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Expansion of Human Induced Pluripotent Stem Cells on Synthetic Substrate in Defined Medium

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ABSTRACT

Human induced pluripotent stem cells (hiPSCs) have the potential to generate patient-specific cells to treat many incurable diseases by cell replacement therapy. However, so far the culture of hiPSCs depends greatly on feeder cells or Matrigel which has safety issues. Thus, chemically defined substrates that could provide niches necessary for cell attachment and proliferation are preferred for clinical application of hiPSCs. Recently, Corning Life Sciences has developed synthetic peptide-functionalized cell culture surface, referred to as Corning® Synthemax™ that support self-renewal and differentiation of human embryonic stem cell (hESC). In this work, we have collaborated with Corning to investigate the attachment, proliferation, and differentiation of hiPSCs on the Synthemax substrate. We demonstrated that iPS cells retained stable proliferation and pluripotency marker protein expression after growing on the Synthemax substrate for ten consecutive passages. Further examination reveals that integrins $\alpha_V\beta_5$ mediates attachment to the substrate. Moreover, we observed hiPSCs colonies were more compact on the Synthemax surface. This may be due to less activation of β-catenin-mediated Wnt signaling pathway in cells on the synthetic peptide surface. In hiPSCs grown on the Synthemax Surface, we also found denser actin filaments in the cell-cell interface and down-regulation of vinculin and up-regulation of zyxin, indicating the reorganization of cytoskeleton structure inside cells in response to cell-matrix interaction.

This thesis is approved for recommendation
to the Graduate Council

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CHAPTER1 INTRODUCTION

Human embryonic stem (hES) cells are valuable for many clinical applications. The emergence of induced pluripotent stem cell (iPSC) technology raises hope of generating patient-specific cells for cell replacement therapy. The clinical application of these cells necessitates the development of new technologies that enable maintaining and differentiating these cells under chemically-defined or xeno-free conditions due to safety concerns over the use of animal-derived products in current human pluripotent stem cell (HPSC) maintenance and differentiation systems. This project focuses on developing xeno-free substrates for long-term HPSC maintenance and directed differentiation for clinical applications.

1.1 Stem cells

1.1.1 Human embryonic stem

Human embryonic stem cells (hESCs) are derived from inner cell mass of blastocysts (**1, 2, 3, 4**) and have the potential to be undifferentiated into any type of cells in the body as listed in Table 1. (**5**). They are able to self-renew indefinitely and the generation of specialized cell type provides the potential for cell replacement therapies to replace damaged and diseased organ or tissue in the patient's body. Therefore, hES cell represents a promising cell source for disease treatment using cell-based therapy, such as Alzheimer's disease (AD), Parkinson's disease (PD), and diabetes. In addition, hES cells can be used for drug discovery, toxicity study, gene therapy, and basic research of development biology. Nevertheless, use of hES cells for research is

ethically controversial since the embryonic stem cells come from surplus of embryos during *in vitro* fertilization (IVF). Immune rejection to the recipients is another major concern using hES cells as a therapeutic source during transplantation.

Table 1 Examples for human embryonic stem cell–derived cell types (5)

Layers	Tissues and cells
Ectoderm	neural precursors, dopamine neurons, motor neurons, retinal cells, keratinocytes melanocytes
Mesoderm	fat, cartilage, skeletal muscle, bone, blood cells, cardiomyocytes
Endoderm	prostate cells, hepatocytes, lung epithelium

1.1.2 Induced pluripotent stem cells

In 1996, a groundbreaking technology so called "Induced pluripotent stem cells technology" or "iPS cell technology" was first publicized by Shinya Yamanaka group at Kyoto University in Japan. This technology allows reprogramming any adult somatic cells into embryonic stem cells-like cells. The reprogrammed cells possess pluripotency as hES cells do (**6, 7, 8, 9**). In the iPS cell technology, four transcription factors, Oct3/4, Sox2, c-Myc, and Klf4 were cloned into retroviral vectors and then traduced into mouse fibroblast cells. The transduced cells showed similar properties of hES cells in morphology, global gene-expression, epigenetic state, teratomas formation, and differentiation ability (**7**). With the same procedures, Yamanaka and his colleagues produced human iPS cells from human fibroblast cells in 2007 (**8**). Meantime,

generation of human iPS cells reprogramming was published in Science from Thomson's group in the United State in 2007 (**9**).

Clearly, the advantages of iPS cells over hES cells are 1) There are no ethical issues since there is no needed to isolate pluripotent stem cells from an embryo. 2) There is no concern about immune rejection to the recipients in cell- or organ- transplantation since the cells can be generated directly from the patient. iPS cells can be generated by biopsy from the patient and then reprogrammed them into pluripotent cells, followed by induced differentiation into a cell type for realization of patient-specific cell-based therapies. Thus, iPS cells raise hopes for treating many otherwise incurable diseases through cell replacement therapies (**10, 11, 12, 13**).

1.2 History of stem cell culture technology

1.2.1 Feeder layer culture

Unlike culturing many specialized cell types, culture of pluripotent stem cells including ES cells or iPS cells is a challenge because special niches are required for adhesion, self-renewl and induced differentiation of these cells. The spontaneous differentiation feature of these cells makes the culture and maintenance complicated. Often, to maintain pluripotent stem cells in undifferentiated condition is one of the major tasks in the long-term maintenance of HPSC lines. At the early stage of hES cell culture, it was found that mouse embryonic fibroblasts (MEFs) as a feeder layer of hES cells are essential for hES cells continuously self-renew in undifferentiated state (**3, 14**). However, it is time-consuming and labor-consuming for preparing MEFs. Importantly, the use of animal cells has great safety concerns in clinical application due to the potential animal virus transmission.

Use human cells as a feeder layer for hES/iPS cell culture was also extensively investigated (**15, 16, 17**). Human fetal muscle (FM), fetal skin (FS), and AFT epithelial cells were used to construct 3 feeder layers for the test of maintenance of HES3 and HES4 cells (**16**). The results show that hES cells grown in the media maintained ES features including morphology of human ES cells, the expression of stem cell surface markers, normal karyotypes and pluripotency (**16**). Human foreskin feeder was used as feeder layer cells to culture three hES cell lines for more than 42 passages and all the hES cells were maintained pluripotent features (**17**). Nevertheless there is a potential of cross contamination, since the feeder layer cells are from different origins. Thus, it is desired to develop feeder-free and serum-free culture system.

1.2.2 Feeder free culture

Currently, Matrigel from BD Biosciences is commonly used as a substrate for hES cells culture in undifferentiated state with combination of serum-free medium without using any feeder cells. Matrigel is a mixture of extracellular matrix proteins extracted from the Engelbreth-Holm-Swarm (EHS) mouse tumor. It is rich in laminin, collagen IV, heparan sulfate proteoglycans, entactin, nidogen and some undefined factors (**18, 19, 20**). Matrigel allows us to mimic the extracellular environment in the body. In the laboratory, Matigel has to be thawed in 4 ºC before coating to a cell culture plate for one hour to form a film on the surface of the cell culture plate. hES cells can be cultured on Matrigel in undifferentiated state for more than 130 population doubling (**21**). Cells retain normal karyotype, expression of hES cells' markers, pluripotency and high telomerase activity (**21**). On the other hand, mTeSR medium from StemCell Technology was formulated for use with Matrige. This combination of the culture system allows maintenance

of hES and iPS cells in serum-free and feeder layer free conditions. Compared to feeder layer cells, preparation of Matrigel coated surface is a relatively easy and inexpensive process.

However, as mentioned above Matrigel is an undefined mixture of ECM proteins produced from animal tumors and there are lot-to-lot variations. In addition, animal derived product may cause pathogenic risks. It raises significant safety concerns over Matrigel use in clinical applications. Hence, there is a great need to develop xeno-free, synthetic surface that capable of providing necessary stem cell niches to allow hES/hiPS cells expansion and differentiation in a xeno-free chemically defined culture system.

1.3 Stem cell microenvironment

1.3.1 Extracellular matrix (ECM)

The extracellular matrix (ECM) *in vivo* contains mainly macromolecules polysaccharides, proteins such as different types of collagens, or proteoglycans. ECM is synthesized, secreted, and degraded by animal cells and distributed in the cell surface or between cells. The macromolecules include collagen, laminin, fibonection, vitronectin, elastin and so on. These substances constitute a complex network structure to support and connect the tissue structures, and to regulate the physiological activities of the cells. Thus, ECM is an important part in animal tissue. It determines the characteristics of connective tissues and plays an important role in cell migration, cell differentiation, cell proliferation, and apoptosis.

1.3.2 Integrin

Integrins are the major proteins of cell surface receptors. They play important roles in the mediation of the cell and extracellular matrix adhesion and transduction information from ECM into the cell. Integrin is a heterodimer formed by α chain (120~185kD) and β chain (90~110kD). So far 18 kinds of α subunits and 9 β subunits have been found. They constitute more than 20 kinds of different combinations of integrin. Generally, integrin proteins are transmemebrane proteins with a short cytoplasmic domain. There are divalent cations domains in both α subunits and β subunits regulating the activity of integrin. The divalent cations are Mg^{2+} which promotes binding and Ca^{2+} which inhibits the binding (**23, 24**).

An integrin on cell surface binds to ECM macromolecules such as collagen, laminin, fibonection, and vitronectin. The amino acid sequence Arginine-Glycine-Aspartic acid (RGD), an adhesion motif, is the most common binding sites between integrin and ECM proteins (**22**). Most cells express more than one kind of integrin which is involved in several life activities. For example, duo to the adhesion ability, integrin can lead to platelet aggregation during the wound healing. In addition, integrin is necessary for some types of cells proliferation (**25, 26**). If the interaction between integrin and ECM is blocked, the cells may fail to attach and spread.

1.3.3 Stem cell niches

Stem cell fate including self-renewal, differentiation, and death is determined by the microenvironment which is also called niches throughout cell-ECM or cell-cell interactions. Recently, stem cell niches are described as dynamic microenvironments that govern the growth and repair of the organism (**27**). For instance, a single injection of fibronectin, a glycoprotein

produced in the body that helps anchor cells in place, can prevent the development of chronic pain that often develops after a spinal cord injury (SCI) (**28**). A one-time injection of fibronectin (50 μg/mL) into the spinal dorsal column (1 μL/min each injection for a total of 5 μL) immediately after SCI inhibits the development of a particular type of chronic painor pain from pressure that would not normally cause pain-which is common in spinal cord injury patients (**28**). Bone marrow mesenchymal stem cell (MSC) not only supports the bone marrow feeder environment for hematopoietic stem cells, but also acts as a niche for itself. Cardiovascular progenitor cell (CPC) niche plays an essential role in maintenance and expansion of CPC in developing human and mouse hearts (**29**). In the ES cell differentiation and during the embryonic development, many phenomena are related to microenvironment, such as different gradient distribution of the protein such as SHH (sonic hedgehog), which can determine the differentiation pathways and development of the embryonic tissue to different lineages. However, stem cell niches and the activation of stem cells by stem cells inches are largely elusive.

