UNIVERSITY OF CALIFORNIA, SAN DIEGO

Expansion of Human Pluripotent Stem Cells with Synthetic Polymer PMVE-alt-MA

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by

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DEDICATION

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LIST OF ABBREVIATIONS

APS	Ammonium persulfate
bFGF	basic fibroblast growth factor
DI	De-ionized
DMEM	Dulbecco's Modified Eagle Medium
EB	Embryoid body
EDTA	Ethylenediaminetetraacetic acid
ESC	Embryonic stem cell
FACS	Flow activated cell sorting
FBS	Fetal bovine serum
hESC	Human embryonic stem cell
hPSC	Human pluripotent stem cell
ICM	Inner cell mass
iPSC	Induced pluripotent stem cell
MEF	Mouse embryonic fibroblast
MEF-CM	Mouse embryonic fibroblast conditioned medium
PAA	Polyacrylic acid
PBS	Phosphate buffered saline
PMVE-alt-MA	Poly(methyl-vinyl-ether-alt-maleic anhydride)
qPCR	Quantitative polymerase chain reaction
RGD	Arg-Gly-Asp
ROCKi	Rho kinase inhibitor
semi-IPN	Semi-interpenetrating polymer network

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ABSTRACT OF THE THESIS

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The differentiation potential of human pluripotent stem cells (hPSCs) promises to treat degenerative diseases with cell replacement therapies. Current culture media and substrates are not only expensive, but are undefined for clinical use because of the various known and unknown animal components that are required to promote proliferation and differentiation. Chemically defined materials are ideal substitutes for hPSC culture. This study focused on the use of PMVE-alt-MA, a synthetic polymer that was previously identified by arrayed screening technology for *ex vivo* hPSCs

maintenance. Synthetic hydrogels and microcarriers endowed with PMVE-alt-MA moieties were used to evaluate hPSC expansion, adhesion, proliferation, colony formation and maintenance. Flow cytometry and realtime PCR were used to characterize the expression of pluripotency markers in both hydrogel and microcarrier cultures. Protein adsorption to PMVE-alt-MA was found necessary for initial cell adhesion. Enzymatic dissociation with Accutase™ was optimized to passage the embryonic stem cell line Hues9 on 9.7% semi-IPN hydrogel for 8 passages and the result was comparable to Matrigel culture. However, polymer coatings on polyacrylamide hydrogel and microcarriers were found to be insufficient for stem cell self-renewal and attachment respectively. Embryoid bodies (EBs) with uniform size and shape were observed in microcarrier suspension but no difference was found in the differentiation pattern when compared to normal suspension EB formation. Our experimental results demonstrated the potential to use PMVE-alt-MA in hPSC expansion, as expected from the arrayed screening technology.

Chapter 1. Introduction

Human pluripotent stem cells (hPSCs, which include human embryonic (hESCs) and induced pluripotent stem cells (iPSCs)) with their ability to divide indefinitely and differentiate into all mature cell types provide an ideal cellular raw material to engineer tissues for cell replacement therapies and to treat previously incurable diseases. Such applications require robust and cost-effective technologies that can optimally support expansion and lineage-specific differentiation of hPSCs. Components of cellular microenvironments, be they chemical, structural or physical, regulate many aspects of cell behavior, including the unlimited potential of hESCs to proliferate and differentiate. However, identifying and controlling the factors that direct limitless expansion in a pluripotent state (also known as self-renewal) and differentiation of hESCs represents a main challenge in designing tractable stem cell culture techniques[1]. A candidate based approach to alter the outcome of cell fates largely ignores the complex interaction between a cell and its microenvironment. Often animal products, which are difficult to isolate, manipulate and modify, are used to support cell growth and promote targeted cellular responses, such as proliferation and differentiation. Current methodology for hPSCs expansion relies on mammalian cellular feeder layers, such as mouse embryonic fibroblast (MEF) or murine sarcoma extracellular matrix (Matrigel), the composition of which is poorly characterized[2]. Without a precise knowledge of the substrates, these cells cannot be used in regenerative medicine. A defined synthetic matrices system that is free of animal components is preferred for clinical use. The strategy of using synthetic combinatorial matrices, the chemical, physical and structural properties of which can be readily engineered, allows us to more precisely control the fate of cells.

Precise surface engineering of cell culture substrates provides control of cell adhesion to materials, cell proliferation, differentiation and molecular signaling pathways[3]. Cell-based assays on controlled micropatterned environments can screen synthetic matrices for their ability to support hPSC growth with the goal of developing fully defined and optimized culture conditions for hPSCs. The selected synthetic matrices can then be evaluated for their ability to sustain long-term self-renewal and promote differentiation of hPSCs in larger scale. A few synthetic materials were recently demonstrated to support the maintenance of hESC pluripotency, viability and growth rate of a limited number of cell lines[4, 5]. With the variations of the achievable passage number in these studies, each new cell line may have to be screened against a panel of defined substrates, hence high density microenvironments arrays serve as the ultimate platform to assay hPSCs.

The objective of this thesis is to examine hPSC cultivation methods with the polymer PMVE-alt-MA, identified from the synthetic polymer microarray technique, by producing hydrogels and coating on beads. The goal is to combine high-throughput screening technology with polymer science and stem cell biology to develop novel synthetic microenvironments for the *in vitro* propagation of hPSCs. We hypothesize that the use of PMVE-alt-MA can promote hPSC self-renewal and growth to produce a large number of cells for clinical use. The precise control of specific cell lines will eliminate existing bottlenecks, thus realizing the full potential of hPSC-based therapies.

1.1. Aims

The specific goals of this study are summarized below.

1) Develop a semi-IPN (PMVE-alt-MA in polyacrylamide) hydrogel cultivating system for hPSC culture.
2) Develop a PMVE-alt-MA coated polyacrylamide hydrogel cultivating system for hPSC culture.
3) Optimize enzymatic cell passaging techniques for the above hydrogel systems.
4) Validate the maintenance of hPSC pluripotency on hydrogel systems by fluorescent microscopy, flow cytometry and qPCR over eight passages.
5) Develop and validate a suspension system with microcarriers for hPSC culture.
6) Test embryoid body (EB) formation and differentiation with coated beads suspension system.

1.2. Experimental Setup

1.2.1. Composite-based hydrogel system

hESCs were seeded on linear PMVE-alt-MA entrapped Polyacrylamide hydrogel network (semi-interpenetrating network), and cultured with MEF-conditioned media (MEF-CM) with bFGF. hESC adhesion and proliferation were studied using two control and six experimental conditions to characterize the effects of FBS protein adsorption (A-C) and the optimal concentration of PMVE-alt-MA/Polyacrylamide.

Control 1A: 9.7% and 11.7% Semi-IPN hydrogel , with no FBS

Experimental 1B: 1%, 4%, 7%, 9.7% and 11.7% Semi-IPN hydrogel, with 10% FBS priming

Experimental 1C: 9.7% Semi-IPN, with 20% FBS

1.2.2. Coating-based hydrogel system

hESCs were seeded on PMVE-alt-MA coated polyacrylamide gel and cultured with MEF-CM with bFGF. hESC adhesion and proliferation were studied using two controls and six experimental conditions to characterize the effects of protein adsorption (A-C) and the optimal concentrations of PMVE-alt-MA coating.

Control 2A: 3% and 4% coating, with no FBS

Experimental 2B: 3%, 4%, 3% (at pH6.0), 4% (at pH6.3) and 10% coating, with 10% FBS priming

Experimental 2C: 4% coating, with 20% FBS

1.2.3. Enzymatic Passaging

Accutase™ (Life Technologies) was used to remove differentiated cells and separate stem cell colonies. To optimize the enzymatic selection method, different agitation and Accutase incubation times (D-F) were tested.

Control 3D: agitate incubation media once after 15 minutes incubation

Experimental 3E: agitate incubation media and aspirate dislodged cells repeatedly for every 3-minute incubation

Experimental 3F: agitate incubation media and aspirate dislodged cells repeatedly for every 1-minute incubation

To determine the optimal number for passaging, cells were counted and passaged at the following densities: 80,000 cells, 100,000 cells and 130,000 cells. Cells were passaged when colonies began to merge.

1.2.4. Suspension Culture

hESCs were seeded on substrate coated microcarriers (Cytodex 1) and cultured with MEF-CM with bFGF in suspension. hESC adhesion and proliferation were studied using three controls and four experimental conditions of suspension.

Control 4G: no coating Cytodex 1 in orbit shaker

Control 4H: Matrigel™ (BD) coating Cytodex 1 in orbit shaker

Experimental 4I: 4% PMVE-alt-MA coating Cytodex 1,
no MEF-CM conditioning in orbit shaker

Experimental 4J: 4% PMVE-alt-MA coating Cytodex 1,
24-hour MEF-CM conditioning in orbit shaker

Experimental 4K: 4% PMVE-alt-MA coating Cytodex 1,
48-hour MEF-CM conditioning in orbit shaker

Control 5H: Matrigel coating, HEK-293 cells, spinner flask

Experimental 5H: Matrigel coating, hESC, spinner flask

Conditions (G-K) represent different coating and priming conditions on cytodex 1. The controls in experiment 4 were used to assess the efficacy of PMVE-alt-MA. Conditions I-K were used to test the efficiency of MEF-CM priming requirement. Experiment 5 was used to test the apoptosis effect of hESC in spinner flask culture.

1.2.5. EB formation and differentiation in suspension

hESCs were suspended with 4% PMVE-alt-MA coated cytodex 1 in EB differentiation media on an orbital shaker. To measure the effect of EB size and differentiation, different initial cell densities were used to obtain EB for quantitative analysis (numbers and sizes) and quantitative PCR (qPCR).

Control 6: without cytodex 1

Experimental 6: with cytodex 1, 500,000 and 250,000 initial cell numbers

1.3. Summary of Results

1. PMVE-alt-MA was successfully polymerized in polyacrylamide to produce a semi-IPN hydrogel for hESC culture for up to 8 passages.
2. 9.7% (w/v) PMVE-alt-MA and 15.5% (w/v) polyacrylamide in semi-IPN hydrogel was shown to effectively maintain hESC pluripotency and support proliferation. The result was comparable to Matrigel culture.
3. Enzymatic selection by using Accutase was optimized to passage hESC on semi-IPN hydrogel.
4. PMVE-alt-MA coating on polyacrylamide hydrogel was shown to culture hESCs for up to eight passages.
5. 4% (w/v) PMVE-alt-MA coating on 10% (w/v) polyacrylamide hydrogel was shown to maintain hESC pluripotency and proliferation.
6. Concentrations, other than 9.7% PMVE-alt-MA in semi-IPN hydrogel, were shown unfavorable for hESC culture due to poor proliferation rate. In addition, poor transparency prevented effective imaging by microscopy.
7. Concentrations and pH values, other than 4% PMVE-alt-MA at pH 2.0, were shown unfavorable in hESC culture due to poor cell attachment or poor cell proliferation.
8. FBS protein adsorption was required for hESC adhesion and pluripotency maintenance on PMVE-alt-MA. 10% FBS solution showed the best attachment and proliferation.

9. hESCs successfully adhered, proliferated, and passaged on Matrigel-coated microcarriers Cytodex 1 for three passages on orbital shaker suspension culture.
10. Pluripotency marker OCT4 decreased over time in suspension culture.
11. MEF-CM incubation increased hESC adhesion to PMVE-alt-MA coated microcarriers.
12. The proliferation of HEK-293 and the death of Hues9-OCT-GFP on Matrigel-coated microcarriers in the spinner flask revealed the cell line dependent effect.
13. More uniform diameters and shapes of EBs were produced by adding PMVE-alt-MA coated microcarriers into traditional EB formation in suspension.
14. Changing microcarriers and cells ratio did not affect EB size or shape.
15. No difference in pluripotency or differentiation levels was found with or without the presence of microcarriers in suspension EB formation.

Chapter 2. Literature Review

2.1. Mammalian Pluripotent Stem Cells

Embryonic stem cells (ESCs) are pluripotent, self-renewing cells that are derived from the inner cell mass (ICM) of blastocysts[6]. The capacity to generate all cell lineages of the developing and adult organism defines the pluripotency of a stem cell, while the ability to proliferate in the same state defines self-renewal. Induced pluripotent stem cells (iPSCs) have been generated by forced expression of OCT4, SOX2 and other transcription factors inside multiple murine and human somatic cells[7, 8]. Although some iPSCs can retain some epigenetic memory from histone modification, fully reprogrammed iPSCs are genetically matched to the donor while their gene expression is no different from ESCs and are thus capable of generating all cells. In general, the pluripotency of stem cells is monitored by a small range of pluripotency markers, such as OCT4 and NANOG. The ability to control and expand the pluripotent stem cell state may lead to advancement in cell replacement therapies. Due to temporal and spatial gradients of various biological cues in current cell culture techniques, larger scale expansion increases these gradients and causes differentiation. Often stem cells in culture may appear to be differentiating, and are manually removed before passaging. Maintaining the pluripotency of stem cells during the cell expansion requires a simple, defined culturing system.

2.2. Stem Cell Culture

Human ESCs (hESCs) have traditionally been cultured on mouse embryonic fibroblast (MEF) feeder layers, which provide cell-to-cell interaction to allow undifferentiated growth of stem cells[9]. Cells are usually batch cultured on tissue culture

plates, and require medium replacement daily and passage every 4 to 7 days. This process is labor intensive. Smooth edges on colonies are a hallmark of hESCs and requires E-cadherin expression of ESCs[10]. Animal serum is not required in the presence of MEF feeder layers, even though MEF conditioned media is necessary. bFGF supports hESCs self-renewal and is often used in hESC culture[11]. Human fibroblast feeder layers may have been an advantage when applying the ESCs in human therapy, but a feeder-free culturing system with Matrigel proves to be easier to avoid mixed cell populations[12]. The composition of Matrigel is measured to be 56% Laminin, 31% Collagen IV, 8% Entactin, and a number of known and unknown growth factors, due to the nature of cell extract. Within the last five years, commercially available feeder-independent defined composition culture media, such as mTeSR1® (Stem Cell Technologies) and StemPro® (Life Technologies), replaces MEF conditioned media from Matrigel culture and brings forth a more defined culturing system[13]. Nevertheless, cell therapy is impossible with the use of animal-derived Matrigel system.

