and conventional suspension culture with regular 24-hour medium replacement were comparable in each density group, the resulted yield was significantly increased in the HD population. This condition also can be observed by a significant increase of the average spheroid diameter of HD culture in comparison with conventional culture. In addition, despite large consumption rate by high cell number, the conditioned culture environment by medium refinement was successfully hinder the negative effect of toxic metabolite which limiting the proliferation up to 5×10^5 cells/ml density.

Table 4. The formulation of differentiation component between two groups of suspension culture

Differentiation stage.	Component	Molecular weight	Conventional	High density
Basal differentiation medium	IMDM/F-12	< 1 kDa	2 ml	Refined
Biopolymer	Low acyl gellan gum	500 KDa	2%	Localized
Definitive Endoderm	Activin-A	24 kDa	100 ng/mL	100 ng/mL
	bFGF	18 kDa	10 ng/mL	10 ng/mL
	TGF β -1	25 kDa	10 ng/mL	10 ng/mL
Foregut Endoderm	BMP-4	34 kDa	10 ng/mL	10 ng/mL
	FGF-4	19.4 kDa	10 ng/mL	10 ng/mL
Hepatoblasts	WIF-1	39.8 kDa	1 μ g/mL	1 μ g/mL
	DKK-1	35 kDa	0.1 μ g/mL	0.1 μ g/mL
Mature iHeps	OSM	25.7 kDa	30 ng/mL	30 ng/mL
	HGF	26 kDa	50 g/mL	50 g/mL

4.5. Conclusion

Based on these findings, we suggest that The dialysis culture system support was successfully maintained the culture condition in the hiPSCs proliferation up to 7 fold expansion rate in well preserved-pluripotency. This experimental results showed the potential for cost-effective hiPSCs expansion methods with efficient growth factor usage.

Chapter 5 – Conclusion and Future Perspectives

5.1. Conclusion

This study provides a simple culture design for expansion and hepatic differentiation of hiPSCs in suspension by investigating several biological factors followed by improvement of culture systems by controlling the culture environment in high cell density. This condition was achieved by continuous basal medium refinement while efficiently reduce the necessity of costly growth factor by accumulating and recycle the remaining suspended macromolecule cytokine/growth factors in the culture medium.

In chapter 1, the necessity of controlled microenvironment was explained. Some advantages of suspension culture can be utilized to optimize the quality of the resulted yields both in PSCs expansion and differentiation. However, some challenges and drawbacks still need to be overcome to get the better final result of cells. Several factors such as excess hydrodynamic stress, accumulation of toxic biomolecule, and lack of nutrition transfer can affect cell growth and reduced the expected physiological function of resulted cells. Therefore, several considerations related to the culture environment were importantly required to expand and directing the differentiation of the PSCs. One of the favorable approaches is by adapting the culture in vivo culture environment to mimics the original in vivo physiological condition of the PSCs culture system, such as the utilization of dialysis membrane.

In chapter 2, the design of small scale dialysis culture system in combination with low acyl gellan gum was improved and tested to provide the proper culture condition by continuous medium refinement, utilizing the accumulated of native or administrated cytokine, and providing the low shear stress environment. This device allowed the high-density culture in spheroid suspension up to 7.8 fold expansion with decent pluripotency and differentiation capability of cultured hiPSCs. This condition was achieved mainly by removal of lactic acid and the important macromolecule deposition in the culture compartment which improving their self maintenance capability.

In chapter 3, the investigation of the size-dependency effect of the hiPSCs spheroid in hepatic differentiation was carried out by formed and differentiated the hiPSCs in a single

spheroid manner. The result showed that the iHeps spheroid derived from more than 250 μm diameter hiPSCs spheroid showing the optimal hepatic differentiation performance. In addition, the cystic like structure resulted from smaller spheroid was observed. Based on the previous research, the appearance of this structure was related to the failure of cellular regulation to suppress the Wnt/ β -catenin in foregut hepatic endoderm stage which resembles the original liver development. The gene profiling also revealed that the increase of Wnt/ β -catenin signaling which upregulated in smaller spheroid can hinder the hepatic lineage maturation.

In chapter 4, the practical application of high-density culture by gellan gum and dialysis support was extended to hepatic differentiation. The initial study was showed the potential of gellan gum utilization in hepatic differentiation of the suspension culture. By considering the previous result, the larger spheroids were used and the hepatic differentiation protocol for three-dimensional hiPSCs spheroid by using stepwise differentiation cytokine cocktail with Wnt/ β -catenin inhibitor in foregut hepatic endoderm was adapted. In current progress at definitive endoderm stage, the high-density culture showing better proliferation with similar performance as that in conventional suspension differentiation.

In conclusion, this system emphasizes the potential of the cost-effective culture platform to improve the high-density hiPSCs with a lower amount of expensive cytokines from an external source.

5.2. Future Perspectives

In future work, this utilization of high-density hiPSCs culture can be performed to increase the differentiation efficiency in the next stage of hepatic differentiation following the analysis of the functional capabilities of the iHeps at stage-specific manner.

The utilization of PSCs self-maintenance by an accumulation of their native cytokines both for expansion or differentiation processes in suspension culture can be considered as a potential factor when developing in vitro culture protocols. However, further examination should be performed regarding the different best condition for expansion or controlling differentiation for each cell type.