1.4 Wnt Pathway

1.4.1 Overview of Wnt Pathway

Wnt/catenin signaling pathway plays a vital role in regulating cellular proliferation, cell fate decision, and organ development (30, 31,32). It has been well understood that Wnt signals modulate β-catenin expression and activate a higher level expression of integrins (33)

Wnt is named after Wg (wingless) and Int (Integration) (**34**). Wingless gene was first found in Drosophila, and plays a role in embryonic development. The adult animal body forming gene Int was first found in vertebrates, located nearby the mouse mammary tumor virus (MMTV)

integration sites. Int-1 gene and the wingless gene are homological. Drosophila wingless gene mutation can lead to the wingless deformity, and mouse mammary tumors in MMTV replication and integration into the genome can lead to the synthesis of one or several Wnt genes. Different Wnt and Wnt ligands are derived from the common ancestor of the various organisms. Wnts interact with ECM molecules to elicit their functions on target cells.

1.4.2 Canonical Wnt pathway

The canonical Wnt pathway describes a series of reactions when Wnt proteins bind with Frizzled receptor family on the cell surface, including the activation of Dishevelled receptor family of proteins and the change of β-catenin levels in the nucleus. Dishevelled (DSH), one of the key components of the cell membrane, is related to the Wnt receptor complex. DSH is activated after Wnt binding to inhibit downstream protein complexes, including axin, GSK-3, and the APC protein. Axin/GSK-3/APC complex can promote the degradation of intracellular signaling molecules of β-catenin. When β-catenin destruction complex was inhibited, β-catenin in the cytoplasm will be stable and part of the β-catenin will transfer into the nucleus to promote the expression of specific genes with the TCF / LEF transcription factor family and induce Wnt target genes (**35**).

1.4.3 Regulation of stem cell by canonical Wnt pathway

Wnt pathways also play important role in maintaining stem cells in undifferentiated state, regulating proliferation of intestinal stem cells, skin stem cells and haematopoietic stem cells (**36, 37, 38**). Activation of canonical Wnt pathway by inhibiton of GSK3β maintains pluripotency and self-renewal of embryonic stem cells (**39**). Overexpression of activated β-catenin and activation

of Wnt pathway leads to not only self-renewal in long-term cultures *in vitro*, but also enhancement of the reconstitution of haematopoietic lineages *in vivo* (**38**). Furthermore, canonical Wnt pathway determines the fate of stem cells. It is reported that the differentiation of neural stem cells into neuronal and glial cells were promoted by adding active Wnt3a (**40**). Stem cell can differentiate into follicular without β-catenin (**41**). Taken together, canonical Wnt pathway regulates not only the expansion of stem cells but also fate of stem cells.

1.5 Synthetic peptide surface

As discussed above, there are concerns of contamination and immunogenic response by the use of mouse feeder cells or Matrigel for the culture of stem cells for clinical application, because both of them are animal origin and have undefined factors. Thus, chemically defined substrates that can provide niches necessary for cell attachment and proliferation are preferred for clinical application of hES/iPS cell. To overcome these issues, development of xeno-free and chemically-defined hES/iPS cell culture system became one of the hottest topics in tissue engineering and regenerative medicine. In animal tissue, ECMs are an important part of niche for maintenance and differentiation of stem cells. Some recombinant ECM proteins have already been investigated to support survival and self-renewal of pluripotent stem cells for several generations (**42, 43, 44, 45**).

Several peptides identified using phage display libraries have been shown to support hESC expansion for three passages (**46**). Another approach that has been attempted is to synthesize biologically active peptides derived from Matrigel components. Since a major element of Matrigel is laminin, three laminin-derived active domains have also been studied for their

capability of supporting hESC self-renewal and proliferation. Nonetheless, these peptide-coated substrates can only support hESC expansions in no more than three passages (**47**). Microarray-based high-throughput screening identified 16 chemically-defined acrylate monomers that can support hESC expansion in no more than five passages (**48**). Corning Life Sciences has recently developed synthetic peptide-functionalized cell culture surface, referred to as Corning® Synthemax™ Surface that support self-renewal and differentiation of hES cells (**45**). This synthetic peptide surface utilized acrylate, a widely used organic synthetic biomaterial, to form fast polymerization. Acrylate-containing carboxylic acid was deposited onto culture vessel surfaces, and then conjugated to peptides containing amines by using 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide/*N*-hydroxysuccinimide (EDC/NHS) to form Peptide-acrylate surface **(**PAS). Five peptides derived from five proteins, including bone sialoprotein (BSP), vitronectin (VN), long fibronectin (sFN), short fibronectin (lFN) and laminin (LM) were synthesized. It was found that peptide surfaces of PAS, sFN, IFN, and LN (but not BSP-PBS and VN-PBS), maintain hES cells at normal morphology and undifferentiated state (**45**). Synthemax Surface was named for the VN-PAS peptide surface developed by Corning.

The ability of Synthemax surface to support hES cells self-renewal and induced differentiation was evaluated by Corning and Geron Corporation (**45**). The surface can support the hES cells renewal (H7) for at least 12 passages with mTeSR1, Knock Out Serum-supplemented medium or TeSR2 without any changes in stem cells characteristics such as stable doubling time, cell viability, normal morphology and karyotype, and expression of pluripotency markers Oct4, TRA-1-60, and SSEA4. In differentiation experiments, teratomas coprising three germ layers (endoderm, mesoderm, and ectoderm) and embryoid bodies were formed by the differentiation of

H7 cultured after 8 passages on the Synthemax. In addition, Cardiomyocytes were directly differentiated from H7 hES cells on Synthemax surface by using a protocol in previous report (**49**).

However, whether this synthetic peptide surface can support growth and differentiation of hiPSC remains elusive. A line of evidence suggests that hiPSCs and hESCs exhibit some differences, despite similar patterns in global transcriptome assessment (**45**). It has been found that a subset of 318 genes differentially expressed between these two types of (**45**). This small set of genes may represent a genetic memory of the ancestor cells from which hiPSCs were derived (**45**). Thus, it is critical to assess whether the Synthemax is suitable for hiPSC maintenance and differentiation. In this work, we investigated the attachment, proliferation, and differentiation of hiPSCs on the Synthemax Surface. The goal of this study was to determine whether hiPSCs can be maintained over long period of time and differentiated on the Synthemax Surface.

CHAPTER 2 MATERIALS AND METHODS

2.1 iPS cell culture

The human iPS cell line IMR90 was acquired from the Wicell Research Institute (Madison, WI). Cells were routinely maintained on growth factor reduced Matrigel (Becton Dickinson Biosciences, Franklin Lakes, NJ) coated dishes in a chemically defined medium mTeSR1 (Stem Cell Technologies, Vancouver, BC, Canada) at 37 ^{o}C with 5% CO_2. The culture medium was exchanged daily. The morphology of cell colonies was examined daily and spontaneously differentiated colonies were removed to ensure maintenance of undifferentiated state of iPS cells. Two methods were applied to retain undifferentiated cells and remove differentiated cells. One method is called pick-to-remove. In this method, the differentiated colonies were physically detached from the culture dish and aspirated along with the spent media. Another method is called pick-to-keep, where the undifferentiated cells and colonies were physically removed and plated in a new plate. To characterize cell growth and differentiation on the Synthemax approximately $5x10^4$ cell/cm^2 iPS cells were plated onto the Synthemax six-well plate (Corning Inc., Corning, NY). Cells seeded in Matrigel coated six-well plate served as a control. Microscopic imaging was performed daily to monitor cell attachment and proliferation. Cell number was counted by Trypan-blue staining in a 24 h time interval. Cell doubling time (t_d) was estimated using equation: $dx/dt=\mu x$; $t_d=\ln 2/\mu$.

2.2 Immunofluorescence staining

Immunofluorescence staining was performed using varied antibodies as primary antibodies and fluorescent dye conjugates as secondary antibodies to detect protein expression. In brief, cells were rinsed twice in 0.5ml/well ice-cold Dulbecco's Phosphate Buffered Saline (DPBS) without Ca^{2+}/Mg^{2+} (Mediatech, Inc. Manassas, VA) at room temperature and fixed by freshly made 0.5ml of 4% paraformaldehyde (Fisher Scientific, Rockford, IL) in PBS (pH 7.4) for 15 min at room temperature with shaking, followed by three times washing with ice-cold DPBS. The samples were incubated for 10 min with 0.5ml per well DPBS (without Ca^{2+}/Mg^{2+}) containing 0.5% Triton X-100 in room temperature with shaking. Cells were then washed with DPBS three times, each for 5 min with shaking. Cells were incubated with 0.5ml per well of blocking buffer (0.05% Tween-20, 0.1% Triton X-100, 1%BSA, 1× DPBS) for 1 hour to block nonspecific binding of the antibodies. After blocking, cells were incubated with primary antibodies (300µl/well) (Table 2) in blocking buffer overnight at 4^{o}C with shaking. After washing the cells three times in 0.5ml/well wash buffer (0.1% BSA, 1×DPBS), each for 5 min. Cells were incubated in fluorophore-conjugated secondary antibodies in the dark for 1 hour at room temperature with shaking. Table 2 lists all the primary and secondary antibodies used in this study. After washing cells three times for 5 min per wash with shaking, cells were labeled with DAPI (diaminophenylindoleas) as well to localize cell nucleus. 4 drops of VECTASHIELD Mounting Medium with DAPI (Vector Laboratories, Inc. Burlingame, CA) were added to each well and incubate for 1 minute. The fluorescence microscopy images were captured by the inverted phase contrast fluorescence microscope Olympus IX71 (MVI, Avon, MA) equipped with a highly sensitive CCD camera (Qimaging, 32-0139-104) using Slidebook imaging analysis software 4.2. (Olympus Imaging America Inc., Center Valley, PA).

Table 2 Primary and secondary antibodies used for immunofluorescence staining

Primary antibody	company	ratio	Secondary antibody	company	ratio
Mouse monoclonal anti-human SOX17	R&D Systems, Minneapolis, MN	1:50	Goat anti-mouse IgG	Sigma, St. Louis, MO	1:100
rabbit monoclonal anti-human FOXA2	Abcam, Cambridge, MA	1:1000	donkey anti-rabbit IgG TRITC	Jackson Immuno	1:50
mouse anti-OCT4	EMD Millipore Billerica, MA	1:100	goat anti-mouse Alexa Fluro 488 IgG1	Invitrogen, Carlsbad, CA	1:200
mouse anti-SSEA4	EMD Millipore Billerica, MA	1:100	goat anti-mouse Alexa Fluro 488 IgG3	Invitrogen, Carlsbad, CA	1:200
Alexa Fluor@488 phalloidin	Invitrogen Eugene,OR	1:40			
rabbit anti-vinculin	Santa Cruz Biotechnology Inc., Santa Cruz, CA	1:50	anti-rabbit IgG - FITC	Sigma, St. Louis, MO	1:150
Rabbit anti-α-actinin	Sigma, St. Louis, MO	1:100			
rabbit anti-zyxin	Sigma, St. Louis,	1:100			

	MO				
rabbit anti-p-FAK	Santa Cruz Biotechnology Inc., Santa Cruz, CA	1:50			

2.3 Western blotting

2.3.1 Protein sample preparation

Cells were cultured on Matrigel coated and Synthemax plates for 48 h. The cells were collected by Typsin EDTA (Mediatech, Inc. Manassas, VA) treatment followed by centrifugation at 300×g for 5 min and washing with DPBS once. The cell pellets were lysed with lysis buffer (50 mM Tris (pH 7.4), 150 mM NaCl, 1 mM DTT, 0.1% SDS, 1% Triton X-100, PMSF) by using a 1 ml syringe (Becton Dickinson, Franklin Lakes, NJ) with 20G$1^1/_2$ needle (Becton Dickinson, Franklin Lakes, NJ) up and down 20 times. Cell lysates were then centrifuged at 21,000×g for 15 min at 4 ^oC. The supernatants were collected and protein concentration was determined using a Pierce BCA protein assay kit (Thermo Scientific Inc., Rockford, IL). Cytoplasmic and nuclear proteins were extracted using a kit from Thermo Scientific. The protein samples were stored at -80^oC for further experiments.