2.2.1. The Use Synthetic Polymer Hydrogels for Stem Cells Expansion

A number of polymer hydrogels have been used in large scale expansion of hESCs, including synthetic and natural polymers[14]. Generally hydrogels do not support cell adhesion; however, certain hydrogels have been designed to present the cell adhesion ligands that manipulate surface adhesion. Researchers have had limited success in designing a completely synthetic substrates composed of semi-interpenetrating polymer network (semi-IPN), polymer hydrogels composed of two non-crosslinked polymer species, to provide structural support and RGD ligand binding mechanism[15]. Linear polyacrylic acid (pAA) chains were modified with synthetic RGD peptides (Ac-

CGGNGEPRGDTYRAY-NH2) that represent active sites in several extracellular proteins for binding multiple integrin receptors, including α1, αv, β1 and αvβ3. These peptide-functionalized linear pAA chains were mixed and polymerized within poly(N-isopropylacrylamide-co-acrylic acid) and proteolytically degradable peptide crosslinker QPQGLAK-NH2. The interpenetrated pAA-graft-RGD chains were shown to promote ESCs adhesion, but ESC pluripotent markers, such as OCT-4 and SSEA-4, could not be maintained for more than 5 days.

2.2.2. The Use of Synthetic Polymer Coatings for Stem Cells Expansion

Synthetic polymer matrices have been the recent focus of hPSCs propagation. The compositionally defined matrix and media that supports hPSCs expansion can be used to determine all the factors that regulate stem cell growth and differentiation. However, only two synthetic matrices PMVE-alt-MA and PMEDSAH have been reported to sustain long-term hPSCs propagation[4, 5]. In both cases, the groups focused on defined matrices, and hence cells were cultured with MEF-conditioned media (MEF-CM). PMEDSAH, which stands for poly[2-(methacryloyloxy)ethyl dimethyl-(3-sulfopropyl)ammonium hydroxide], was coated on tissue culture polystyrene dishes by surface-initiated graft polymerization, which UV ozone activated the culture surface prior to the polymerization of methacrylated-based monomers. Pluripotency of BG01 and H9 hESC lines in MEF-CM was confirmed at passage 25 by qPCR markers NANOG, OCT4 and SOX2. PMEDSAH serving as defined matrices remained a challenge because long-term propagation (<10 passages) was reported not compatible with serum-free defined media such as StemPro and mTeSR. PMVE-alt-MA, on the other hand, was off-the-shelf linear polymer coated on glass surfaces, and was able to sustain pluripotency of HUES1,

HUES9, and iPS with MEF-CM and StemPro for 5 passages. Optimizing the conditions of defined synthetic matrices is crucial to develop a reproducible culture system for hPSCs expansion.

2.2.3. Synthetic Polymers Screening Technology

The ability to screen thousands of commercially available polymers is the key to identifying classes of biomaterials that are suitable for cell expansion. Simultaneously monitoring and assaying multiple cellular markers are essential requirements for high throughput screening of arrayed matrices. Material-cell interactions, cell growth, and cell characterization were the common sequential order in screening[16, 17]. Dr. Langer's group used a microarray printer to spot and polymerize 576 different combinations of 25 different acrylate, diacrylate, dimethacrylate and triacrylate monomers on top of an epoxide coated glass slide[16]. About 70% of the spots showed good attachment and growth of a robust cell type, C2C12, but only 24 different combinations of matrices allowed the growth of hESCs (H9). Prior to optimization, it showed that a large library was required to identify the compatibility of hPSCs. The same research group used OCT4-GFP transgenic hESC to further investigate polymer surface roughness, elastic modulus and wettability to map cell behavior. Two hit polymers of distinct chemical structures were verified to promote hESCs and hiPSs growth and maintained pluripotent markers. Most importantly, adsorbed proteins from human serum were required to interact with the appropriate polymer surface chemistry to optimally promote pluripotency. Prediction of chemical functional groups remained difficult to quantitatively relate to the biological performance of cells. Large-scale experiments will be required to investigate the long-term robust culture of hPSCs.

2.2.4. Poly(methyl vinyl ether-alt-maleic anhydride) (PMVE-alt-MA)

Synthetic polymer PMVE-alt-MA was identified by arrayed synthetic polymers screening technology for its support of long-term attachment, proliferation and self-renewal of Hues1, Hues9, and iPSCs[5]. The hPSCs cultured on PMVE-alt-MA maintained their characteristic morphology, expressed high levels of pluripotency markers, and retained a normal karyotype. A range of different molecular weights of PMVE-alt-MA was tested for the effect on proliferation and self-renewal of hPSCs. From the range of $2.16x10^5$ Da to $2.40x10^6$ Da, the molecular weight $1.25x10^6$ Da was shown to be the best in hPSC culture.

2.3. Suspension Culture Systems

Bioreactors have been used to produce cells or cell products in large scale because of their high cell volume density and easy-to-monitor homogenous microenvironment. In the bioprocessing industry, cells are used to synthesize products such as antibodies, enzymes and viruses. Recently, reports on the expansion of hESCs in scalable stirred-suspension bioreactors demonstrated feasible methods to cultivate cells as aggregates and in microcarrier suspension, as well as differentiation[18-20]. The number of cells used in cell therapy falls in the range of tens of millions to a few billion[21]. Suspension culture can produce such quantities of cells and direct differentiation of stem cells in a number of reports. Concentrations of 10^6-10^7 mammalian cells/mL were common in conventional stirred-suspension bioreactors[22]. A few liters of working volumes would be sufficient to provide 10^9 stem cells or stem cell-derived cells in clinical use. The minimal labor involved in stem cell productions requires a high level of robustness in a completely

defined system, but also ensures the scale-up process, monitoring and better control of culture variables. Since the cells are actual products, the operation condition of bioreactors may directly impact the stem cell self-renewal and propagation probability. Therefore, derivation of new hPSC lines may sometimes be required to optimize the operation protocols of bioreactors.

2.3.1. Free Suspension

Standard static tissue culture requires passaging from new substrates when confluency is reached. hPSCs are particularly sensitive to dissociation in the process of passaging and cryopreservation[23]. Therefore, the transition from hPSC static culture to stirred-suspension culture always requires the additive named Rho Kinase inhibitor (ROCKi) to increase the survival rate of single hPSC. For example, hESC H9 was enzymatically dissociated from Matrigel and agitated in magnetic bar stirring bioreactors at the rate of 100rpm in mTeSR. H9 grew in aggregates and were passaged by using Accutase every 6 days. In 20 days, these cells maintained a normal rate of growth, pluripotent markers (79% OCT4+, 50% TRA-1-60+) by flow cytometry, and were capable of differentiating into all 3 germ layers. Another report has shown high pluripotency (~90% positive in SSEA-4, TRA-1-60 and TRA-1-81) in two out of three cell lines (HES2 and H7, not HES1), but 58% cell death (in comparison to 21.4% in monolayer culture) after 20 weeks of culture in similar magnetic stirring systems[20]. Cell death may be avoidable by optimizing the culturing conditions.

hESC colonies are often required to partially dissociate into smaller clumps to remain viable during passages. Inoculation of small hESC clusters with variable size results in an uneven distribution and is further magnified by cell proliferation in

bioreactors. The underlying reason for excessive colony agglomeration was due to the high levels of E-cadherin expression of hESCs. Microcarrier culture of a fixed cell density environment may serve as better control in cell passaging. Using microcarrier-free stirred-suspension bioreactor culture system has worked well with only a selected number of cell lines, thus further investigation is necessary to maintain pluripotency and cell propagation at the same time.

2.3.2. Microcarriers

Microcarriers are usually spherical particles made of various biocompatible materials, including glass, cellulose, and plastics, with a range of diameters between 100-300um. Due to their high surface area to volume ratio, microcarriers are extensively used to scale up anchorage-dependent cells. This is one possible method to assist hPSCs expansion with the use of microcarriers as substrate. Often microcarriers are coated with cell adhesion substrate to increase cell attachment. Researchers have modified the surface of commercially available microcarriers Cytodex 3 (crosslinked dextran, denatured collagen on surface) with either Matrigel or MEFs to enhance hESC attachement and inhibit differentiation[19]. Suspension was performed on ultralow attachment 6-well plates at 2ml/well and 2-3x10^6 cells/well with MEF-conditioned media on an orbit shaker inside the incubator. The growth rate and pluripotency of two hESC lines (H1 and H9) were indistinguishable to Matrigel-coated tissue culture. Other researchers have also used manufactured microcarrier Hillex II (crosslinked polystyrene, modified with cationic trimethyl-ammonium) to expand hESC ESI-017 line for 6 passages and 14-fold increase in number while undifferentiated markers OCT4 and Tra1-81 were still expressing[24]. Feeder free microcarrier culture was proved to be feasible in principle.

Optimizing the passage protocol was an issue in hESC microcarrier culture. Researchers have observed the growth of hESCs in multiple layers on microcarriers and the agglomeration of multiple microcarriers into larger cell masses[25]. Not only did they cause inconsistent growth and differentiation, but the larger clusters could not be efficiently passaged as small clumps. Appropriately designed culture systems, such as varying volume, cell and microcarriers concentrations, could reduce agglomeration while maintaining a significant growth rate of stem cells. Lastly, defined medium mTeSR1 was shown to produce low attachment and survival of hESCs on mirocarriers. Further investigations are needed on the passaging technique and the use of culture medium to achieve completely defined system.

2.4. Embryoid Bodies Formation

Linear differentiation within the embryoid body (EB) closely resembles the development of an embryo. In suspension, ESCs aggregate into spherical cell clusters, which consist of an outer layer of endoderm cells within 2-4 days, and further differentiate to form a cystic void with an inner layer of columnar ectoderm-like cells[26]. The culture of hESCs in a three-dimensional configuration often is associated with their differention because hESCs propagate as colonies on flat substrates[22]. Germ layers derivation within EBs is a common method to test for the differentiation potential of hESCs. Lineage-specific differentiation can be further induced by differentiation media to generate a number of cell types, including hematopoietic, neuronal, myogenic and cardiac cells. EBs can be generated by one of the following 3 common methods: 1) hanging drop, in which cells within a hanging fixed volume droplet aggregate into one EB; 2) suspension culture, in which ESCs are dispersed on low-attachment culture plate

and cell aggregates from collision and proliferation; 3) spin EB, in which cells in solution are centrifuged into polymeric wells and aggregate into EBs. Even though hanging drop and spin EB produce homogeneous size EBs, scalable issue and dead cells aggregation appear respectively. Suspension culture is the most scalable method to produce quality EBs; but heterogeneous EB size and morphology may limit homogenous differentiation.

Investigations have been done to correlate EB size with directed differentiation[27]. Several groups have reported the EB differentiation efficiency in different ranges of size due to the distinct methods in EB formation. In general, EB of 100-300um diameter had all lineage markers highly turned on and differentiated into neuronal lineage in serum-free condition[28]. It is clear that uniform EB size and defined conditions can enhance the yield of lineage-specific differentiation.

Chapter 3. Materials and Methods

3.1 Cell Culture

Mouse embryonic fibroblast (MEF) and HEK-293 was cultured in 1x high glucose DMEM, 10% fetal bovine serum, 1% (v/v) L-glutamine penicillin/streptomycin. Cultures were incubated at 37°C with 5%CO_2 in humidified atmosphere. MEF conditioned medium (MEF-CM) was produced by 24 hours of MEF culturing in Hues Medium (1x Knockout DMEM, 10% (v/v) Knockout Serum Replacement, 10% (v/v) human plasmanate (Talecris Biotherapeutics), 1% (v/v) non-essential amino acids, 1% (v/v) penicillin/streptomycin, 1% (v/v) Gluta-MAX, 55uM 2-mercaptoethanol (Sigma)). hESC was maintained on 2% Matrigel (BD) coated tissue culture dish and cultured with MEF-CM supplemented with 10ng/ml bFGF, which was changed daily. Matrigel coated tissue culture dishes were prepared by mixing 2% Matrigel (v/v) in DMEM-F12 media on ice and coating the dish in 37°C incubator for 5 min. All media components are purchased from Invitrogen unless indicated otherwise. hESC was routinely passaged by 5 min exposure of Accutase (Millipore) and followed by one rinse of Hues media between centrifugation at 200x g. Cells were resuspended and plated 1:10.

3.2. Hydrogel Systems

3.2.1. Synthesis of PMVE-alt-MA Semi-IPN Hydrogel

The dry powder of linear PMVE-alt-MA (Mn=1.25x10^6Da) was purchased from Sigma. The polymer was first dissolved in DI water for 6 hours at 37°C, frozen in -80°C for 12 hours, then lyophilized and mortared to powder before used. Semi-IPN gels in experiment 1 (1%, 4%, 7%, 9.7% and 11.7% (w/v)) were synthesized using 15.5% (w/v) polyacrylamide (PAm) by Ammonium Persulfate (APS) and TEMED polymerization.

The mixture was transferred to 1mm thick glass casting cassette for overnight polymerization in sterilized area. Semi-IPN gel was then incubated for 2 hours in 70% ethanol, 2 days in DI water and 2+ days in PBS. 1 day incubation of 10% FBS (Gibco) in DMEM was required prior to cell culture.

3.2.2. Synthesis of PMVE-alt-MA coated Polyacrylamide Gel

Similar to section 3.2.1, 1mm thick 10% PAm was polymerized by APS and TEMED. PAm gel was then incubated for 2 hours in 70% ethanol, 2 days in DI water and 2+ days in PBS before PMVE-alt-MA coating. 1 day incubation of 4% (w/v) PMVE-alt-MA in 1xPBS and 1 day incubation of 10% FBS (Gibco) in DMEM was required prior to cell culture.

3.2.3. OCT4-GFP Reporter Cell Line

Hues9-OCT4-GFP reporter line used in experiment 1, 2 and 3 was previously described[5]. GFP gene was turned on by the transcriptional control of an OCT4 promoter to provide real-time measurement of pluripotency. GFP expression was stable in MEF-CM throughout the experiments and the cells appeared to retain pluripotency under microscopy and flow cytometry.

3.2.4. Optimization of Enzymatic Selection

Accutase was used to dissociate stem cell colonies on hydrogels in experiment 3 and on Matrigel. Culture media was removed and rinsed with PBS prior to Accutase incubation at 37°C. For hESCs on Matrigel, it required 1 min Accutase incubation, a brief agitation, and then aspiration to remove some differentiated cells. Differentiated cells dissociated prior to most pluripotent stem cell, possibly due to the lower expression of E-cadherin and the spreading on substrate. To completely dissociate the colonies into single

cells, 5 extra min of Accutase incubation was necessary. For Hues9-OCT4-GFP on hydrogel, they attached strongly to the substrate, thus multiple short Accutase incubations were required to remove differentiated cells until stem cell colonies dislodged from the hydrogel. The optimal enzymatic selection of hESC was tested in experiment 3. 5 extra min of Accutase incubation was needed to dissociate the stem cell colonies.