The high-density culture by dialysis and gellan gum biopolymer can be extended in larger scalable hiPSCs expansion or differentiation might be important to obtain a large number of expected yields for the actual purpose. Several studies showed the feasibility to utilize large scale dynamic dialysis suspension culture for pluripotent stem cells culture and differentiation, such as slow turning lateral vessel (STLV)⁸⁰ and stirred bioreactor with dialysis perfusion¹⁷⁵. However, the oxygenation has still become a problem in these bioreactors regarding the small surface area, which can be better achieved in our miniaturized device.

Therefore, in order to enhance the better oxygenation, nutrient transfer, toxic metabolite removal, full utilization of cellular self-maintenance, and spheroid uniformity we proposed the O-shaped dialysis culture system in combination with low acyl gellan gum to achieve low shear stress culture environment for the hiPSCs expansion and differentiation.

This scaling up method is necessary to realize the clinical and industrial application such as liver regeneration as described in chapter 1 which required a large number of cells per batch.

Table 5. The cost estimation for actual liver regeneration therapy (required cells : 6×10^{10}) based on the previous study in comparison with the current study and our proposed future study

Dialysis based culture system	Volume	Expansion Yield	Total cost for the hiPSCs expansion	The total cost of the hepatic DE differentiation
Slow turning lateral vessel (STLV) (Come <i>et al.</i> , 2008)	1 L	5×10^6 cells/mL	¥ 8,320,000	¥ 76,800,000
Stirred tank bioreactor with dialysis (Nath <i>et al.</i> , 2017)	100 mL	1×10^6 cells/mL	¥ 41,600,000	¥ 384,000,000
The miniaturized multicompartiment HD system in this study	2 mL	32×10^6 cells/mL	¥ 1,300,000	¥ 12,000,000
The proposed HD culture with O shaped dialysis (using rotational culture)	20 mL	32×10^6 cells/mL	¥ 1,300,000	¥ 12,000,000

In addition, this HD culture with dialysis support and gellan gum utilization also can be potentially applied for various types of the cell for actual mass production for regenerative medicine or industrial application.

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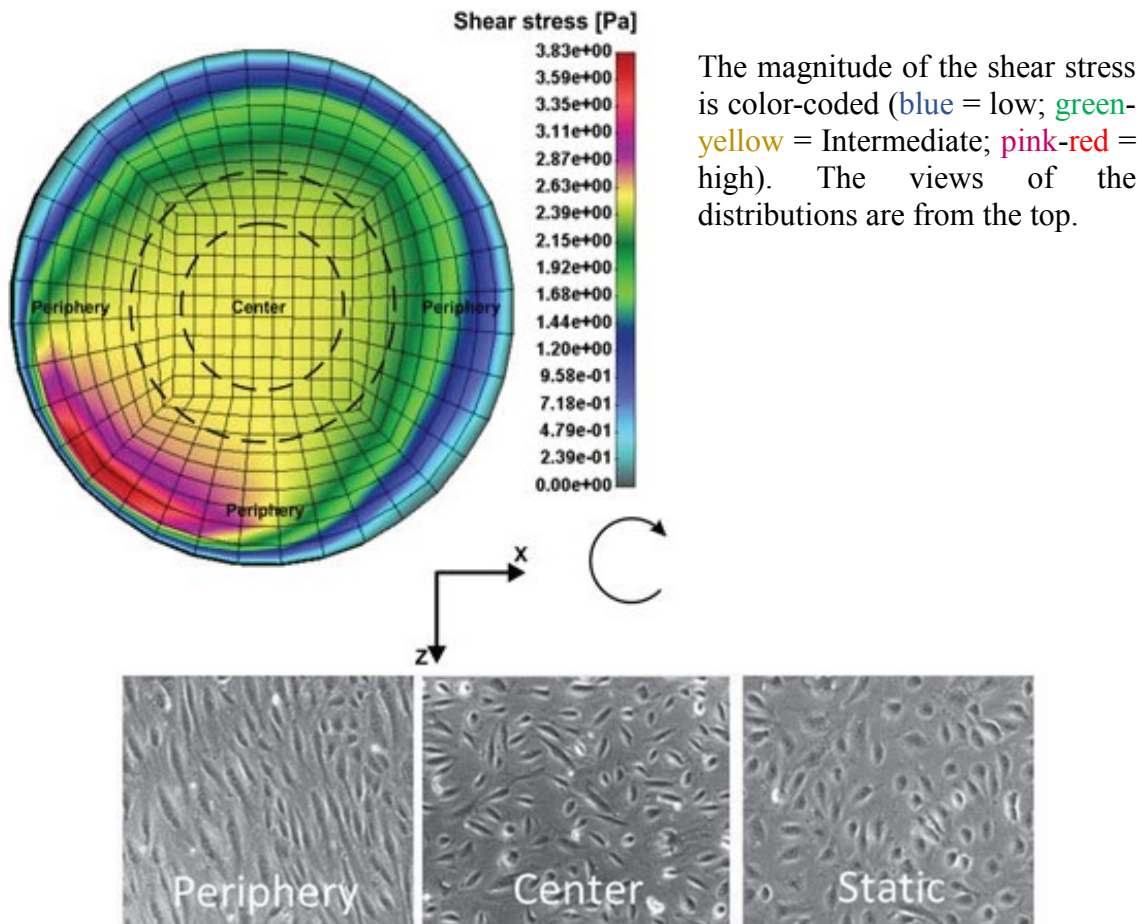
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Appendix - The rheological movement in round shape culture dish during rotational culture



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Hydrodynamic shear stress distribution among different region of 6 well plates tissue culture dish during rotational movement. The result of computational simulation was confirmed by different morphology of adherent culture of HUVEC cells in rotational culture