2.3.2 SDS-PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis)

Certain amount of cellular protein samples were mixed with 2×Laemmli loading buffer (Bio-Rad Laboratories, Inc., Hercules, CA) containing 5% of β-mercaptoethanol and heated at 98°C for 5

minutes. After heat treatment, the samples were centrifuged at 21,000 ×g for 5 min. The samples were loaded into wells of a 4-20% Mini-Protein® Precast gel (Bio-Rad Laboratories, Inc., Hercules, CA) and electrophoresis was run in the Tris/Glycine/SDS running buffer (Bio-Rad Laboratories) at 200 V for 30 min. Magic Mark™ XP Western Standard (Invitrogen, Carlsbad, CA) was used as a protein standard.

2.3.3 Immuno blotting and detection

PVDF nitrocellulose membrane was prewetted in methonal for 1 min and then soaked in a transfer buffer (24.8 mM Tris, 192 mM Glycine, and 20% v/v methanol). After SDS-PAGE electrophoresis, the gel was carefully removed from the cassette and embedded into a transfer cassette in the following order: a sponge, filter paper, 0.45 µm nitrocellulose membrane (Bio-Rad Laboratories), the gel, filter paper, and a sponge. Protein transferring was performed in the Tetra Cell (Bio-Rad Laboratories). The Tetra Cell was filled with a transfer buffer with an ice box to keep membrane transferring at low temperature. Transfer was conducted at 100 V for 1 hour. After washing the membrane with Tween-PBS (1x PBS, 0.05 % Tween-20) buffer twice for 5 minutes with shaking, the membrane was incubated in a blocking buffer (1x PBS, 0.05 % Tween-20, 5% non-fat milk) for 1 hour with shaking at room temperature. The membrane was incubated with primary antibodies (Table 3) in blocking buffer for 1 hour or overnight at 4^{o}C with shaking. After three times washing with Tween-PBS buffer, the membrane was incubated with corresponding secondary antibodies (Table 3) conjugated to horseradish peroxidase for one hour with shaking, followed by wash three times. Lastly, the membrane was incubated for 1 minute in a Super Signal West Substrate Working Solution (Thermo Scientific Inc., Rockford,

IL). Protein expression was detected using a Molecular Imager ChemiDoc XRS System (Bio-Rad Laboratories) and PDQuest Analysis software from Bio-Rad Laboratories, Inc.

Table 3 Primary and secondary antibodies used for Western blotting analysis

Primary antibody	company	ratio	Secondary antibody	company	ratio
rabbit anti-vinculin	Santa Cruz Biotechnology Inc., Santa Cruz, CA	1:200	anti-rabbit IgG HRP	Sigma, St. Louis, MO	1:1000
Rabbit anti-α-actinin	Sigma, St. Louis, MO	1:1000			
rabbit anti-zyxin	Sigma, St. Louis, MO	1:1000			
Rabbit anti-human β-catenin	Sigma, St. Louis, MO	1:2000			

2.4 Integrin blocking assay

In order to examine the involvement of intgrins in cell attachment in the synthetic peptide surface (**50**), IMR90 cells were detached by dispase treatment followed by gentle scraping. Collected cells were washed by CMRL-BSA medium containing L-glutamine, pyruvate, 0.35% BSA, CMRL 1066 (Mediatech, Inc. Manassas, VA). Approximately 70,000 cells were incubated in the presence of or absence of anti- human integrin antibodies in 1 ml CMRL-BSA medium. A

total of six samples were carried out. They were control (without antibody), anti-α_5, anti-β_1, anti-α_6, anti-$\alpha_V\beta_5$, and anti- all the 4 antibodies (total). 10 µg of 1mg/ml integrin antibodies (EMD Millipore Billerica, MA) were added to each sample. For the total of 4 antibodies mixture, 10 µg of each type of anti-integrin antibodies were used. The cells were then seeded to the wells of Synthemax plate and cultured for 1 h at 37^{o}C in cell culture CO_2 incubator. Cells were also seeded to Matrigel-coated plate for comparison. After incubation, cells were washed 3 times by CMRL-BSA medium, followed by fixation using 100% ethanol (0.5 ml/well) for 5 minutes. Cells were then stained by 0.4% crystal violet in methanol (0.5 ml/well) for 5 minutes and washed by deionized H_2O at least 5 times. At least 7 regions were randomly selected and colony numbers were counted under a microscopy using the 10x objective lens. Images were taken by an inverted phase contrast fluorescence microscope Olympus IX71 equipped with a highly sensitive CCD camera and Slidebook imaging analysis software 4.2 (Olympus Imaging America Inc., Center Valley, PA). The experiments were repeated at least three times independently.

2.5 Definitive endoderm differentiation from human iPS cell

Differentiation of IMR90 cells into definitive endoderm (DE) was conducted as described in our previous work (**51**). Briefly, cells were seeded onto Synthemax plate and cultured in the mTeSR1 medium. Cells were fed with differentiation medium when cells reached 40-50% confluence. DE medium contains of RPMI1640, nonessential amino acids, sodium pyruvate, B27 (Invitrogen), 1mM sodium-butyrate (Sigma), and 4 nM activin A. After 24 hour of differentiation, sodium butyrate concentration was reduced to 0.5mM in the differentiation medium. The medium was exchanged every other day until day 7 post differentiation.

2.6 Quantitative real time–polymerase chain reaction (qRT-PCR) analysis

To detect the expression of two DE marker genes, SOX17 and FOXA2, in DE tissue differentiated from iPS cells, total RNA were extracted from the cells using a RNA extraction kit RNeasy Plus Mini from QIAGEN (Valencia, CA). TaqMan qRT-PCR was performed using QuantiTecT Muptiplex RT-PCR NR Kit (Qiagen) according to the manufacturer's protocol. The cyclophilin (Applied Biosystems, University Park, IL), a human housekeeping gene, was served as endogenous control for normalization. RNA from adult human pancreata (Stratagene, La Jolla, CA) was used for comparison and normalization to detect relative mRNA expression level of DE cells. No reverse transcription control, and no template control samples were also performed to ensure the absence of genomic DNA amplification in the qRT-PCR assay and no false positive signal produced in the detection and analysis. The primer-probe pairs (**52**) were used as below:

Sox17 forward (5' to 3'): CAGCGAATCCAGACCTGCAGACCTGCA,

Sox17 reverse (5' to 3'): GTCAGCGCCTTCCACGACT,

Sox17-probe (5'FAM to 3'-Tam): ACGCCGAGGGCTACTCCTCC

Foxa2 forward (5' to 3'): CCGACTGGAGCAGCTACTATG,

Foxa2 reverse (5' to 3'): TACGTGTTCATGCCGTTCAT,

Foxa2- probe (5'FAM to 3'-Tam): CAGAGCCCTCGGCACTGCC

2.7 Statistical analyses

Data were presented as mean ± standard deviation. The statistical analysis was performed based on the Student's *t*-test using a one-tailed algorithm. The significance was determined at p 0.05.

CHAPTER 3 RESULTS AND DISCUSSION

3.1 Characterization of iPS cells attachment and proliferation on synthetic peptide surface

To evaluate the attachment and proliferation of iPS cells on synthetic peptide surface**,** iPS cells IMR90 were seeded on the Synthemax plate. Cells grown on a Matrigel coated conventional tissue culture plate served as a control for comparison. We observed that cell attachment time is different between the two types of surfaces. The cells on Synthemax surface need more time to attach to the surface. After culture for 2 days, colony morphology of cells on synthetic peptide surface were more round compared with that on Matrigel coated surface (Fig.1). Also the colonies grown on Synthemax plate are smaller throughout the culture.

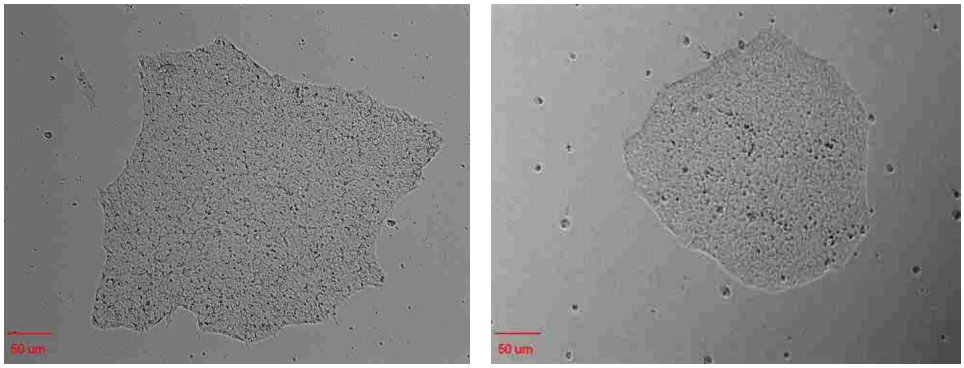


Figure1. IMR90 cell colony grown on Matrigel (MG) and Synthemax surface (SM).

Fig.2 shows a typical time course of iPS cell proliferation on both Matrigel- and synthetic peptide-coated surfaces. In these experiments, approximately $5x10^4$ cell/cm^2 IMR90 cells were seeded into a well of Synthemax surface modified six-well plate. The same number of cells were used to seed to a Matrigel coated six-well plate. As shown in Fig. 2, the kinetics of cell growth

indicated that cells grown on Synthemax are equivalent to that on Matrigel. Equation 1 was used to calculate the doubling time:

$$X=X_0e^{\mu t} \quad (1)$$

Where X is the amount of cells; X_0 is the amount of cells at time 0; μ is the specific growth rate; t is the culture time.