3.2.5. Optimization of Passaging on Hydrogels

Dissociated stem cells from hydrogel were collected by 200x g centrifugation, and resuspended in MEF-CM. Prior to each passage, viability and number of cells were measured by BioRad TC10 automatic cell counter with trypan blue. 35,000-57,000cells/cm² (or 80,000-130,000cells) was passaged to 1-day FBS-primed hydrogels in experiment 3. Cell density was adjusted to optimize passaging for every 7 days, at the time when the colonies began to merge.

3.3. Characterization of Long-Term Culture of hESC on Hydrogels

Hues9-OCT4-GFP on hydrogels was initially passaged every 4-7 days depending on colony density. GFP expression level was monitored throughout the experiments with fluorescent microscopy. Characterizations, including flow cytometry and quantitative PCR (qPCR), were performed after six sequential passages.

3.3.1. Flow Cytometry

All cells were collected as described in 3.2.4 and further dissociated into single cells by pipetting. After 200x g centrifugation, the cell pellet was resuspended in ice cold FACS buffer (1x PBS, 10%BSA, 5uM EDTA) and incubated on ice. A cell strainer of 40-μm pore size was used to achieve single cell suspension for FACS (BD FACSCantoII). GFP signal was quantified to represent the endogenous expression level of pluripotent

marker OCT4. Hues9 and Hues9-OCT4-GFP passaged on Matrigel were used as the baseline controls of GFP negative and GFP positive respectively in comparison to Hues9-OCT4-GFP passaged on hydrogels. Data are presented as the number of cells versus the intensity of GFP level.

3.3.2. RNA isolation and qPCR

RNA was isolated from cells using TRIzol (Invitrogen), treated with DNase I to remove DNA, and then reverse transcribed to cDNA by qScript cDNA Supermix (Quanta Biosciences). Quantitative realtime PCR was performed using TaqMan probes and TaqMan Fast Universal PCR Master Mix (Applied Biosystems) on a 7900 HT Real Time PCR machine. TaqMan gene expression assay primers of OCT4 and NANOG were used to quantify pluripotency, while gene expression was normalized to 18S rRNA level. The expression level of each gene was calculated as $\Delta C_t^{target} - \Delta C_t^{18s}$.

3.4. Microcarrier Culture

3.4.1. Siliconization of Glassware

Hydrolyzed microcarriers attach strongly to any plastic or glass surfaces. All glassware (15ml glass test tubes or 25ml magnetic stirring flask (Bellco)) requires siliconization to prevent electrostatic binding to microcarriers. Clean and dry glassware was rinsed with Sigmacote (Sigma) and dry autoclaved to increase the strength of siliconization. PBS rinsing was required to remove residual biotoxic Sigmacote prior to microcarrier sterilization. Sigmacote can be reused.

3.4.2. Sterilization of Cytodex 1

Sterilization of microcarrier Cytodex 1 was performed in 15ml glass test tubes. Surface area of Cytodex 1 (Sigma) was measured by dry weight to 0.018g (equivalent to

the surface area of one 10cm tissue culture plate) in each glass tube. Cytodex 1 was prepared by overnight hydration in PBS at 4°C, three times PBS rinsing to remove small particles, and was kept in 10ml PBS before wet autoclaving at 115°C for 20 min. Centrifugation was needed to avoid decanting low density microcarriers in suspension. Sterilized Cytodex 1 with PBS was sealed and kept at 4°C for less than 2 weeks before used.

3.4.2. Matrigel coating of Cytodex 1

Cytodex 1 in experiment 4 and 5 was coated with 2% Matrigel in DMEM/F12 (Invitrogen). PBS in the glass test tube was decanted and replaced with 5ml ice-cold Matrigel solution. Coating step was performed in sealed test tubes which were placed on a rocker in cold room for 24 hours. Matrigel solution was removed and rinsed with culture media three times prior to cell seeding. Matrigel coated Cytodex 1 can be kept at 4°C for up to 7 days.

3.4.4. PMVE-alt-MA Coating of Cytodex 1

Cytodex 1 in experiment 4 and 6 was coated with PMVE-alt-MA solution (4% (w/v) lyophilized PMVE-alt-MA in 1x PBS). PBS in sterilized Cytodex 1 test tube was decanted and replaced with 5ml ice-cold PMVE-alt-MA solution. Coating step was performed in sealed test tubes on a rocker in cold room for 24+hours. PMVE-alt-MA solution was acidic and required rinsing prior to cell seeding. PMVE-alt-MA solution was rinsed five times with culture media, or until color of culture media remained unchanged. PMVE-alt-MA coated Cytodex 1 was incubated for different amounts of time in MEF-CM in experiment 4 to determine the necessity of protein adsoprtion for cell adhesion.

3.4.5. Seeding and Passaging on Coated Cytodex 1 in Microcarrier Suspensions

Microcarriers with HEK293 cells and Hues9-OCT4-GFP were suspended in ultra-low attachment 6-well plates (CellStar) on orbital shaker or magnetic bar stirring flask (Bellco) in experiment 4 and 5. $4x10^5$ cells and 0.003g of sterile Cytodex 1 were mixed in 5ml of appropriate media in each well on a 6-well plate on orbit shaker. The motion of orbit shaker at 90rpm enhanced the attachment of cells to the coated microcarriers. Microcarriers with attached cells were either continuously cultured on the 6-well plate in experiment 4 or transferred to stirring flask in experiment 5 after 24 hours. 80% of culture media was replaced everyday in experiment 4 and cells were passaged two times on Cytodex 1 for 5-6 days. Hues9-OCT4-GFP was removed from the microcarriers with 10 min Accutase incubation. Number of cells was counted by BD TC10 cell counter and $5x10^5$ cells, together with stripped microcarriers, were centrifuged and transferred to freshly coated Cytodex 1. Microcarriers with cells were cultured 6 days in stirring flask in experiment 5 and 80% of culture media was replaced on day 3. 20ml of appropriate media was stirred at the rate of 18rpm (lowest) to 30rpm (highest) to suspend the microcarriers.

3.5. EB Formation in PMVE-alt-MA Coated Cytodex 1 Suspension

hESCs agglomerate in ultra-low attachment plate on orbit shaker. A standard protocol used 500,000 Hues9 per well on 6-well plate in Hues EB differentiation media (1x Knockout DMEM, 10% (v/v) Knockout Serum Replacement, 1% (v/v) non-essential amino acids, 1% (v/v) penicillin/streptomycin, 1% (v/v) Gluta-MAX, 55uM 2-mercaptoethanol (Sigma), 5uM Rocki (Sigma)). To determine the effect of micocarriers and initial cell density on EB size, the same amount of PMVE-alt-MA coated Cytodex 1

per well, as described in 3.4.4., was added to 250,000-500,000cells in Hues EB differentiation media. EBs of diameter>100um were collected by 100um cell strainer at day 7. The distribution of EB diameters was measured from still images on day7 and mRNA expression levels of OCT4, NANOG and Nestin were determined by qPCR.

Chapter 4. Results

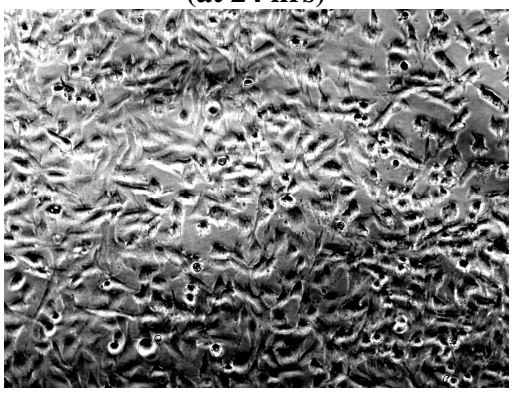
4.1 Characterization of Initial Attachment on PMVE-alt-MA Semi-IPN Hydrogel

Five concentrations of semi-IPN gel were attempted from the method described in the semi-IPN protocol. For a fixed polyacrylamide concentration at 15.5%, PMVE-alt-MA concentration was varied to obtain 1%, 4%, 7%, 9.7%, and 11.7% polymer content. 1% PMVE-alt-MA gelled immediately after mixing with APS and TEMED. 4%, 7%, 9.7% and 11.7% semi-IPN gels were cast in 1mm thick glass cassette by following the semi-IPN protocol. In the first protein adsorption test, 10% FBS was used for conditioning and Hues9-OCT4-GFP cells were then seeded onto all four concentrations of semi-IPN gels. However the opacity of 4% and 7% was too high to allow light microscopy while the 9.7% and 11.7% semi-IPN were translucent and allowed light and fluorescent microscopy. Microscopy images (Figure 1) showed that 9.7% PMVE-alt-MA support cell adhesion better (about 25% area with cell adhesion in 24 hours) than 11.7% PMVE-alt-MA (about 10% area with cell adhesion in 24 hours). Hues9-OCT4-GFP did not proliferate on 11.7% gel in the culture for more than 6 days. 9.7% PMVE-alt-MA semi-IPN gel was chosen for further experiments because GFP expression of Hues9-OCT4-GFP remained for more than 5 days (Figure 2).

To determine the efficacy of FBS proteins adsorption, 0% and 20% FBS priming were also tested with Hues9-OCT4-GFP (Figure3). Only small areas (<5% surface area) of non-FBS conditioned PMVE-alt-MA semi-IPN (in Figure 3A) had differentiated-like or fibroblast-like cell attachment. 80% of the surface area of 20% FBS priming (in Figure 3C) had cell attachment but proliferation of these cells was slow. In contrast, cells

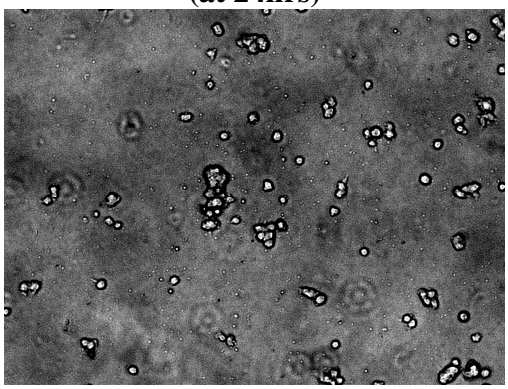
growing on 10% FBS priming for 24 hours appeared to have normal colonies formation on about 25% surface area of PMVE-alt-MA semi-IPN hydrogel.

9.7% PMVE-alt-MA semi-IPN (at 24 hrs)



(A)

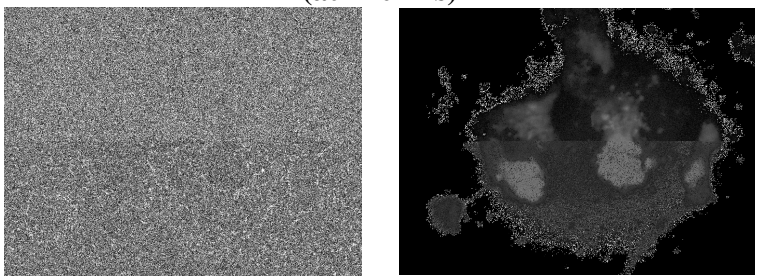
11.7% PMVE-alt-MA semi-IPN (at 24hrs)



(B)

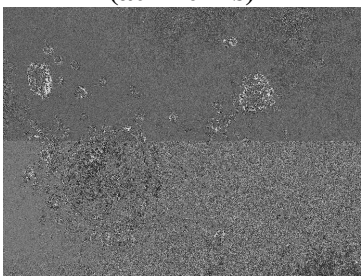
Figure 1. 24 hours Post-Seeding of Hues9-OCT4-GFP onto 10% FBS Primed PMVE-alt-MA semi-IPN hydrogel. (A) Cells on 9.7% PMVE-alt-MA semi-IPN attached to approximately 25% of the hydrogel surface and **(B)** cells on 11.7% PMVE-alt-MA semi-IPN attached to approximately 10% of the hydrogel surface. Hues9-OCT4-GFP appeared to be spreading in (A) and contracting in (B).

9.7% PMVE-alt-MA semi-IPN (at 120 hrs)



(A) (B)

11.7% semi IPN (at 120hrs)



(C)

Figure 2. 120 hours Post-Seeding of Hues9-OCT4-GFP onto 10% FBS Primed PMVE-alt-MA semi-IPN hydrogel. (A) Proliferation of stem cells colonies and **(B)** OCT4-GFP signal were visible on 9.7% PMVE-alt-MA semi-IPN gel. **(C)** Slow proliferation of stem cells was observed on 11.7% PMVE-alt-MA semi-IPN gel.

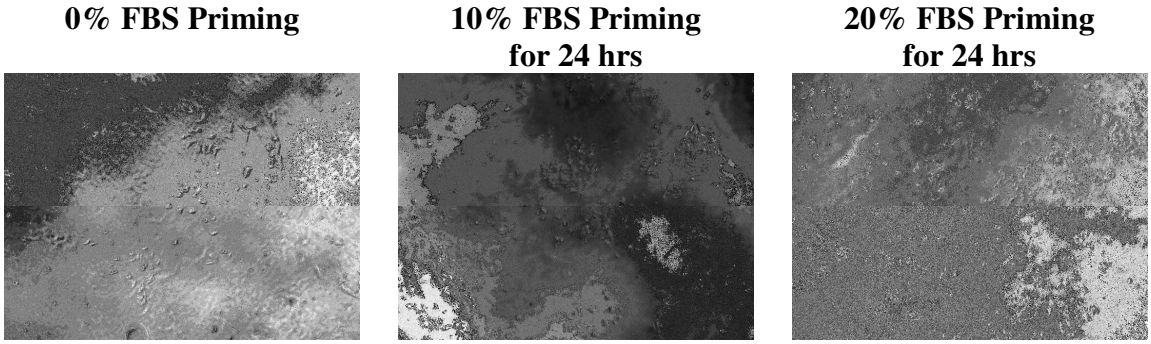


Figure 3. Comparison of 48 hours Post-Seeding of Hues9-OCT4-GFP onto (A) no FBS priming, (B) 10% FBS priming for 24 hours, and (C) 20% FBS priming for 24 hours. Cells only attached to 5% area in (A) and appeared to be fibroblast-like. Colonies were visible in (B) and their morphology was embryonic-like. Cells attached to 80% surface area in (C) and appeared to be contracted.

Table 1. Time Table of hESC passage on PMVE-alt-MA semi-IPN hydrogel

	Days on Semi-IPN hydrogel	Accumulated Days
Seeding	5	5
Passage 1	4	9
Passage 2	8	17
Passage 3	5	22
Passage 4	4	26
Passage 5	8	34
Passage 6	5	39
Passage 7	7	46
Passage 8	7	53

4.2 Characterization of Long-Term Growth on PMVE-alt-MA Semi-IPN Hydrogel

4.2.1. hESC Morphology and Endogenous OCT4 Expression

Hues9-OCT4-GFP was passaged on 10% FBS primed PMVE-alt-MA semi-IPN hydrogel for 8 passages and a total of 53 days under the protocol described in 3.2.1. Flow cytometry and qPCR were performed to monitor the changes in pluripotency markers at the end of passage 6, 7 and 8. The morphology and GFP expression both represented

normal hESCs characteristics at different time points of cells on PMVE-alt-MA semi-IPN gel (Figure 4).

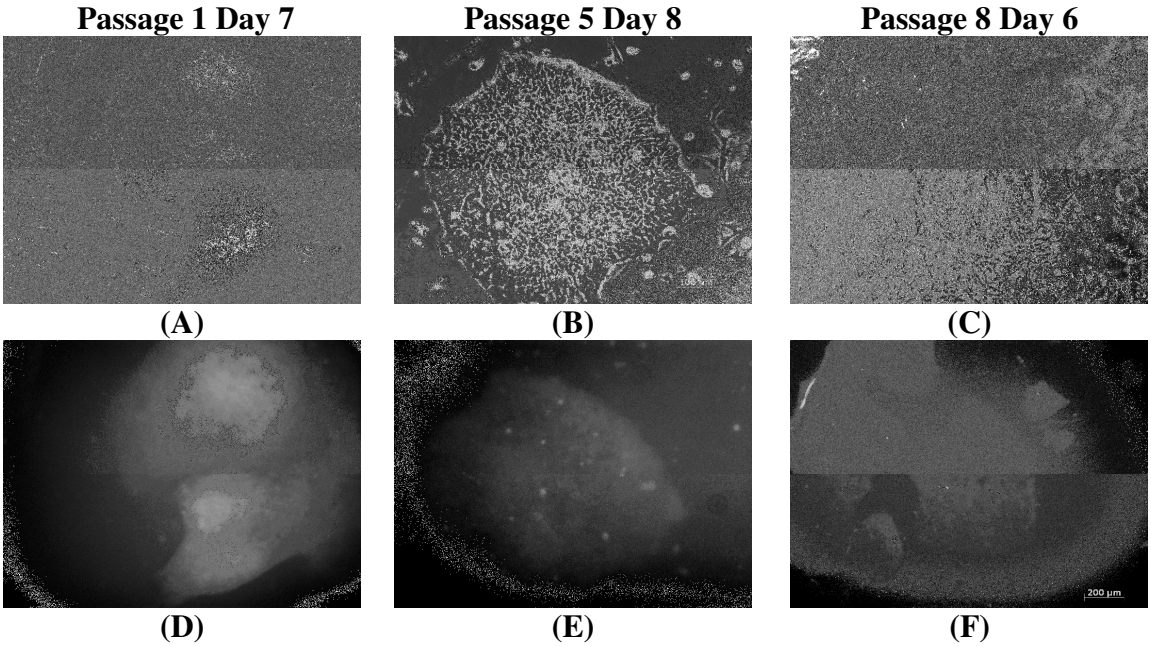


Figure 4. Morphology and OCT4-GFP expression of Hues9-OCT4-GFP cells on PMVE-alt-MA semi-IPN hydrogel at different time points of passaging. Phase contrast and fluorescent images at **(A,D)** Passage 1 Day 7, **(B,E)** Passage 5 Day 8 and **(C,F)** Passage 8 Day 6 were shown here. hESC were accessed for their (A-C) characteristic morphology and (D-F) maintatenance of pluripotency (OCT4). Normal colony formation on semi-IPN hydrogel was observed at the end of each passage. Some differentiated cells were growing around the colonies as normally seen in Matrigel plate culture. Fluorescent images of GFP expressions aligned with the colonies

4.2.2. Flow Cytometry of hESC on PMVE-alt-MA Semi-IPN Hydrogel

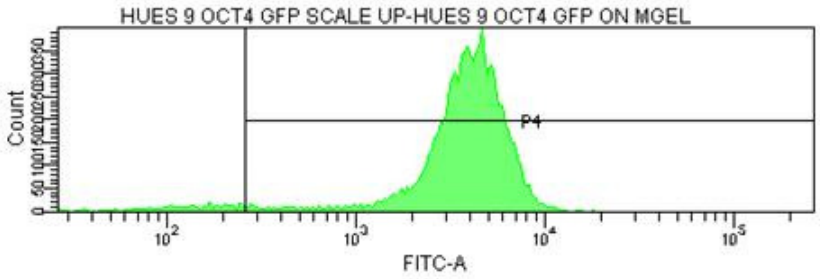
Expression of OCT4-GFP on PMVE-alt-MA semi-IPN gel was comparable to Hues9-OCT4-GFP passaging on Matrigel. The OCT4 expression level, represented by the GFP (FITC) intensity, of Hues9-OCT4-GFP passaged on Matrigel was similar to those cultured on PMVE-alt-MA semi-IPN gel for 6-8 passages (Figure 5). The GFP positive gate (vertical line in Figure 5A-D) was set by non-GFP expressing Hues9. The populations of cell passaged or collected for qPCR were 86.5%-96% GFP positive. The

peaks of all flow cytometry diagrams were located at the same intensity, thus they suggested that the population of stem cells on semi-IPN gel were highly pluripotent.

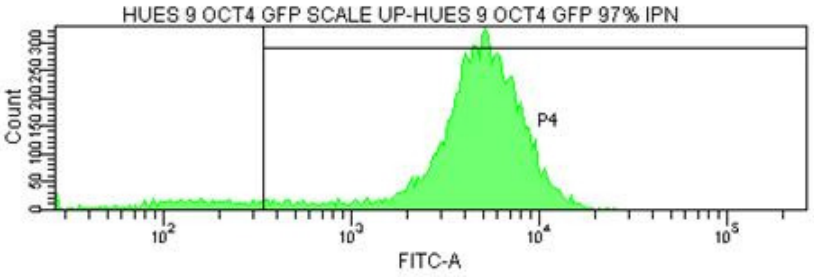
4.2.3. mRNA Expressions of OCT4 and NANOG

Quantitative PCR was used to measure the mRNA expression of pluripotency markers OCT4 and NANOG of hESC cultured on PMVE-alt-MA semi-IPN hydrogel. Cells were collected at the end of passage 6, 7 and 8, and were compared to Hues9-OCT4-GFP cultured on Matrigel. All mRNA levels were normalized to endogenous control 18S. The level of OCT4 was lowered by 1.5-3 folds ($\Delta\Delta$Ct) and the level of NANOG was lowered by 1-2.5 folds ($\Delta\Delta$Ct) in cells cultured on PMVE-alt-MA semi-IPN gel (Figure 6).

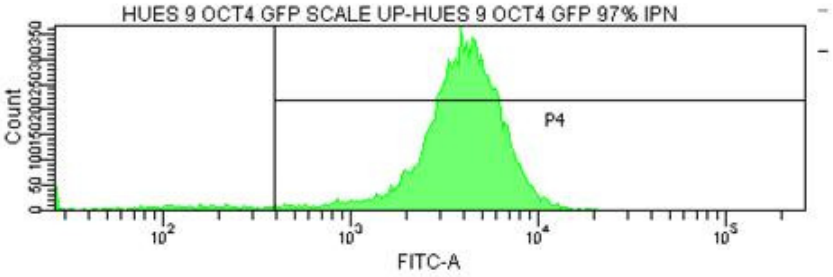
(A) On Matrigel 95.0% OCT4-GFP Positive



(B) End of Passage 6 94.6% OCT4-GFP Positive



(C) End of Passage 7 96.0% OCT4-GFP Positive



(D) End of Passage 8 86.5% OCT4-GFP Positive

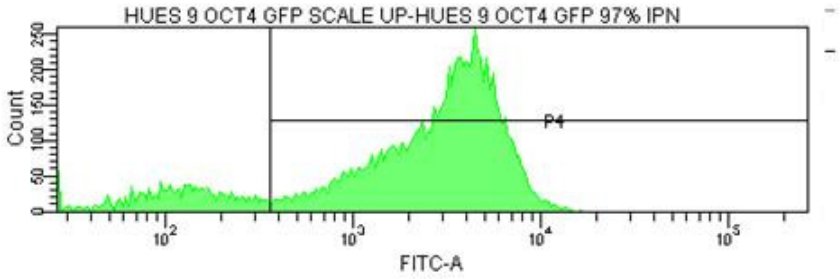


Figure 5. Flow cytometry diagrams of Hues9-OCT4-GFP long-term cultured on Matrigel and PMVE-alt-MA. (A) The OCT4 expression level, represented by the GFP (FITC) intensity, of Hues9-OCT4-GFP on Matrigel. Flow diagrams of cells cultured on Semi-IPN gel at the end of **(B)** passage 6, **(C)** passage 7, and **(D)** passage 8 were shown. The peaks of all flow diagrams, including (A) on Matrigel, were located at the same level of GFP expression. The result suggested the long-term effect on pluripotency of Hues9-OCT4-GFP were similar on PMVE-alt-MA semi-IPN gel to on Matrigel.

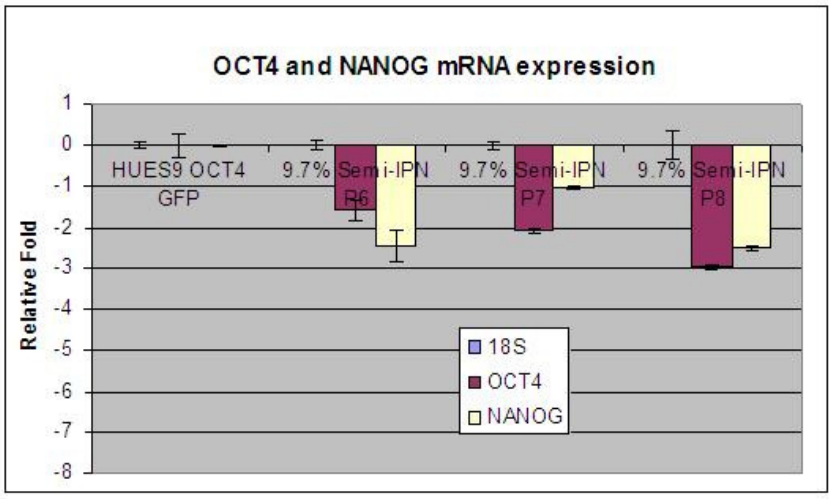


Figure 6. mRNA expression levels of OCT4 and NANOG of long-term Hues9-OCT4-GFP culture on PMVE-alt-MA semi-IPN gel. 18S, OCT4 and NANOG (left to right) expressions were grouped together. Control Hues9-OCT4-GFP, and Hues9-OCT4-GFP of passage 6, 7, and 8 on 9.7% PMVE-alt-MA semi-IPN were listed from left to right. Relative fold was measured by ΔΔCt of endogenous control 18S and the target gene. The decrease in measured mRNA expression may be resulted by the mixed population of differentiated cells in pluripotent cells. All values are presented as mean ± standard error of the mean.

4.3. Characterization of Initial Attachment on PMVE-alt-MA Coated Polyacrylamide

Five conditions of PMVE-alt-MA solution were used to coat polyacrylamide gel as described in the hydrogel coating protocol. For a fixed 10% polyacrylamide hydrogel casted in 1mm glass cassette, PMVE-alt-MA coating solution was varied to obtain 3%, 4%, 3%(at pH6.0), 4%(at pH6.3), and 10% polymer content. 10% FBS was used for proteins adsorption on PMVE-alt-MA coating. Hues9-OCT4-GFP was then seeded onto all five conditions of coating. pH values of PMVE-alt-MA was normally acidic (~pH2.0) in PBS and the increased pH to about ~pH6 should neutralize the charges on the polymeric side chains. Microscopy images (Figure 7) showed that Hues9-OCT4-GFP was attached and proliferated on 4% PMVE-alt-MA coating at 48 hours post-seeding.

Cells were initially attached to the thin layer of polymer created by the 10% coating, but later dissolved in the media. At day 2, no cells were attached to 10% coating, and cells only attached but not proliferated on elevated pH coating.

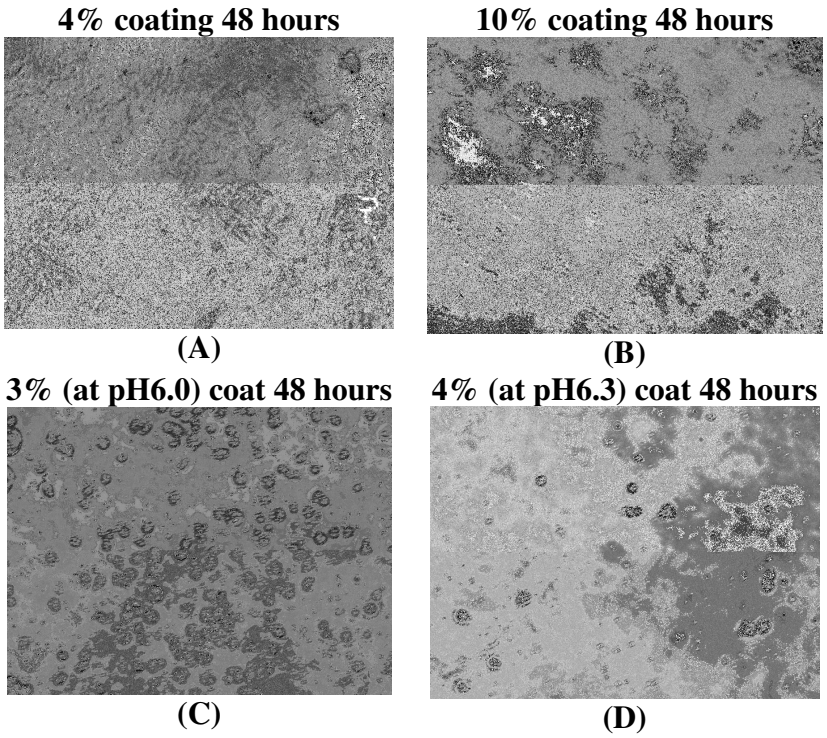


Figure 7. 48 hours Post-Seeding of Hues9-OCT4-GFP onto 10% FBS Primed PMVE-alt-MA coating. (A) 4% coating supported proliferation and attachment of hESCs **(B)** 10% coating did not support attachment. No proliferation was observed on **(C)** 3% (at pH6.0) and on **(D)** 4% (at pH6.3).