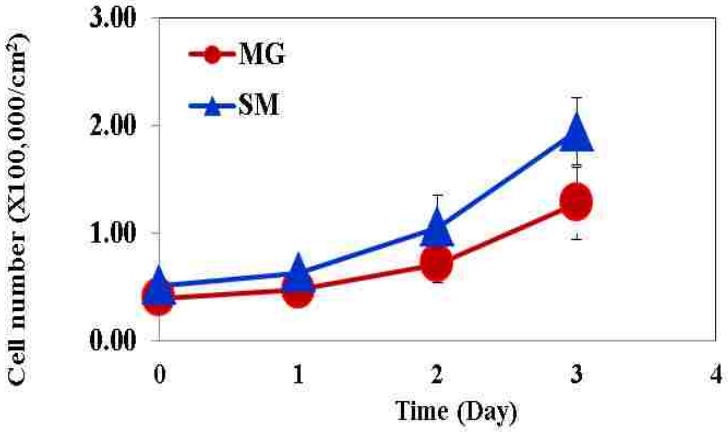
$$Ln(X) =Ln(X_0) +\mu t \quad (2)$$

When $X= 2X_0$,

So, doubling time= $Ln2/\mu$ (3)

By counting cell number X and X_0, μ can be obtained from the slope of the linear equation. After substituting μ to the equation, doubling times can be calculated by Equation 3. Accordingly, the doubling times of cells on Synthemax and Matrigel coated plate are 44.05±1.45 hours and 42.98±7.86 hours, respectively. The results suggested that there is no significant difference in specific growth rate when cells are grown on Synthemax and Matrigel surfaces. However, we observed that the colonies on Matrigel are bigger than on Synthemax. This was verified with immunofluorescence staining results described in Fig. 11 &12.

A



B

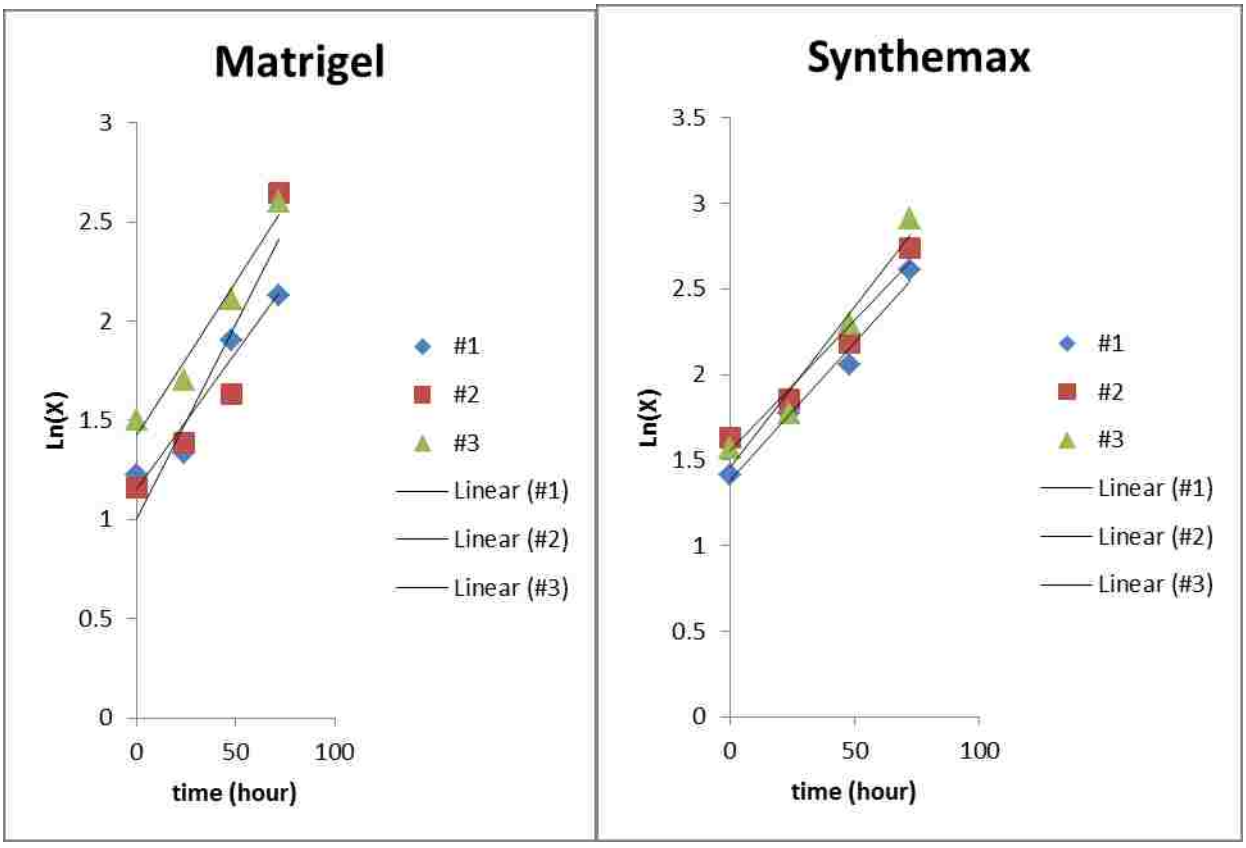


Figure 2. (A) Growth curve of iPS cells on Synthemax and Matrigel-coated plates. (B) Estimation of the specific growth rate μ. Three independent experiments were conducted to calculate the slope μ.

In another experiment, we tested cell proliferation capacity on Synthemax surface. As shown in Fig. 3, after culture cells on the Synthemax for more than three days, cells reached exponential proliferation phase with approximately 14 million cells at day 6 in a well of six-well plate. No differentiated cell colonies were found. It should be pointed out that cells expanded on Matrigel-coated surface have to be subcultured on day 3~4 after plating, as relative larger colonies formed on the Matrigel coated surface on day 3~4 and colony-colony merge should be avoided in order to prevent cells from spontaneous differentiation. While cell colonies are smaller on Synthemax surface, which allows expansion of iPS cells for longer time before subculture. For this reason, we were only able to examine Matrigel-coated surface culture by 4 days of culture. The result obtained from Fig. 3 suggests that the productivity of iPS cell expansion is actually much higher than that on Matrigel-coated surface.

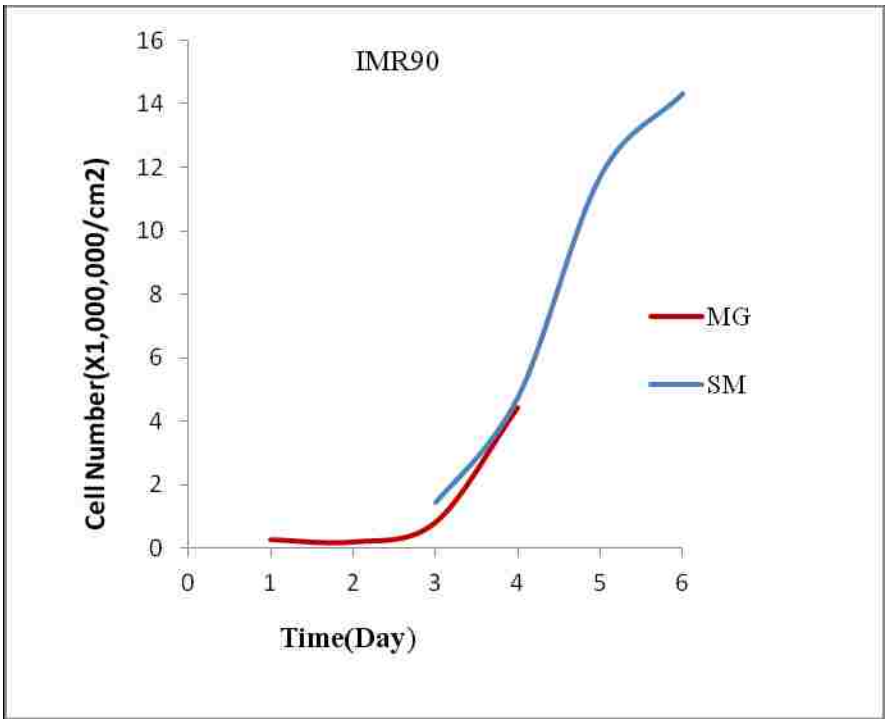


Figure 3. Capability of iPS cell growth on synthetic peptide surface.

Furthermore, in order to characterize how many passages can the synthetic peptide surface support iPS cell self-renewal under undifferentiated state, we detected the expression of pluripotency markers after 10 consecutive passages. High expression of stem cell specific markers OCT4 and SSEA4 could be detected after 10 consecutive passages (Fig.4), suggesting the cells maintained in undifferentiated state during passages. However, after 13 passages, some spontaneous differentiation of iPS cells was observed (Fig.5). From these, we could see Synthemax plate could maintain iPS cells in undifferentiated state over multiple passages.