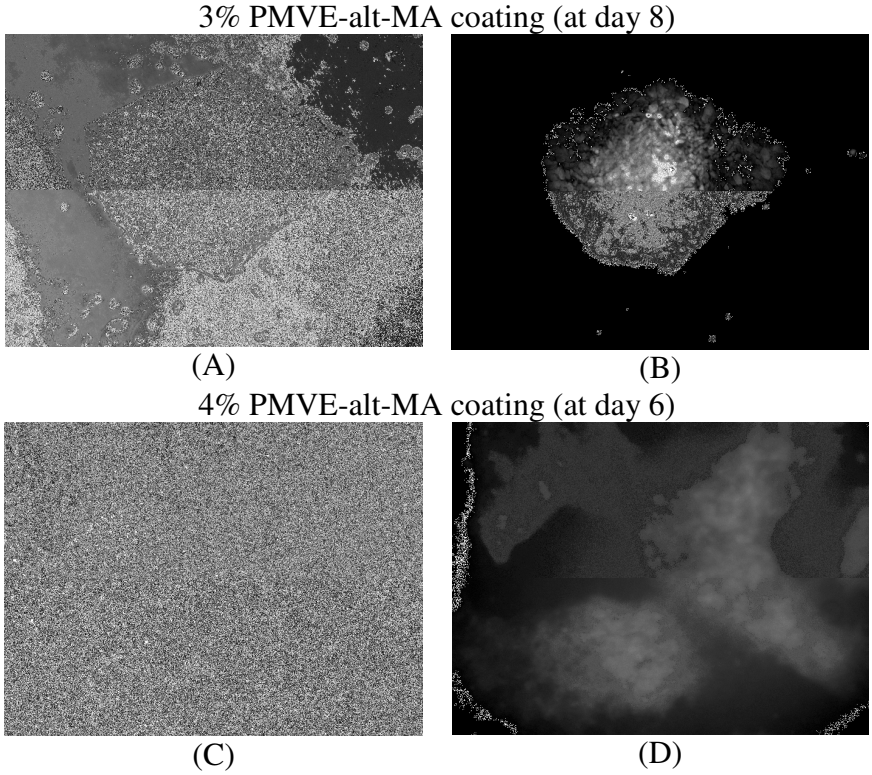


Figure 8. Proliferation of Hues9-OCT4-GFP on 10% FBS Primed PMVE-alt-MA coating. (A) Only a few hESC attached and proliferated on 3% PMVE-alt-MA coating and (**B)** OCT4-GFP signal was visible at day 8. **(C)** hESC colonies were embedded in differentiated cells and **(D)** OCT4-GFP signal revealed the location of stem cell colonies at day 6. Compared to 3% coating, more cells were attached to 4% coating and proliferation was also faster on 4% coating.

4.4 Characterization of Long-Term Growth of hESC on Coated Hydrogel

4.4.1. hESC Morphology and Endogenous OCT4 Expression

Hues9-OCT4-GFP was passaged on 10% FBS primed PMVE-alt-MA coated polyacrylamide hydrogel for 8 passages and a total of 5 days under the protocol described in 3.2.2. Passage time was identical to passages on PMVE-alt-MA semi-IPN described in table 2, except passage 8 was collected at day 9. Flow cytometry and qPCR were performed to monitor the changes in pluripotency markers at the end of passage 6, 7 and 8. The morphology and GFP expression were both similar to normal hESCs

characteristics at different time points of cells (Figure 9). In contrast, more differentiated cells were seen on PMVE-alt-MA coated hydrogel than on Matrigel or PMVE-alt-MA semi-IPN gel.

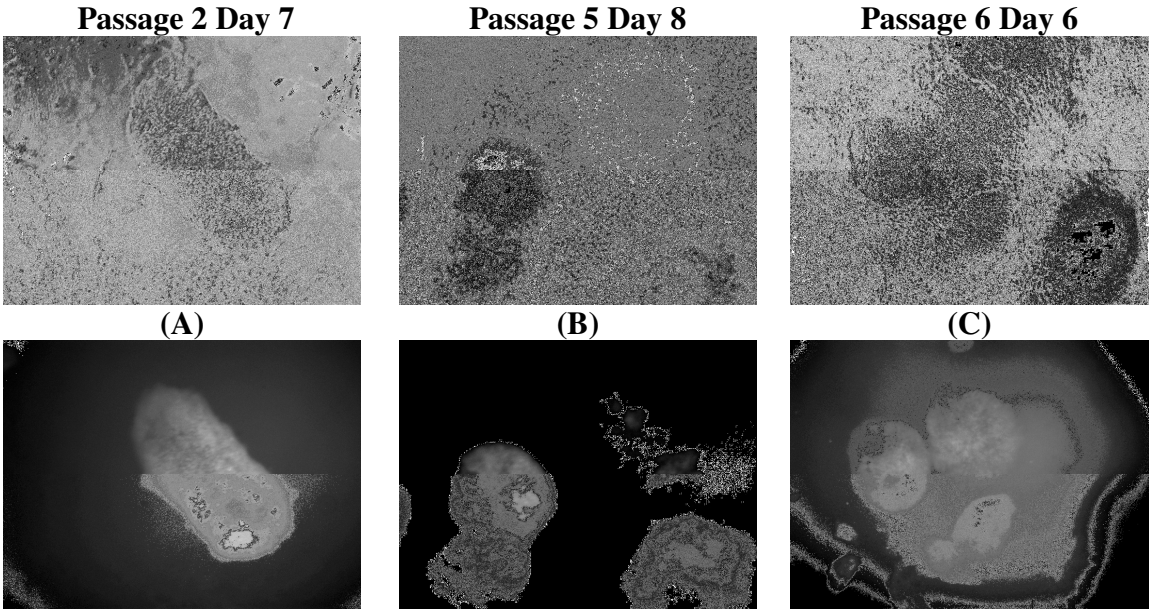


Figure 9. Morphology and OCT4-GFP expression of Hues9-OCT4-GFP cells on PMVE-alt-MA coated Polyacrylamide at different time points of culturing. Phase contrast and fluorescent images at **(A,D)** Passage 2 Day 7, **(B,E)** Passage 5 Day 8 and **(C,F)** Passage 6 Day 6 were shown here. hESC were assessed for their (A-C) characteristic morphology and (D-F) maintatenance of pluripotency (OCT4). Normal colony formation on PMVE-alt-MA coating was observed in all time points. More differentiated cells were growing around the colonies than normally seen in Matrigel plate culture. Fluorescent images of GFP expressions aligned with the colonies to confirm the pluripotency of hESC.

4.4.2. Flow Cytometry of hESC on PMVE-alt-MA Coated Polyacrylamide Hydrogel

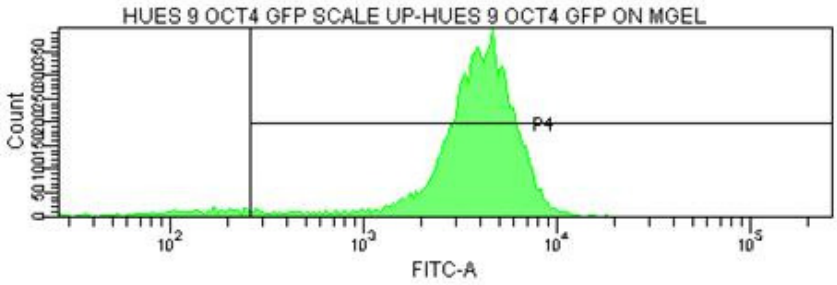
Expression of OCT4-GFP on PMVE-alt-MA coated hydrogel was different than Hues9-OCT4-GFP passaging on Matrigel. A population of hESC retained the OCT4 expression level similar to cells growing on Matrigel, as the population peaks were located at the same region. Two distinct peaks at the end of passage 6 represented the mixture of differentiated and undifferentiated cells. Because of the difficulty to separate

differentiated cells from undifferentiated cells by enzymatic selection method (described in 3.2.4), 58.3% GFP positive cells were passaged from passage 6 to passage 7 (figure 10B). However, 82.0% GFP positive cells were passaged from passage 7 to passage 8 (figure 10C). Enzymatic selection was not reliable on PMVE-alt-MA coated polyacrylamide hydrogel. Cells were cultured on passage 8 for 9 days (figure 10D), and extended culture caused the differentiated of hESC.

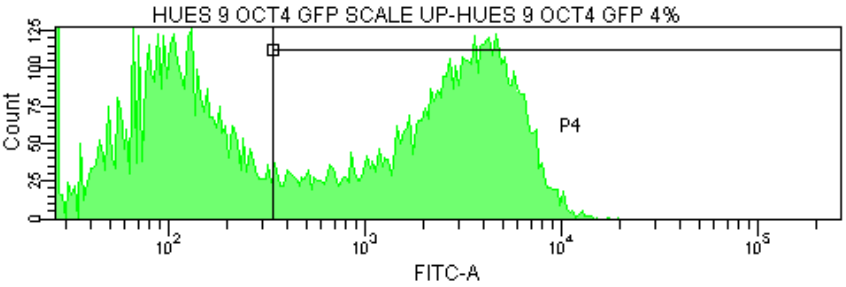
4.4.3. mRNA Expressions of OCT4 and NANOG

Quantitative PCR was used to measure the mRNA expression of pluripotency markers OCT4 and NANOG of hESC cultured on PMVE-alt-MA coated polyacrylamide hydrogel. Cells were collected at the end of passage 6, 7 and 8, and were compared to Hues9-OCT4-GFP cultured on Matrigel. All mRNA levels were normalized to endogenous control 18S. The result did not reflect the pluripotency of hESC population on coated PMVE-alt-MA due to the mixture of differentiated and undifferentiated cells. The result of passage 7 (P7) was more representative because the 82.0% of the cells were OCT4 positive in flow cytometry (Figure 11).

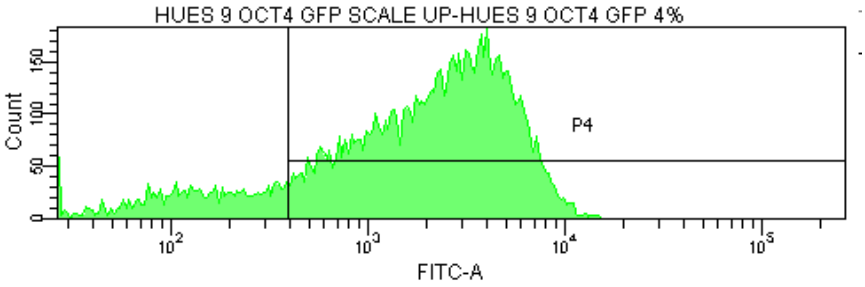
(A) On Matrigel 95.0% OCT4-GFP Positive



(B) End of Passage 6 58.3% OCT4-GFP Positive



(C) End of Passage 7 82.0% OCT4-GFP Positive



(D) End of Passage 8 33.0% OCT4-GFP Positive

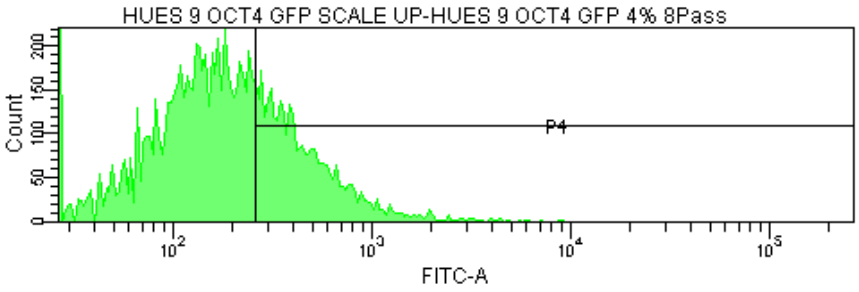


Figure 10. Flow cytometry diagrams of Hues9-OCT4-GFP long-term cultured on Matrigel and PMVE-alt-MA coating. (A) The OCT4 expression level, represented by the GFP (FITC) intensity, of Hues9-OCT4-GFP on Matrigel. Flow diagrams of cells cultured on 4% PMVE-alt-MA coating at the end of **(B)** passage 6, **(C)** passage 7, and **(D)** passage 8 were shown. The peaks of flow diagrams of (B) and (C) were aligned to the peak in (A), and that suggested hESC remained the same level of pluripotency as of culturing on Matrigel. The shift of the population peak in (D) suggested that passages at every 7 days were required to avoid differentiation. The result suggested the long-term effect on pluripotency of Hues9-OCT4-GFP could be retained for 7 passages.

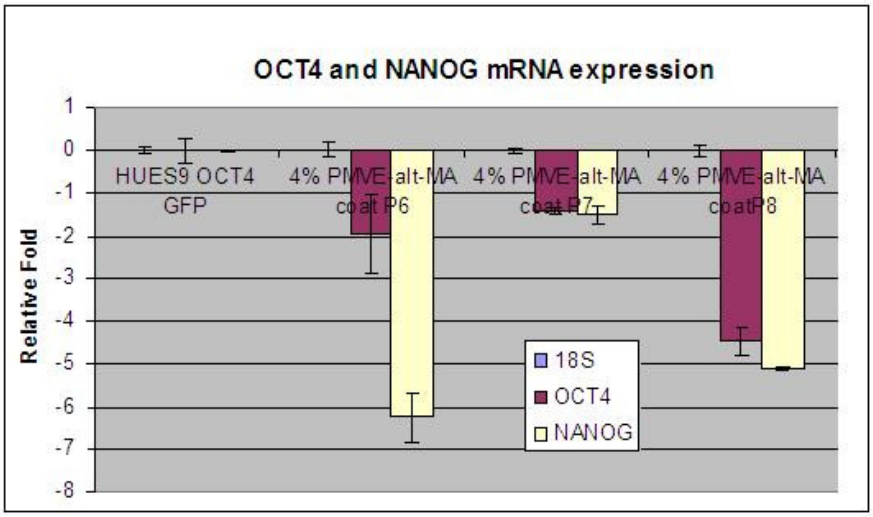


Figure 11. mRNA expression levels of OCT4 and NANOG of long-term Hues9-OCT4-GFP culture on PMVE-alt-MA coated polyacrylamide hydrogel. Relative fold was measured by ΔΔCt of endogenous control 18S and the target gene. All values are presented as mean ± standard error of the mean. The result did not reflect the pluripotency of hESC population on coated PMVE-alt-MA due to the mixture of differentiated and undifferentiated cells. The result of passage 7 (P7) was more reliable because 82.0% of the cells were OCT4 positive in flow cytometry.