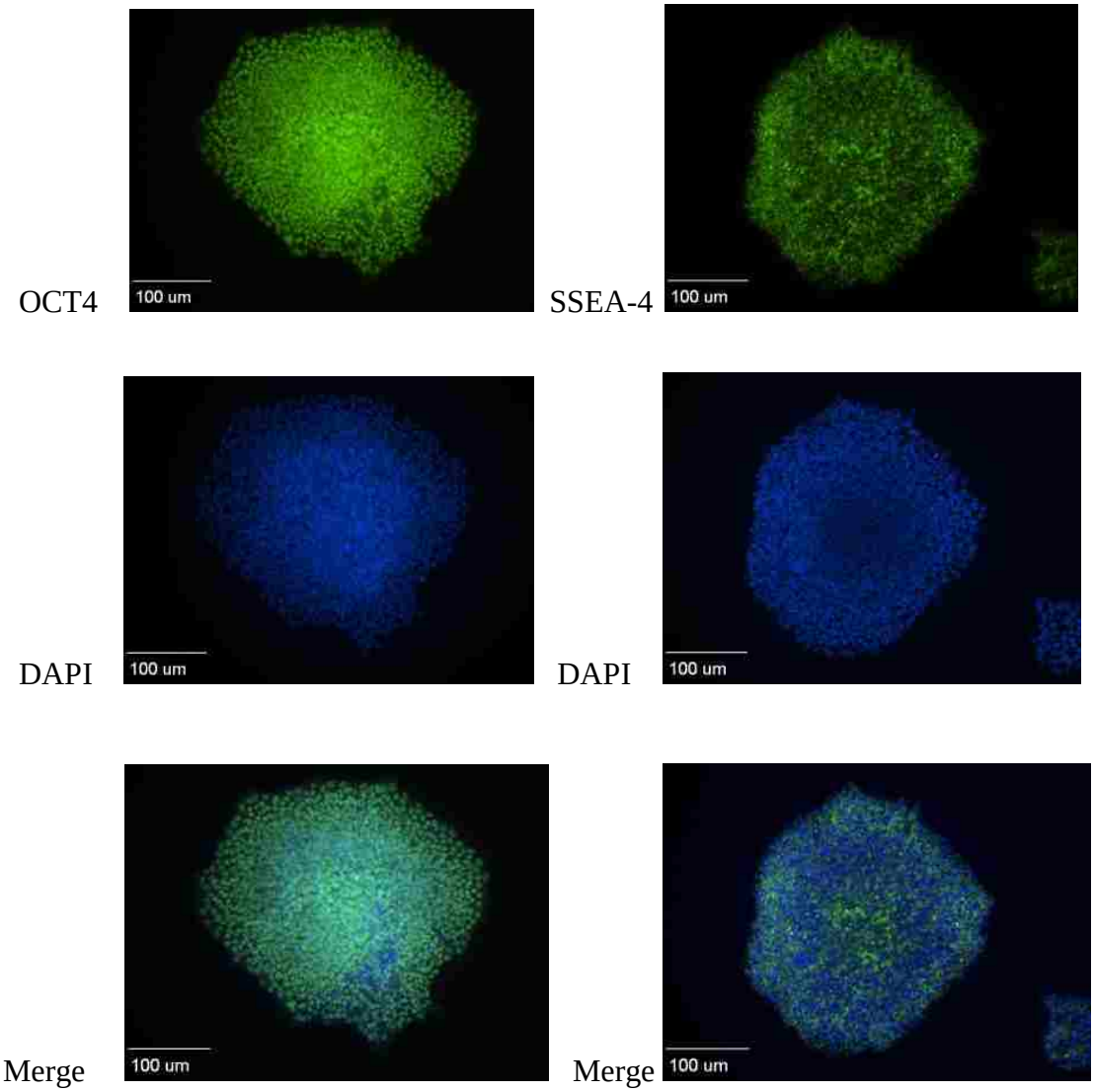


Figure 4. Fluorescence microscopy images of anti-OCT4 and anti-SSEA4 labeled iPS cells. IMR90 were maintained in undifferentiated state on Synthemax surface for 10 passages. Scale bar: 100 µm. Mouse anti-human OCT4 (1:100) and mouse anti-human SSEA4 (1:100) were used as primary antibodies. Goat anti-mouse Alexa Fluro 488 IgG1 (1:200) and goat anti-mouse Alexa (Alexa Fluro 488 IgG3 (1:200) were used as secondary antibodies.

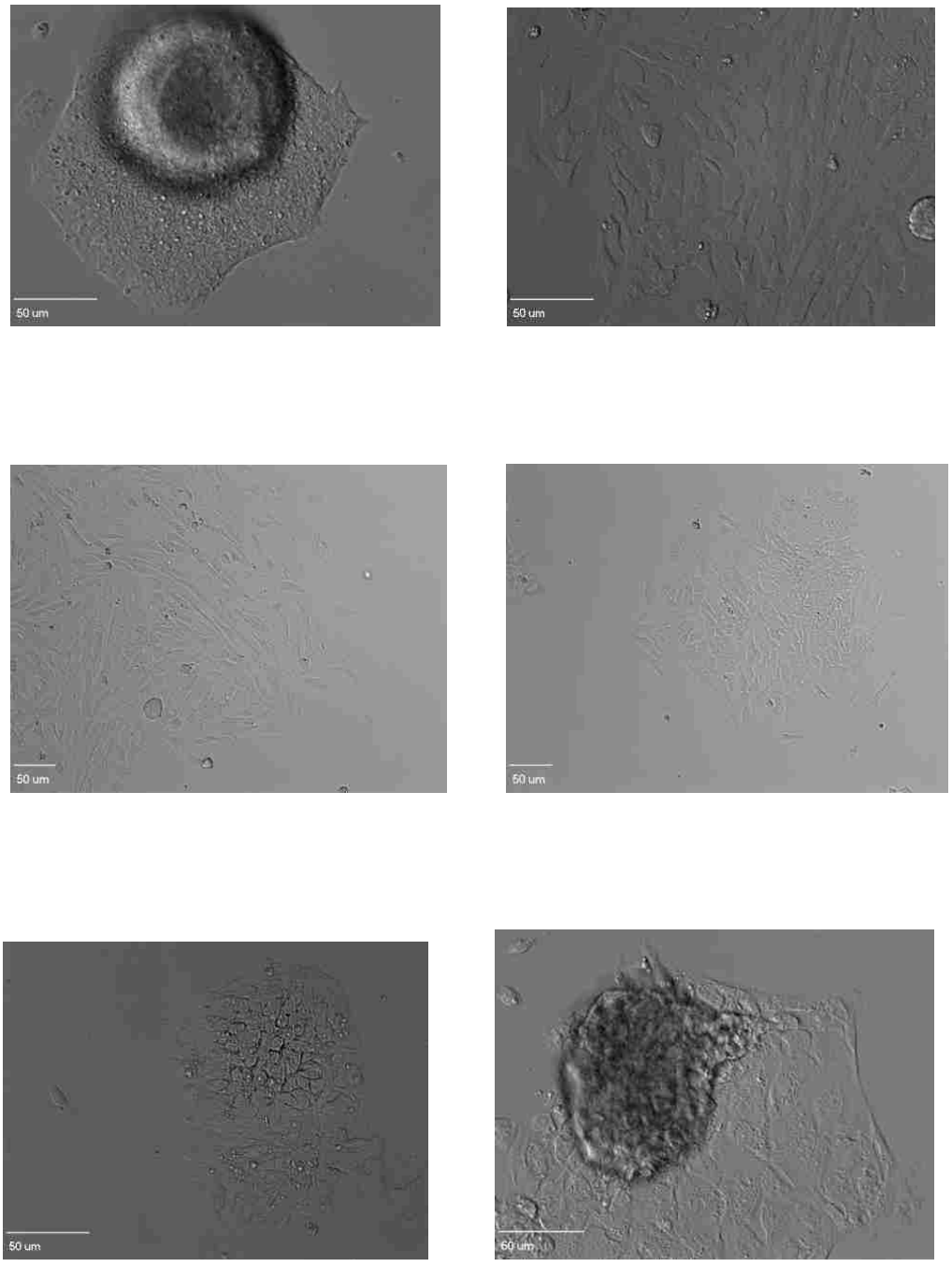


Figure 5. Distinct differentiated colonies grown on Synthemax plate after 13 passages under bright field. Scale bar: 50 µm.

3.2 Differentiation on Synthemax plate

We next investigated whether iPS cells retain their ability to differentiate into a specific lineage such as a DE (Definitive endoderm) lineage. The differentiation of DE lineage is the most critical first step in hESC pancreatic differentiation (**53**). Thus, demonstration of iPSC directed DE differentiation on peptide surface could help develop a xeno-free differentiation system to generate transplantable β-cells from iPS cells for diabetes therapy**.** Synthemax plate has been proved to allow differentiation of human embryonic stem cells into three germ layers (**45**). Here, we examined DE differentiation of iPS cells on the Synthemax surface as we mentioned in the Introduction that hES cells and iPS cells are identical on many aspects but not the same. As shown in Fig. 6A, the DE morphology was observed after day 5 post induction of the differentiation. DE marker genes, Sox17 and Foxa2 in cells differentiated on both synthetic peptide- and Matrigel-coated surfaces after 6 days' differentiation were analyzed by Taqman qRT-PCR analysis. No expression of Foxa2 and Sox 17 could be detected in undifferentiated IMR90 cells on both plates (Fig. 6B & C). Both Foxa2 and Sox 17 expressed at similar levels and no significant difference could be observed between the differentiations on the two types of surfaces. This experimental result indicates that Synthemax surface is as good as Matrigel coated surface for induced differentiation of iPS cells into DE lineage. Further confirmation by immunofluorescence (Fig. 6D) shows the expression of FOXA2 and SOX17 in differentiated iPS cells on Sythemax. Our results indicate that the Synthemax Surface provides the appropriate niche environment that supports both the expansion and the directed differentiation of hiPSCs.

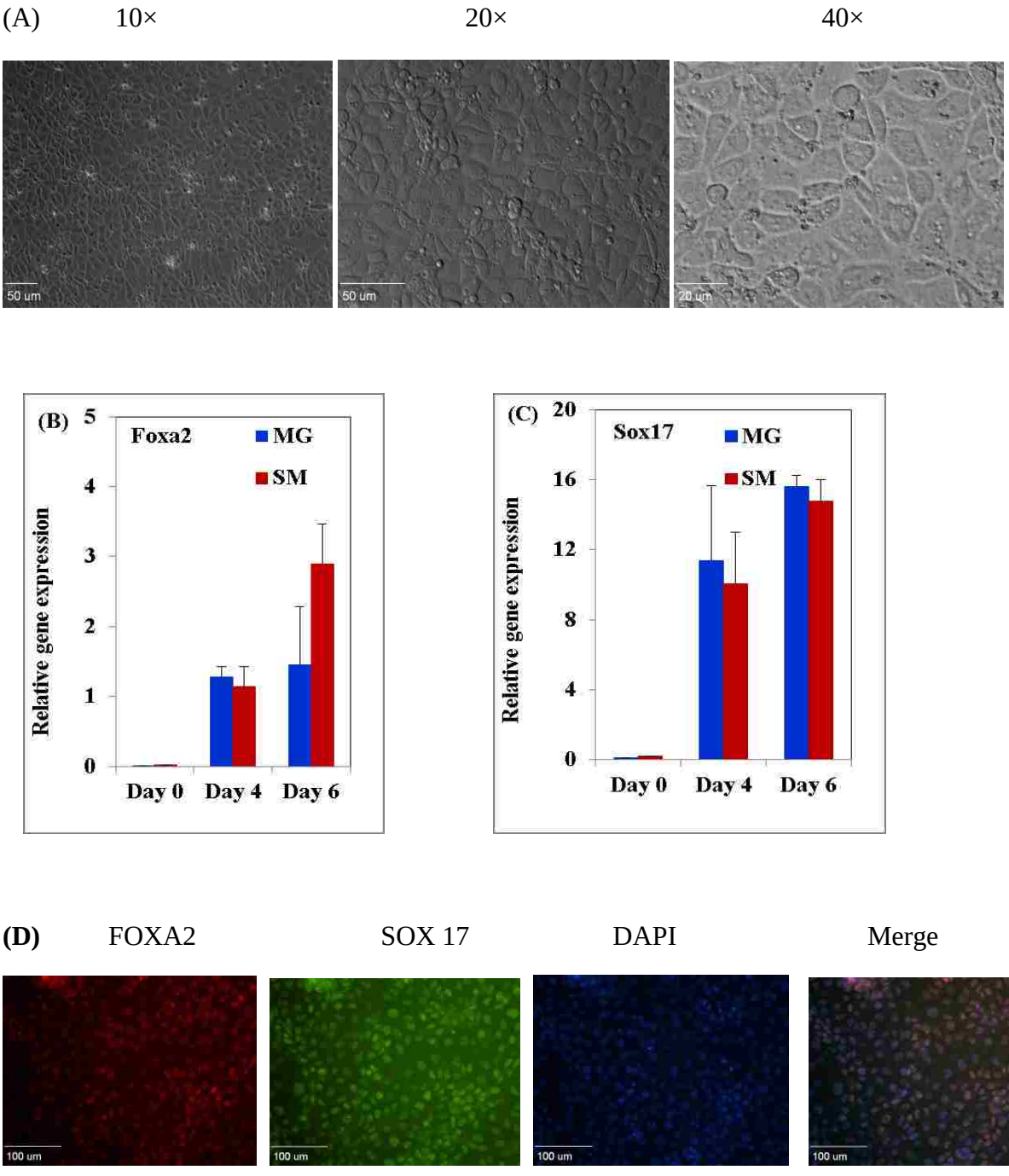


Figure 6. Definitive endoderm (DE) marker gene and protein expression in IMR90 cells differentiated on MG and SM. (A) Morphology of DE after 6 days of differentiation. (B) Foxa2, and (C) Sox17 mRNA expression detected by qRT-PCR. Data were presented as mean ±SD. (D) Immunofluorescence detection of DE marker. Mouse monoclonal anti-human SOX17 (1:50) and goat anti-mouse IgG FITC (1:100) were used as primary and secondary antibodies for SOX17. Rabbit monoclonal anti-human FOXA2 (1:1000) and donkey anti-rabbit IgG TRITC (1:100) were used as primary and secondary antibodies for FOXA2.

3.3 Functional role of integrins in iPS cell attachment

Synthemax Surface is made of the VN-PAS surface as mentioned in the Introduction. It is reported that $\alpha_V\beta_5$ integrin mediated adhesion to vitronectin (VN), so we conducted integrin inhibition assay by blocking various integrins with anti-integrin antibodies before seeding the cells to confirm $\alpha_V\beta_5$ mediated adhesion on Synthemax plate. Seeding cells on Matrigel coated plates was used as control. As a result, blocking of integrin $\alpha_V\beta_5$ shows 93% inhibition of the attachment on the Synthemax plate (Fig. 7B), but on Matrigel coated plates no significant reduction of attachment could be detected. In addition, the blocking of integrins α_5, α_6, and β_1 only reduced the cell adhesion to the Synthemax plate by 20, 6, and 11%, respectively. The four integrins antibodies together nearly completely abolished the attachment of iPS cells to the Synthemax Surface. According to the mechanism study on the cell-matrix interaction shown in Fig. 7B, only one integrin is available for cell adhesion and spreading if an iPS cell interacts with Synthemax surface. This is because the synthetic peptide surface was made by single peptide sequence which was derived from vitronectin. Therefore, only integrin $\alpha_V\beta_5$ expressed from a cell can bind to the peptide sequence. These results are consistent with our hypothesis that recognition of recombinant vitronectin protein by integrin $\alpha_V\beta_5$. By contrast, the cell seeded on Matrigel coated plates has much less affection by integrin blocking. Among the integrins α_5, α_6, β_1 and $\alpha_V\beta_5$, integrin β_1 blocking has the biggest reduction which is about 40%, meaning the role of β_1 is more important for the attachment to Matrigel. This result is consistent with the report that integrin β_1 is required for hiPSCs adhesion and proliferation on Matrigel-coated surfaces (**47**). The combination of antibodies against integrins α_5, α_6, β_1 and $\alpha_V\beta_5$ resulted in a 62% reduction of cell adhesion to the Matrigel surface. These results suggest that multiple integrins are involved in mediating hiPSCs adhesion to the Matrigel surface.

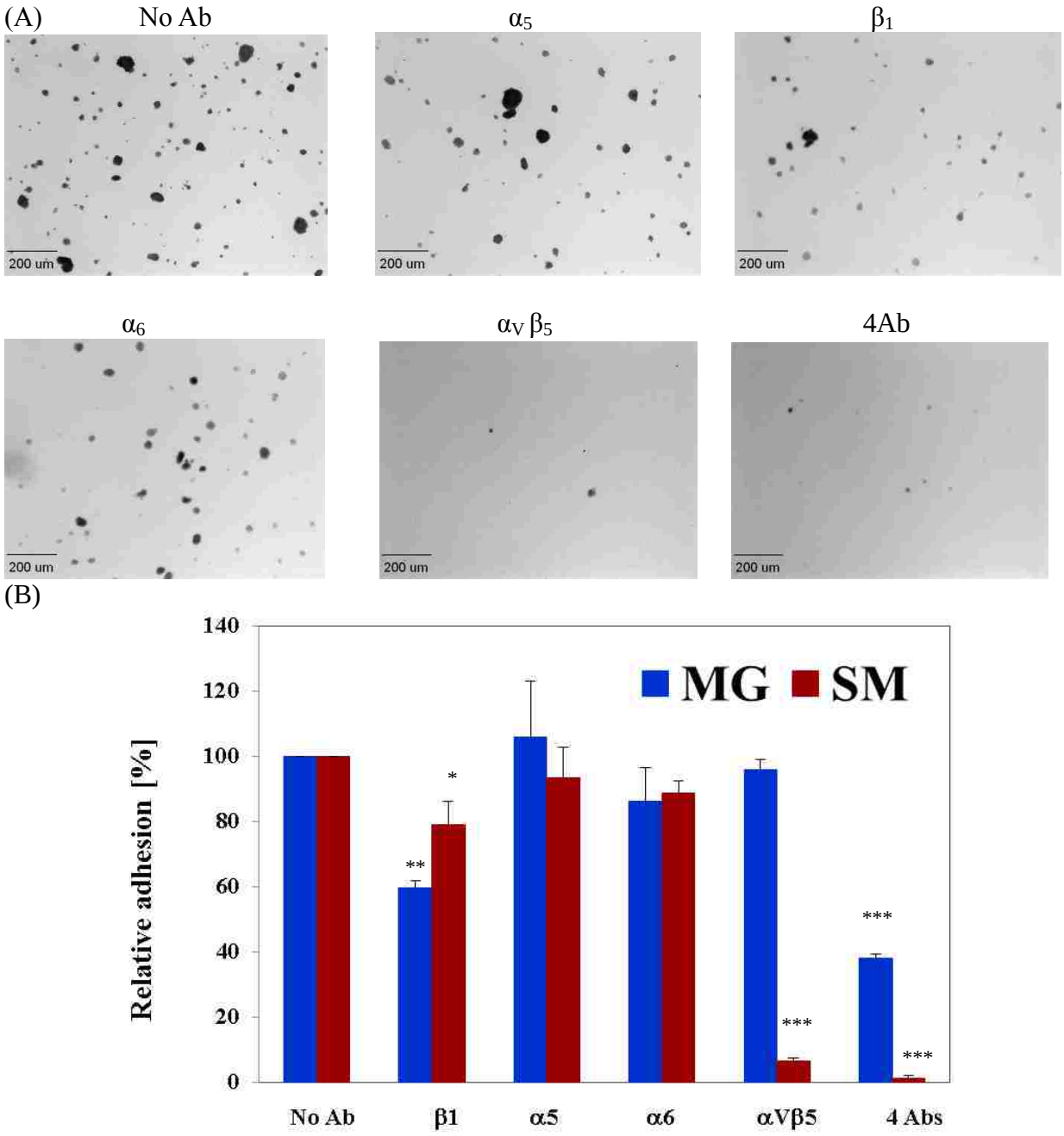


Figure 7. The role of integrins in promoting iPS cell adhesion to MG and SM substrates. (A) Micrographic images of cell attachment on SM surface without and with integrin antibodies blocking. Scale bar: 50 mm. (B) Relative iPSC attachment on MG and SM. Data are presented as the mean ± SD (n=14). *: p=0.037; **: p=0.0059; ***: p<0.0001. Symbols: Ab, antibody; MG, Matrigel surface; SM, Synthemax surface. Dilution of antibodies: 1:40

3.4 Wnt pathway

As described in the Introduction, Wnt pathway plays important roles in hES/iPS cell expansion and differentiation. In order to investigate whether or not this important signaling pathway was indeed affected by the substrates of surface matrix, we detected the nuclear translocation of β-catenin in iPSCs grown on SM surface and compared to MG-coated surface. Proteins in the cytoplasm and in the nucleus were extracted separately. β-actin was used as an internal control for the western blot assay. As revealed in Fig 8, less β-catenin was translocated from cytoplasm to nucleus when cells were grown on SM compared with that on MG. The translocation of less β-catenin to the nucleus suggested less activation of β-catenin-mediated Wnt signaling pathway in iPS cells grown on SM. The experimental results indicated that the lack of multiple integrins for iPSCs attachment and proliferation may lead to the down-regulation of Wnt signaling and thus support iPSCs proliferation in limited period of time as discussed above shown in Fig. 3, 4, and 5.

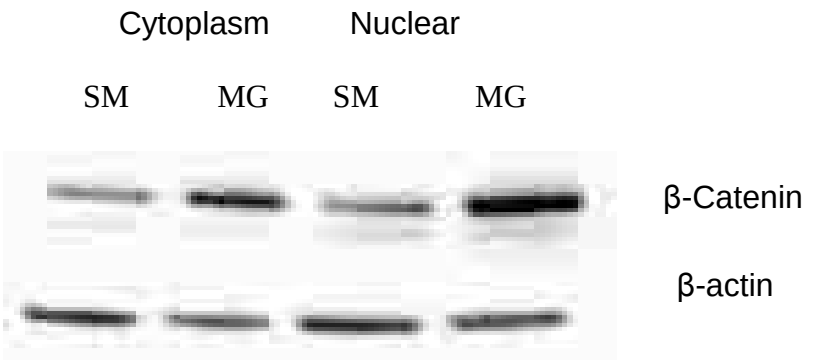


Figure 8. Cytoplasm and nuclear β-catenin expression in iPSCs grown on Matrigel (MG) and Synthemax (SM) surfaces. Cytoplasm and nuclear proteins were extracted separately for Western blot analysis. Results shown are western blotting analysis from two independent experiments. Antibodies: Rabbit anti-human β-catenin (1:2000), anti-rabbit IgG HRP (1:1000).

3.5 Organization of the cytoskeleton structures

Cytoskeleton plays an important role in integrin related signaling transduction pathways (**54, 55**). The cytoplasmic domains of integrins bind to the cytoskeleton through adapter proteins like vinculin, α-actinin, and phosphorylated-focal adhesion kinase (p-FAK). In order to investigate how the substrate affects the organization of the cytoskeleton structures, we examined the expression of cytoskeleton related proteins such as actin filaments (F-actin) and vinculin during iPS cell proliferation on the Synthemax substrate and compared to the Matrigel-coated surface. Due to phalloidin binds specifically at the interface between F-actin subunits we investigated F-actin polymerization in iPS cells grown on the Synthemax substrate immune-stained with phalloidin at 48 hour after culture. As shown in Fig. 9, the actin filament network of cells on Synthemax surface is much different from that on Matrigel–coated surface. Cells grown on synthetic peptide surface showed the accumulation of denser and broader actin filaments between the cell-cell interfaces. Vinculin is another cytoskeletal protein that is involved in linkage of the cytoplasm to the focal adhesions. Expression of vinculin on both types of substrates was shown in the Fig.10A & B. Western blot analysis revealed low level of vinculin expression in cells grown on Synthemax surface (Fig. 13A).