4.5. Optimizations of Enzymatic Selection and Passaging on PMVE-alt-MA Semi-IPN Gel

Generally Hues9 and other hESCs on Matrigel dissociated into single cells by enzyme Accutase in less than 5 mins. Differentiated cells had weaker attachment and they dissociated in Accutase within 1-3 mins. Undifferentiated cells could be enriched by this enzymatic selection. Over 90% of Hues9-Oct4-GFP collected by this method were GFP positive (Figure 10A).

Hues9 had stronger attachment to PMVE-alt-MA than to Matrigel. Undifferentiated cells dissociated by Accutase took place in 15 mins. Agitation and multiple aspirations could decrease the amount of dissociation time to minimize Accutase contact. When agitation and aspiration were performed every 3 mins of incubation, the

resulting cell populations were 50-90% GFP positive. When the same protocol was performed every 1 min of incubation, the resulting population was 85+% GFP positive. Therefore, more often the aspirations were performed, the higher percentage of undifferentiated cells was collected.

The optimal number of cells used in each passage was important to yield a higher number of cells at the optimal density at a fixed number of days. Seedings of 80,000 and 100,000 expanded 4-5 folds in number over a 7-day period. Seeding of 130,000 became confluent in 4 days with an approximate expansion of 2 folds.

4.6. Microcarrier Culture with Matrigel Coated Cytodex 1

4.6.1. hESCs Adhesion and Proliferation on Matrigel Coated Cytodex 1

Hues9-OCT4-GFP was able to adhere and grow on Matrigel coated Cytodex 1 (Figure 12). $4x10^5$ cells were suspended with coated Cytodex 1 on the orbit shaker. In less than 2 hours, all of the cells were attached the surface of microcarriers. Cells continued to proliferate and the entire microcarrier surface was covered with cells. In contrast, uncoated Cytodex 1 did not support hESC attachment (Figure 13).

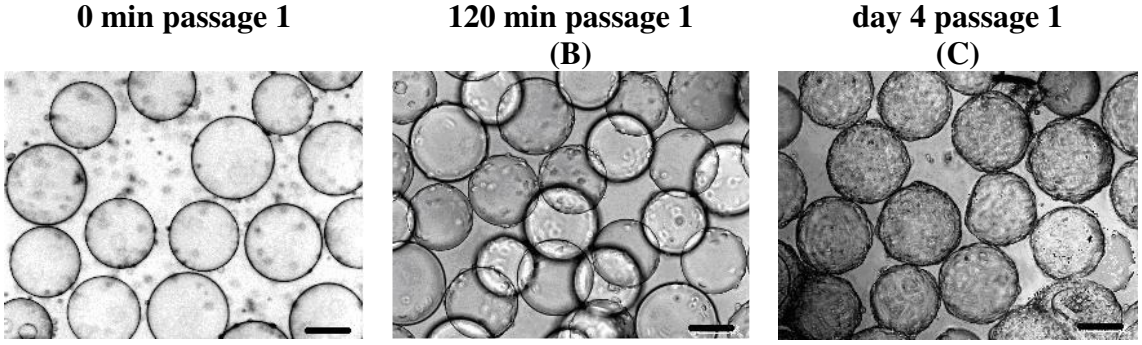


Figure 12. Adhesion and proliferation of hESCs on Matrigel coated Cytodex 1. (A) Hues9-OCT4-GFP were suspended in orbit shaker with Matrigel coated Cytodex 1. **(B)** All hESCs attached to microcarriers in less than 120 min. **(C)** Smooth surface was observed at day 4 post-seeding when the extracellular matrix layer was secreted by hESCs. Scale bar = 100 μm.

hESCs aggregated 2 days post-seeding

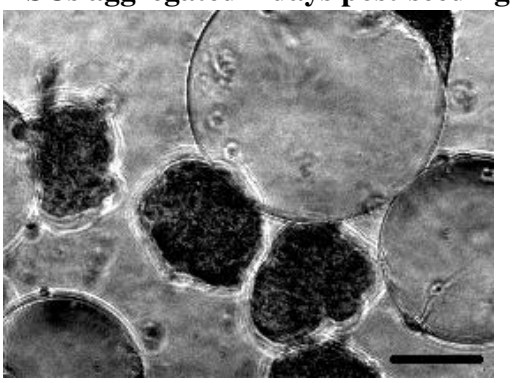


Figure 13. No adhesion of hESCs on uncoated Cytodex 1. This day2 post-seeding image showed that cells aggregated. hESCs required suitable substrate for attachment. Scale bar = 50 μm.

4.6.2 hESCs Passage on Matrigel Coated Cytodex 1

Passaging cells from cultured microcarriers to new microcarriers was described in 3.4.5. hESCs from passage 1 were growing in layers and densely packed (Figure 14A). Similar observations were previously described by other researchers[24]. hESCs from later passage were confluent on microcarriers but they were loosely packed (Figure 14B). Even though ESC colonies were observed, these replated day5 passage 3 cells onto Matrigel appeared to be partially differentiated as monitored by poorly defined colony boundaries (Figure 14C). Morphology of these cells on microcarriers was different than Matrigel culture and was not expected.

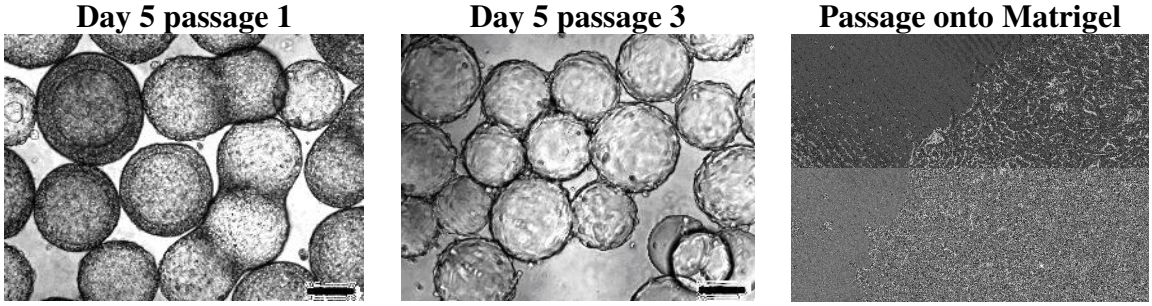


Figure 14. Passaging hESCs on Matrigel coated Cytodex 1 and replating on Matrigel. (A) Hues9-OCT4-GFP was growing in layers on Matrigel coated Cytodex 1 at day 5 post-seeding. **(B)** 3 passages on hESCs were cultured on microcarriers on orbit shaker. **(C)** hESCs from the end of passage 3 were replated on Matrigel plate. The morphology of hESCs was slightly changed, where the boundary of ESC colony was less defined. Scale bar = 100 μm.

4.6.3. Decreased Expression of the Marker of Pluripotency over Multiple Passages

Flow cytometry was used to monitor the endogenous OCT4 expression over the time period of 3 passages. Samples from multiple time points were used to monitor the changes in OCT4 expression during each passage. OCT4 expression remained around 90% positive during passage 1, and lowered to 50-80% during passage 2 and 3. A significant drop in numbers of OCT4-GFP positive cells was seen at each passage. Similar results were found when microcarriers coating and preparation was performed at either 4°C or 37°C. The decrease of OCT4 signal suggested that the coating of Matrigel was not directly related.

Flow diagrams of OCT4-GFP signal at day4 and day5 of passage 1 of Matrigel coated Cytodex 1 were used to compare with Hues9-OCT4-GFP growing on Matrigel plate (Figure 16). A slight decrease in GFP level was observed on cells at day 4 passage 1. A bigger decrease in GFP level was measured on cells at day 5 passage 1. Flow cytometry revealed the early decline of OCT4 in passage 1

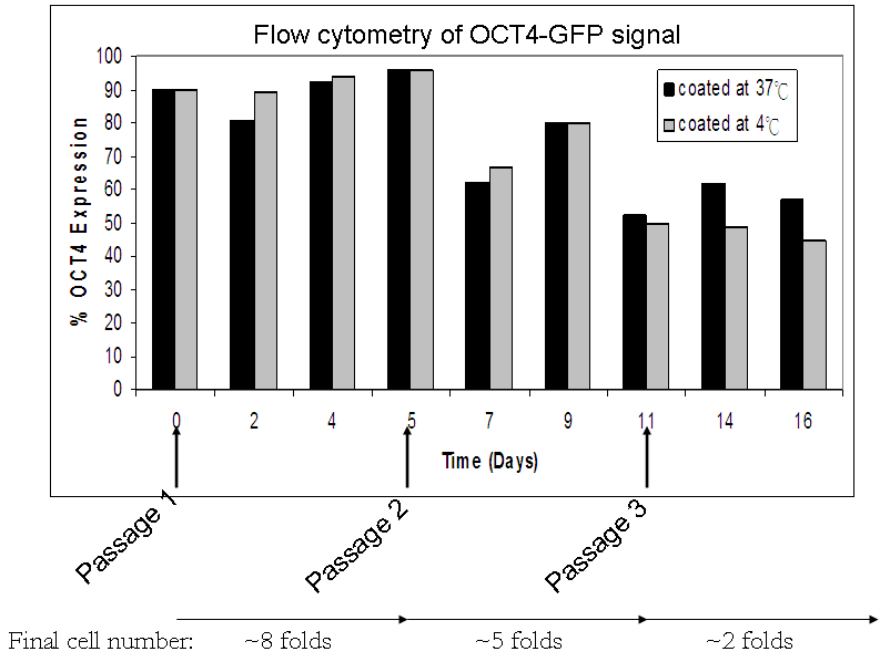


Figure 15. OCT4 expression and proliferation of cells over 3 passages on Matrigel coated Cytodex 1. Biological replicates were used to monitor the change of OCT4 expression at the selected time points. Hues9-OCT4-GFP were passaged on day 0, day 5 and day 11 and numbered passage 1, 2 and 3 respectively. 4°C and 37°C coatings both produced similar results in OCT4 expression. The expression of the marker of pluripotency OCT4 decreased at each passage. The numbers of cells collected from each passage decreased from initially 8 fold expansion, to 5 folds at the end of passage 2 and to 2 folds at the end of passage 3.

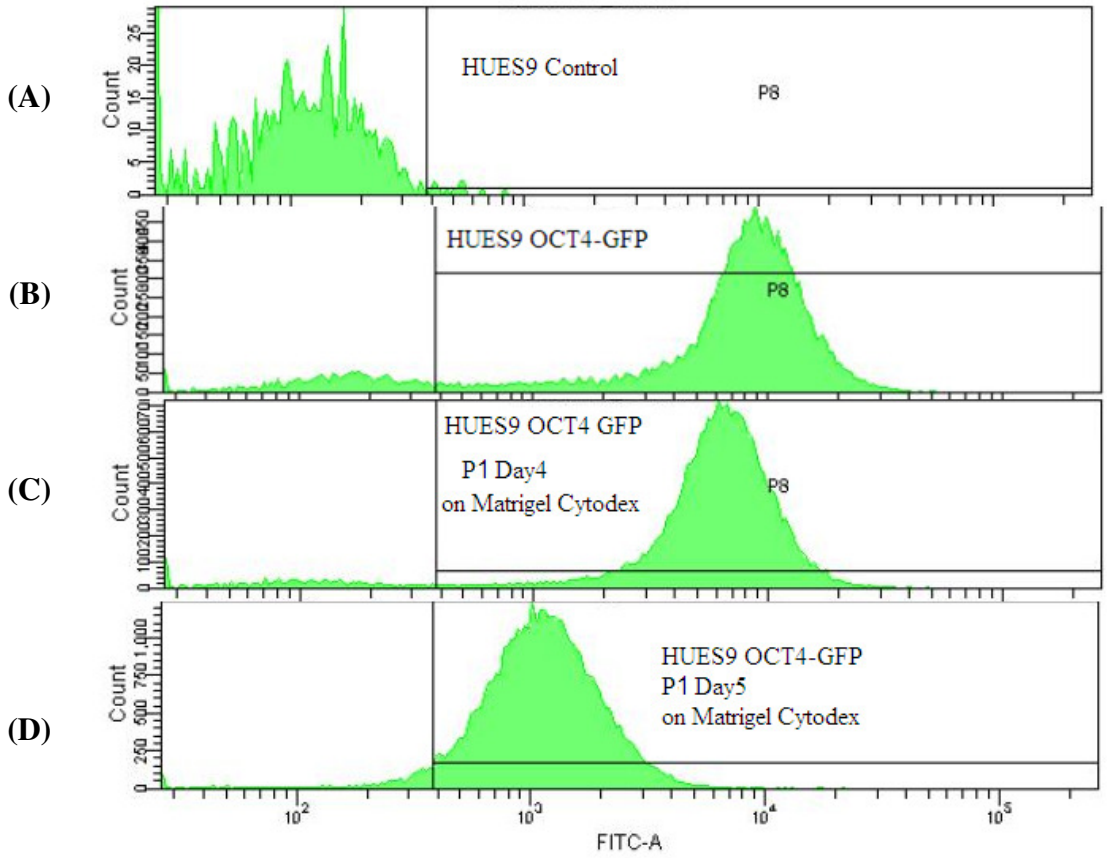


Figure 16. Pluripotency of hESCs decreased during passage 1 in microcarrier culture. **(A)** The gate indicated by the vertical line was fixed by the non-transfected Hues9 control. **(B)** Normal Hues9-OCT4-GFP expressed a stable level of OCT4 signal. **(C)** A slight decrease of OCT4 signal in the entire population of Hues9-OCT4-GFP at passage 1 day 4. **(D)** A drastic decrease of OCT 4 signal in the entire population of Hues9-OCT4-GFP at passage 1 day 5, one day after (C).