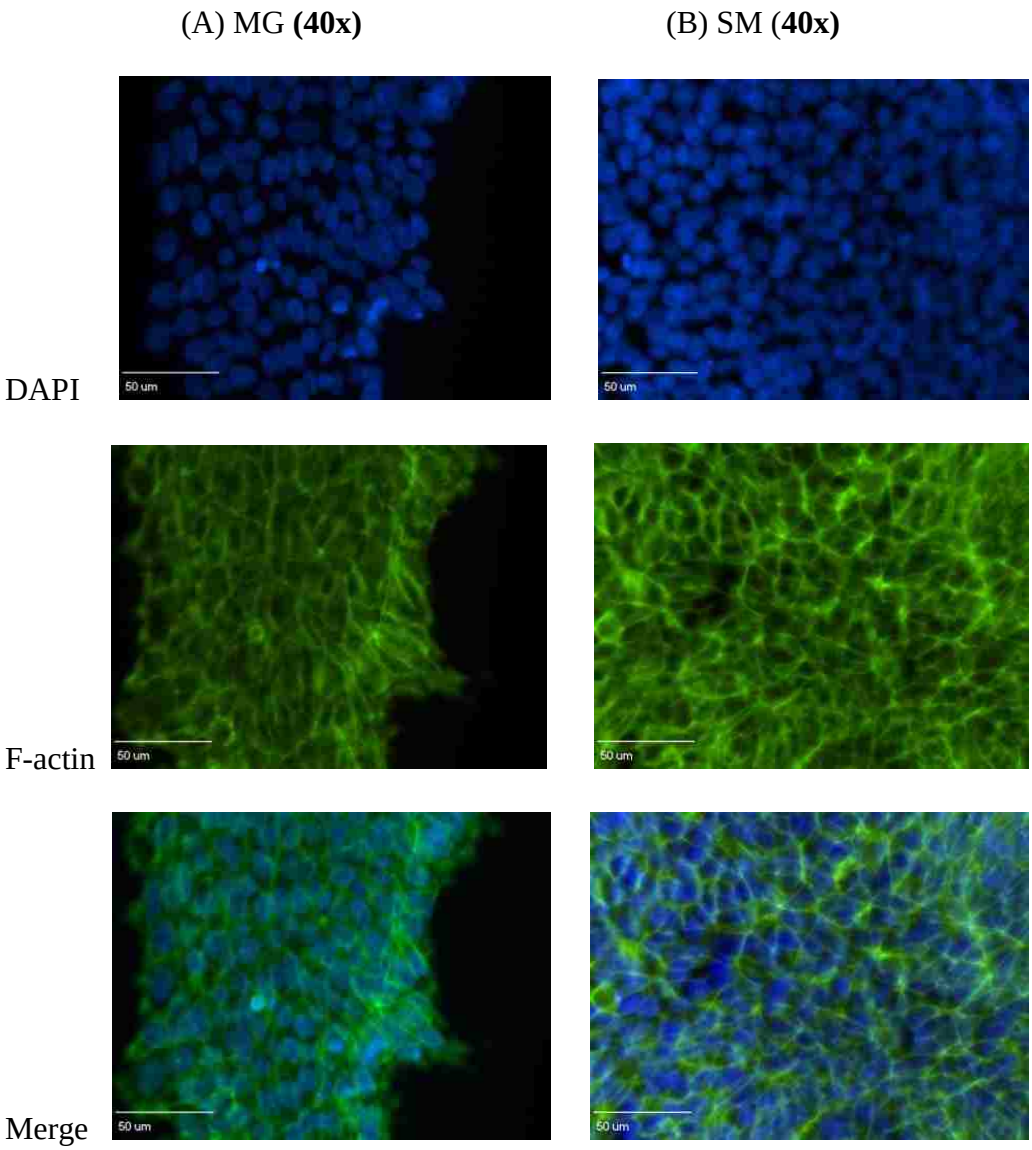


Figure 9. Micrographic images of F-actin expression in cells grown on MG (A) and SM (B) surface. Scale bar: 50µm. Magnification: 40×. Antibody: Alexa Fluor 488 phalloidin (1:40).

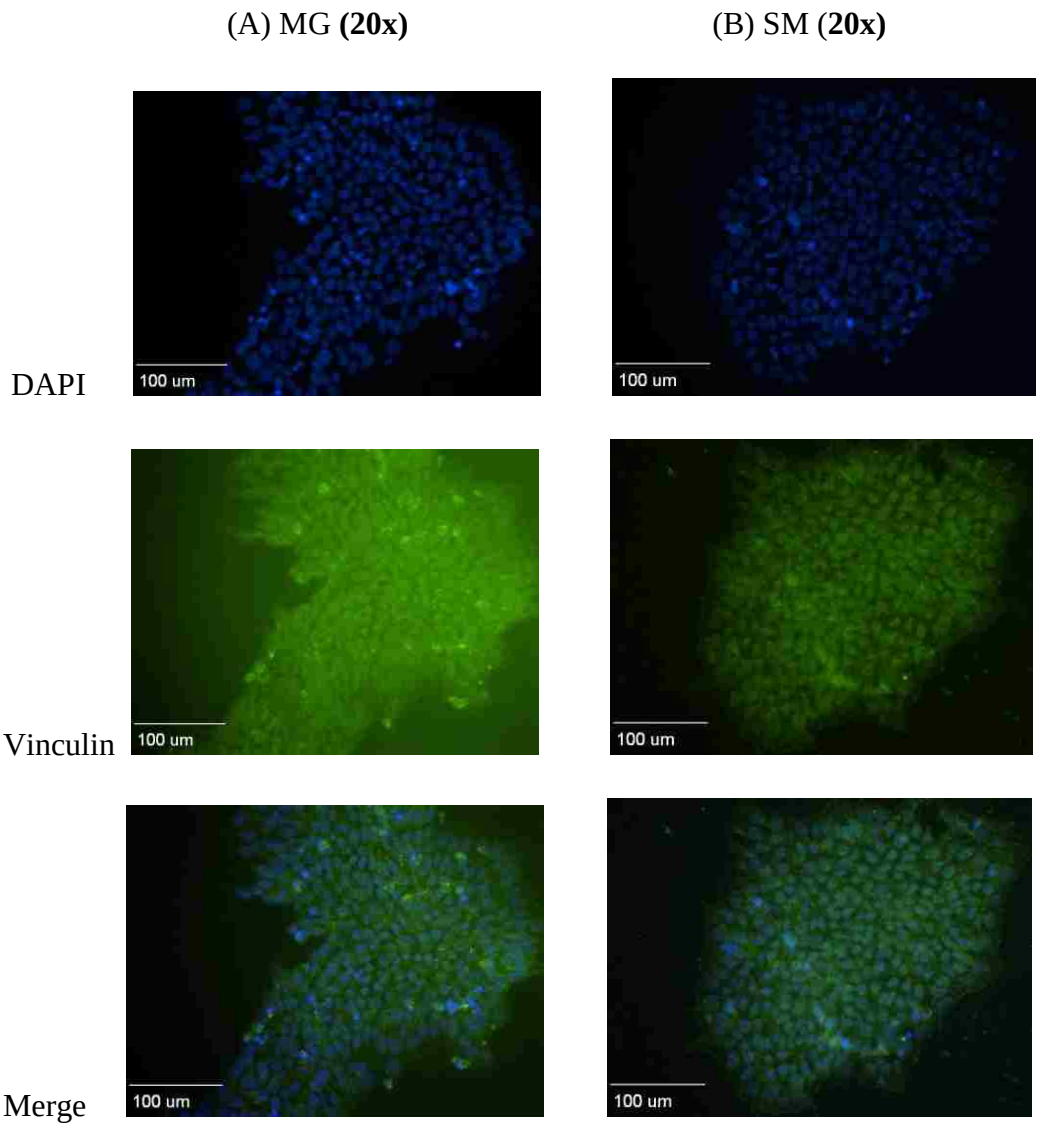


Figure 10. Micrographic images of vinculin expression in cells grown on MG (A) and SM (B) surface. Scale bar: 100µm. Magnification: 20×. Rabbit anti human vinculin (1:50) and mouse anti-rabbit IgG –FITC (1:150) were used as primary and secondary antibodies.

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In addition, we found that there is a higher expression of zyxin protein in cells grown on Synthemax surface (Fig. 11). Western blot assay revealed a significant up-regulation of zyxin in cells grown on the Synthemax surface (Fig. 13C). Zyxin is a zinc-binding phosphoprotein that concentrates at focal adhesions and along the actin cytoskeleton. Since zyxin is directly involved in cell spreading and proliferation and is inversely correlated to differentiation (**56**), the up-regulation may contribute to cell attachment and proliferation on the Synthemax surface. Moreover, we examined α-actinin expressions in cells grown on the Synthemax and Matrigel substrates (Fig.12) and we observed nearly similar level of α-actinin expressions on both types of surfaces. In the attempt of studying role of p-FAK on iPS cell-synthetic peptide surface interaction, significant difference of p-FAK expression were not be detected between the cells cultured on Matrigel and Synthemax surfaces (Fig. 14). While the mechanism of these changes in cell cytoskeletal proteins is unclear, it may indicate a reorganization of cellular molecules and focal adhesions, which facilitates the spreading and self-renewal of iPS cells on substrates, such as peptide surface used in this work.