4.7. MEF-CM Conditioning for PMVE-alt-MA Coated Cytodex 1 in Suspension Culture

4.7.1. No hESC Adhesion to Unconditioned PMVE-alt-MA Coated Cytodex 1

No cells were attached to PMVE-alt-MA coated Cytodex 1 when Hues9-OCT4-GFP was cultured with MEF-CM with 10ng/ml bFGF in orbit shaker (Figure 17). Instead, hESCs agglomerated into clusters and continuously grew in size. The cell clusters were

regular in size and spherical in shape at day 3. The result was different than using Matrigel coated Cytodex 1 with the same conditions.

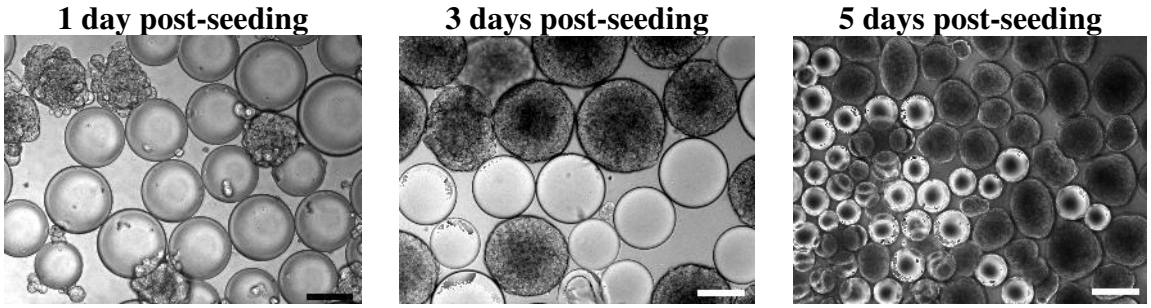


Figure 17. hESCs agglomerated in suspension when unconditioned (no FBS priming) PMVE-alt-MA coated Cytodex 1. (A) Slightly irregular shape cell clusters were found in between unconditioned microcarriers at day 1. **(B)** hESCs grew into larger spherical clusters at day 3. **(C)** hESCs cluster continuously grew in irregular shape at day 5. Scale bar = 100 μm.

4.7.2. MEF-CM Conditioning Promoted Cell Adhesion

Mixed results were obtained when MEF-CM was used to condition PMVE-alt-MA. One out of two replicates of 24-hour and three out of four replicates of 48-hour MEF-CM conditioning showed Hues9-OCT4-GFP attachment and proliferation (Figure 18). hESCs agglomerated in clusters if they were not adhered to microcarriers. However, a small number of hESCs was still found attached to the microcarriers in the latter cases. In summary, MEF-CM conditioning promoted cell adhesion as not seen in non-conditioned PMVE-alt-MA coated microcarriers.

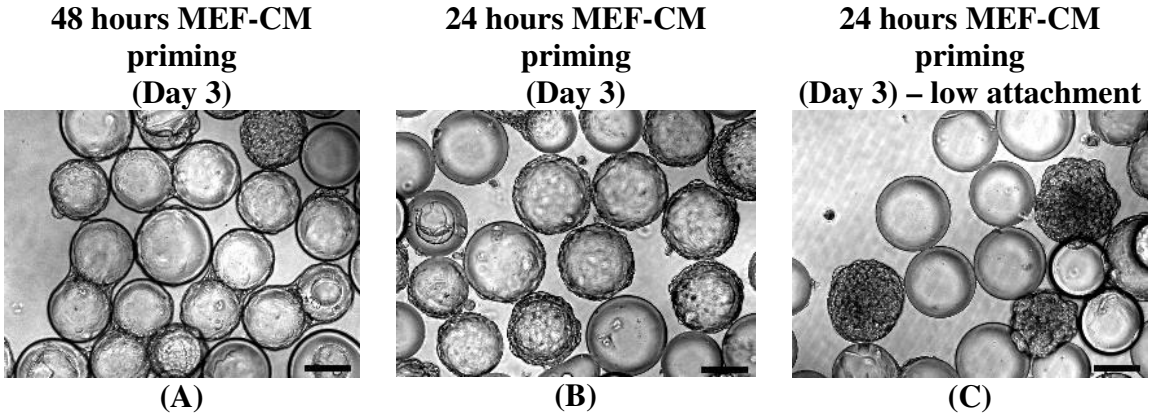


Figure 18. hESCs attached to some FBS conditioned PMVE-alt-MA coated Cytodex 1. (A) 48 hours and **(B)** 24 hours FBS conditioning of PMVE-alt-MA allowed Hues9-OCT4-GFP attachment and proliferation. **(C)** The replicate of (B) 24 hours FBS conditioning did not support cell attachment. hESCs were growing in clusters. Scale bar = 100 μm.

4.8. hESC Suspension Culture in Magnetic Stirred Bar Spinner Flask

4.8.1. Cell Attachment in Spinner Flask Suspension

hESCs were able to attach to Matrigel coated microcarriers in the motion of orbit shaker. However, spinner flask suspension did not support cell attachment from the mixture of hESCs and Matrigel coated microcarriers. Only a few cells were bound to microcarriers when HEK-293 cells were used. In summary, cells were required to attach to microcarriers on orbit shaker prior to spinner flask culture.

4.8.2. Cell Death in Spinner Flask Suspension

Spinner flask was initially stirred at 30rpm to create sufficient suspension of microcarriers. Pre-attached hESCs on the microcarriers were completely detached in 2 days. The reduced stirring speed at 18rpm was enough to suspend the majority of microcarriers. hESCs detachment from the microcarriers at 18rpm was seen at 48 hours and completed at 72 hours. The negative effect of spinner flask suspension was alleviated by reducing stirring speed. The viability of the freely suspended cells was found to be

10% using trypan blue staining. On the other hand, HEK-293 cells were proliferating in the same suspension condition and agglomerated with other microcarriers to create big clusters. This cell line specific effect has been reported by other researchers[25].

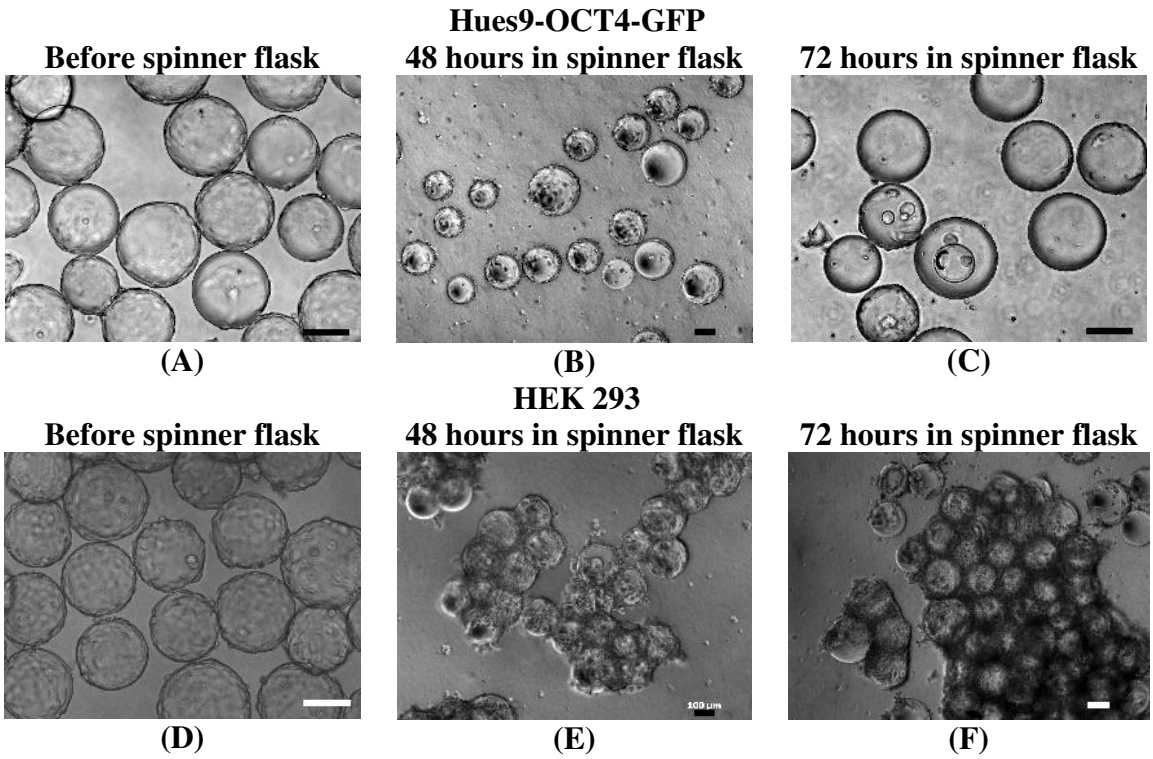


Figure 19. Differences in Matrigel coated Cytodex 1 culturing of hESC and HEK-293 cells in spinner flask stirring at 18rpm. (A) Prior to transferring into spinner flask, Hues9 was attached to the microcarriers. **(B)** Dead hESCs detached and suspended in media at hour 48. **(C)** All hESCs were detached from the microcarrier at hour 72. **(D)** 293 cells were attached to the microcarrier before transferring to spinner flask. **(E)** 293 cells continued to propagate on microcarriers at hour 48. **(F)** Microcarriers were agglomerated into cell clusters at hour 72. Not all cell types were able to sustain in spinner flask culture. Scale bar = 100 μm.

4.9 Embryoid Body (EB) Formation

4.9.1. Producing EB with Microcarriers

Around half a million cells per well were generally used for suspension EB formation in a 6-well low binding plate on the orbit shaker. Differences were found by adding microcarriers in suspension EB formation (Figure 20). EBs with uniform size and

spherical shape were produced in the presence of PMVE-alt-MA coated Cytodex 1. Without microcarriers, the resulting EB shape and size was non-uniform and widely distributed. In general, there were more small (size < 90 μm) than large (size > 100 μm) cell clusters. Similar results were found by using 250,000 and 500,000 initial cells. The presence of PMVE-alt-MA coated microcarriers, but not the number of initial cells, controlled the formation of EBs.

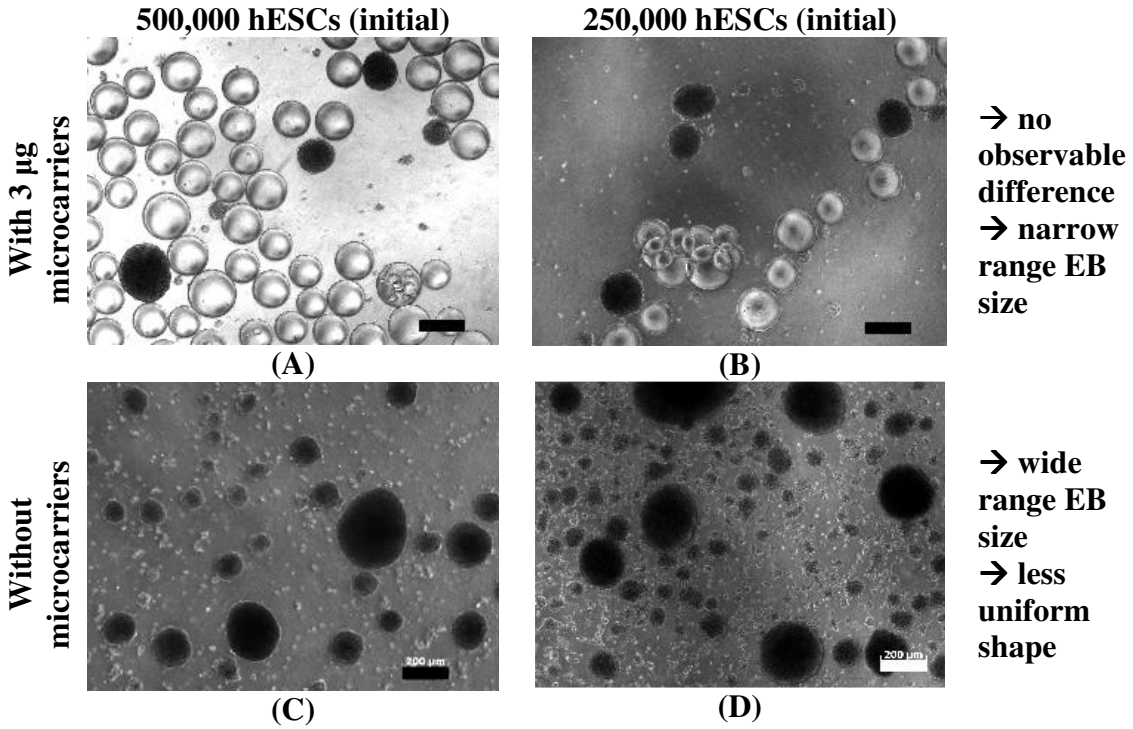


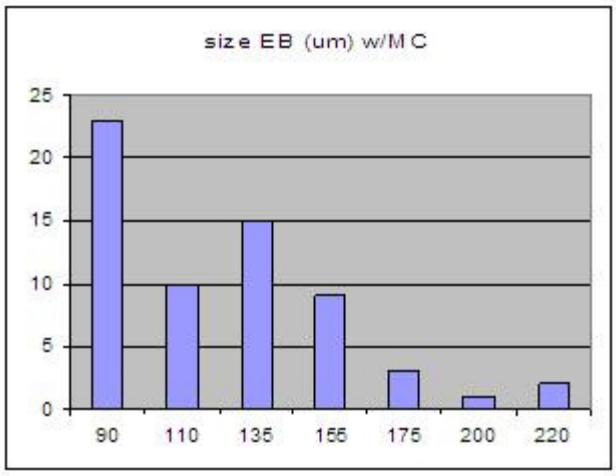
Figure 20. Different sizes and shapes of embryoid bodies (EB) at 120-hour by using suspension formation with and without microcarries. In the presence of microcarrier in suspension, **(A)** higher number of hESCs (500,000) and **(B)** lower number of hESCs (250,000) produced EB with spherical shape and narrow range of sizes. In regular suspension EB formation, **(C)** 500,000 cells and **(D)** 250,000 cells produced a wide range of EB size and less regular shapes EBs. Fewer cells and EBs were produced in (A) and (B) than in (C) and (D). Scale bar = 200 μm.

4.9.2. Distribution of EBs Diameters

When using EB formation as a directed differentiated method, similar sized EBs were collected for a better homogenous differentiation. The sizes of EBs were measured from the still microscopy to produce the distribution of EB diameters (Figure 21). The diameters of EBs were ranging from 90 μm to 220 μm, with the use of PMVE-alt-MA coated microcarriers in suspension. In the case of EB formation without microcarriers, EBs larger than 90 μm were collected by cell strainer to represent the current technology for homogenous differentiation. The diameters of EBs without the use of microcarriers were ranging from 90 μm to 370 μm. With the use of microcarriers, EBs were more homogenous in size.

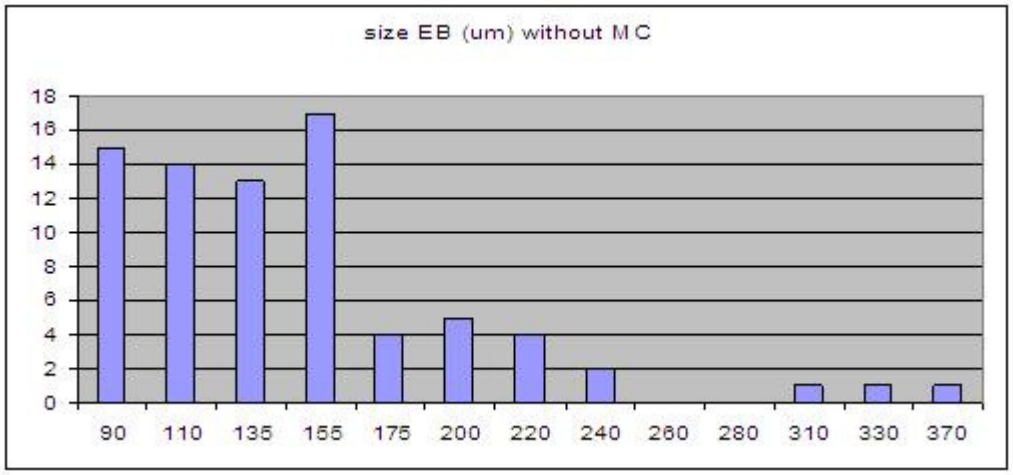
4.9.3. mRNA expression in EBs formed with microcarriers

mRNA expressions of pluripotency markers OCT4 and NANOG and neural differentiation marker Nestin were compared to analyze the differentiation of EBs (Figure 22). No difference was found from the differentiation patterns of EB formation with and without microcarriers. EBs collected from both methods showed that OCT4 and NANOG expressions were decreased by about 6 folds and 8 folds respectively, while Nestin expression was increased by 3 folds.



(A)

Note: small EBs (size<90μm) were removed by cells strainer in (B).



(B)

Figure 21. Distribution of EB diameters in suspension EB formation at day 7. (A) With the use of microcarriers, the resulting EB diameters were in the range of 90-220 μm**. (B)** In typical suspension EB formation, very large EBs (diameter > 240 μm) were formed. EB diameters were rounded down to the values listed in the diagram.

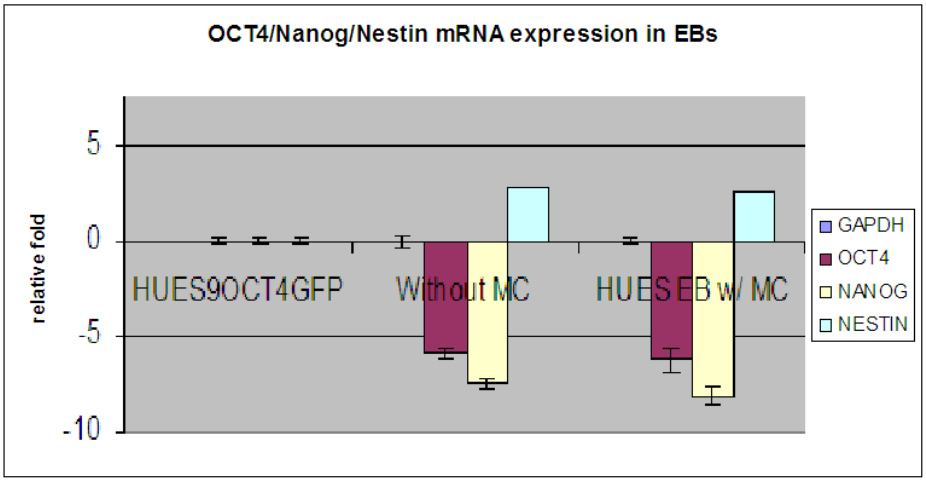


Figure 22. qPCR analysis of the markers of pluripotency and neural lineage in EBs formed in the presence of microcarriers at day 7. GAPDH, OCT4, NANOG and Nestin (left to right) expressions were grouped together. Hues9-OCT4-GFP passaged on Matrigel, EBs collected from normal suspension formation, and EBs collected from suspension using microcarriers were listed from left to right. EB smaller than 90 μm were removed by cell strainer in normal suspension formation The resulting expression patterns of EBs were similar with and without the use of microcarriers in suspension formation. Relative fold was measured by ΔΔCt of endogenous control GAPDH and the target gene. All values are presented as mean ± standard error of the mean.

Chapter 5. Discussion

Controllable and scalable culture methods to produce genetically stable and pluripotent stem cells are the current focus in culture technology development. Robust and cost-effective technologies require chemically defined culture conditions, including culture media and substrates. This study investigated the novel use of a synthetic polymer, PMVE-alt-MA, as a substrate in larger-scale hydrogel and suspension hESC culture. Semi-IPN hydrogel and coated hydrogel were developed for long-term maintenance of the proliferation and pluripotency of hESCs. The negative effect of using microcarriers as the suspending substrate was found to induce differention. The intermediate result of this experiment re-directed the use of PMVE-alt-MA to produce homogenous size and shape of EBs for the hESC differentiation process.

PMVE-alt-MA was successfully polymerized in polyacrylamide to produce a semi-IPN hydrogel that was suitable for long-term hESC culture. The ratio of PMVE-alt-MA and Polyacrylamide in semi-IPN hydrogel was evaluated, and the best hydrogel was able to sustain long-term hESC culture. Morphology and expression of pluripotency markers were comparable to the positive control of plate culture on Matrigel in the 8-week study. Furthermore, hESC attached more strongly to semi-IPN hydrogel than to the Matrigel substrate. Since undifferentiated cells expressed E-cadherin for cell-cell adhesion, Accutase was optimized to remove differentiated cells, which were often individualized and widely spread, from the undifferentiated cells in colonies.

PMVE-alt-MA was also shown to support long-term hESC culture by coating a polyacrylamide hydrogel. 4% PMVE-alt-MA supported self-renewal of hESC by polyacrylamide surface adsorption. In higher percentage coating (10%), cells attached to

the dissolvable polymer and later on removed together with the polymer layer. PMVE-alt-MA coating of pH 6.0 did not support hESC attachment. Decreased positive charges on neutral polymer could cause insufficient protein adsorption for hESC adhesion. For PMVE-alt-MA coating, pH value and polymer concentration played an important role in cell adhesion.

In contrast to semi-IPN hydrogel, PMVE-alt-MA coating promoted the proliferation of both undifferentiated cells and differentiated cells (shown in Figure 9). Differentiated cells were passaged to the new substrate when the enzymatic selection was not sufficient to reduce the number of differentiated cells. Manual selection of undifferentiated colonies was often performed in current hESC culture. But labor intensive methods were not suitable for large scale hESC expansion.

Interactions between the substrate and cell surface integrins were required for initial hESC adhesion. Non-specific adsorption of proteins in FBS on PMVE-alt-MA has introduced reproducible results of hESC binding, even though the mechanism of how this polymer bound to proteins was not clear. However, undefined FBS should be avoided in the future studies involving chemically-defined media.

Adhesion, proliferation and passaging of hESCs were shown on Matrigel-coated Cytodex 1. Flow cytometry data not only showed the rapid drop of OCT4 level after each passage, but also the initial decrease of OCT4 within the first passage in orbit shaker suspension culture. Furthermore, lower cell yield resulted at each passage. Passaging could cause damage to fragile hESCs[2], but the decrease of pluripotency markers in the first passage was a more significant issue in long-term maintenance of stem cells. Poor

attachment and proliferation were also observed when defined media StemPro was used to replace undefined MEF-CM in the microcarrier culture.

In the scale-up spinner flask culture, the substantial proliferation of HEK-293 cells revealed the cell line dependent effect in suspension culture because all hESCs growing in the same condition died slowly. Some hESC cell lines were shown to tolerate the high shear rate in suspension culture, while some were shown to fall into differentiation or apoptosis [25, 29]. Conventional microcarrier culture for mammalian cell propagation was not suitable for long-term propagation of hESC.

In contrast, PMVE-alt-MA coated microcarriers were not conditioned with FBS. The use of MEF-CM incubation produced mixed results of hESC adhesion to polymer coated microcarriers. Low reproducibility in this experiment suggested insufficient protein adsorption by using only MEF-CM. Furthermore, hESCs were agglomerated in the suspension with non-conditioned polymer coated microcarriers. The unusual uniform sizes and shapes of cell clusters have been sought by many researchers as an ideal method to promote homogenous differentiation. The identical result was successfully made by switching the hESC culture media to EB differentiation media. EBs produced in microcarrier suspension were more spherical and homogenous in size, in comparison to conventional suspension EB formation. However, from qPCR analysis of pluripotency and neural differentiation markers, no difference was found with or without the presence of microcarriers in suspension. Other differentiation markers are needed to conclude whether this EB differentiation method could promote homogenous differentiation.

Chapter 6. Conclusion

This study demonstrated that the off-the-shelf polymer - PMVE-alt-MA - can be chemically and biologically feasible for hESC expansion. More specifically, a semi-IPN hydrogel developed here was shown to support hESC adhesion, long-term proliferation, and passaging. The cost-effective PMVE-alt-MA has the potential to replace expensive and undefined biologically substrate for hPSC culture. A completely defined synthetic culturing system that is free of animal components is necessary for the clinical use of hPSC. Microcarrier culture for hESC expansion may not be possible; recently an increasing number of publications have suggested shear sensitivity as the causal effect on hESCs. hESCs on microcarriers, on the other hand, could be used to direct differentiation of those shear sensitive cells, such as mesenchymal stem cells and endothelial cells. PMVE-alt-MA coated microcarriers were demonstrated to produce spherical shape and uniform size EBs. Further investigation of suspension EB with microcarriers could improve homogenous differentiation.

Appendix: Additional Figures

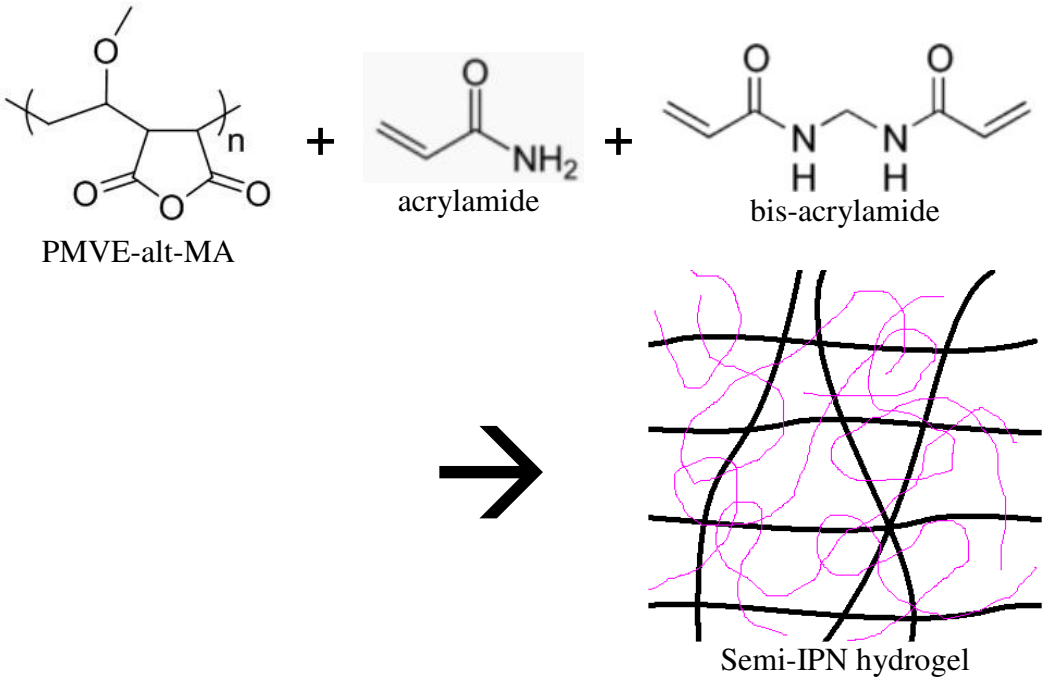


Figure A23. Formation of PMVE-alt-MA Semi-IPN hydrogel. PMVE-alt-MA was trapped in the polymer produced by acrylamide and bis-acrylamide. The solid black line represent the polyacrylamide network and the thin red lines represent PMVE-alt-MA.

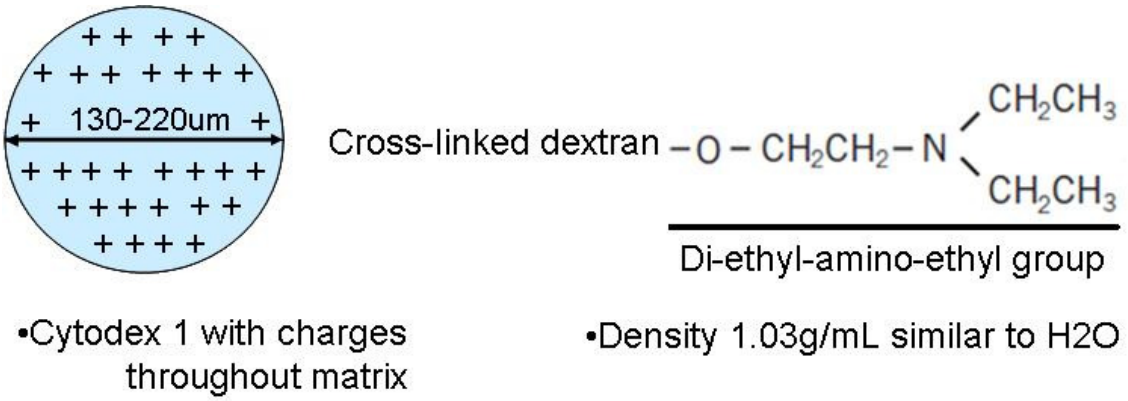


Figure A24. Specifications of Cytodex 1. Details of Cytodex 1 were shown.

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