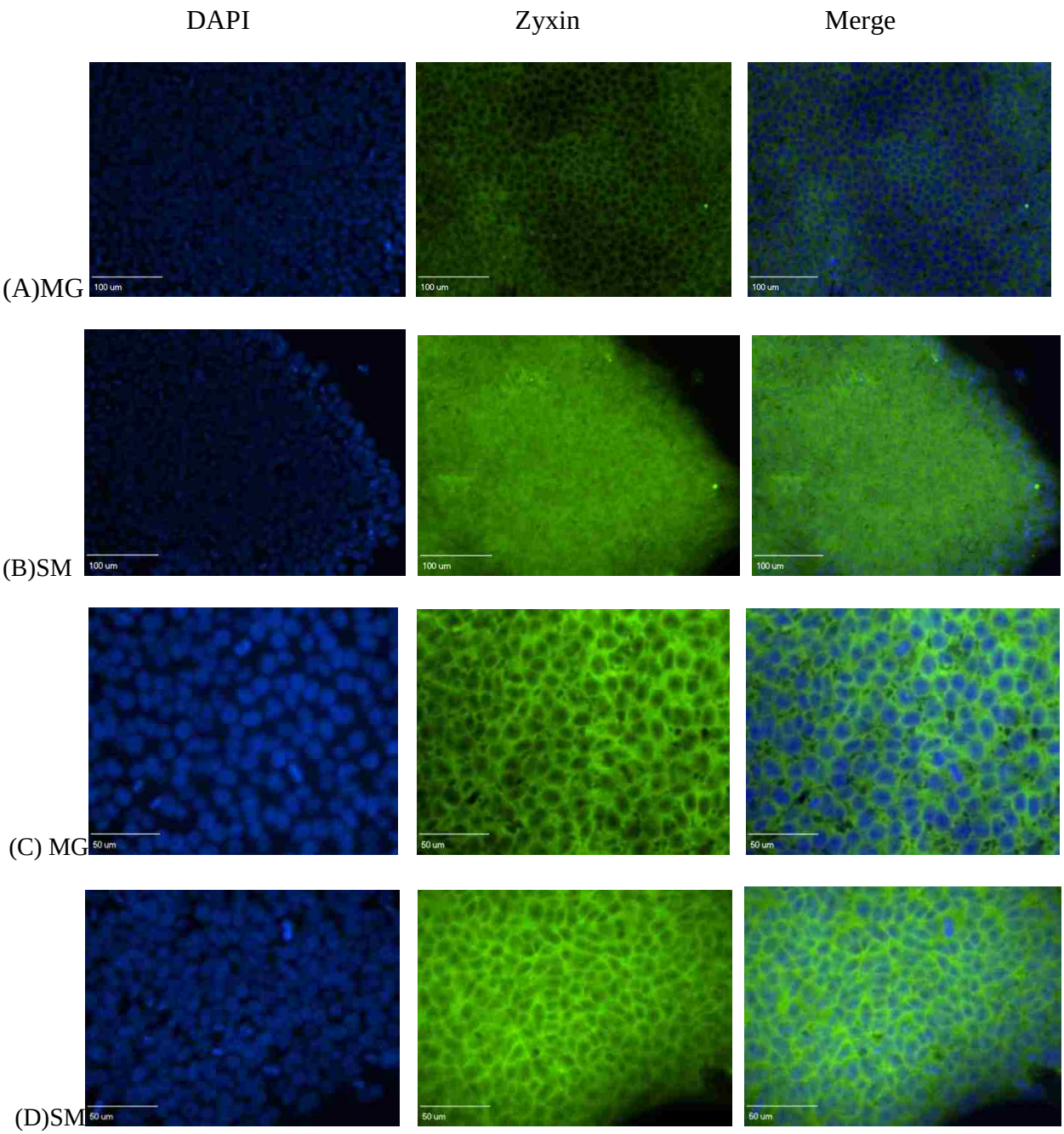

Figure 11.Micrographic images of Zyxin expression in cells grown on MG (A&C) and SM (B&D) surface. Scale bar: 100µm for A&B; 50µm for C&D. Magnification: 20× for A&B; 40× for C&D. Rabbit anti human zyxin (1:100) and mouse anti-rabbit IgG –FITC (1:150) were used as primary and secondary antibodies.

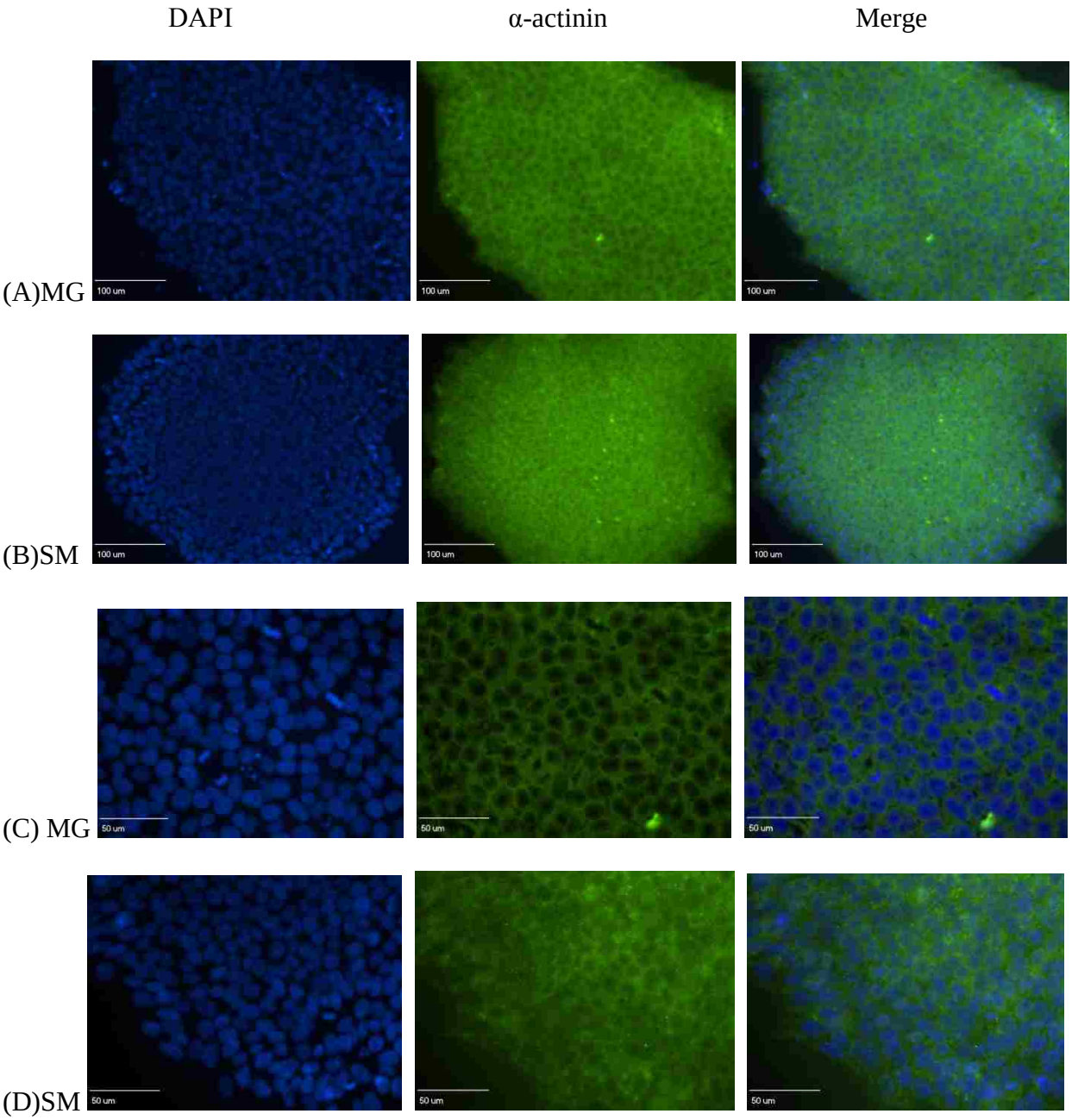

Figure 12.Micrographic images of α-actinin expression in cells grown on MG (A&C) and SM (B&D) surface. Scale bar: 100μm for A&B; 50μm for C&D. Magnification: 20× for A&B; 40× for C&D. Rabbit anti human α-actinin (1:100) was used as primary antibodies and mouse anti-rabbit IgG –FITC (1:150) as secondary antibodies.

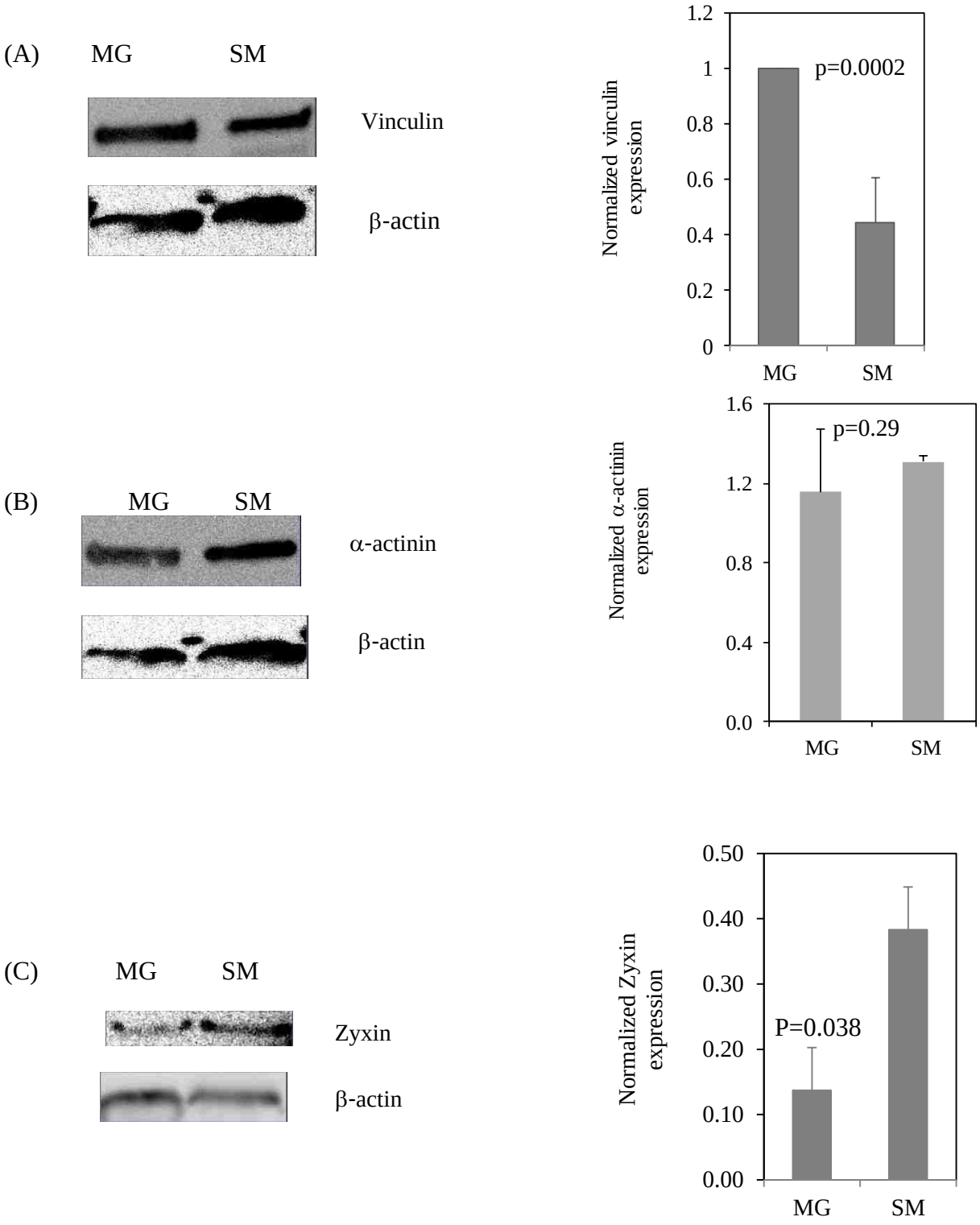

Figure 13. Cytoskeletal protein expression in iPS cells grown on Matrigel (MG) and Synthemax (SM) surfaces. Cells were harvested at 48 h post seeding and total proteins were extracted for Western blot analysis. (A-C) Vinculin, α-actinin, and zyxin expression detected by Western blot

analysis and relative protein expression using β-actin as a loading control respectively. Semi-quantification of protein expression was performed by Kodak 1D gel imaging software. At least three independent experiments were performed and data were presented as mean ± SD. Rabbit anti-human vinculin (1:200), rabbit anti-human α-actinin (1:1000), rabbit anti-human zyxin (1:1000) were used as primary antibodies, respectively. Mouse anti-rabbit IgG HRP (1:1000) was used as secondary antibodies. Bands shown are representative results from three experiments.

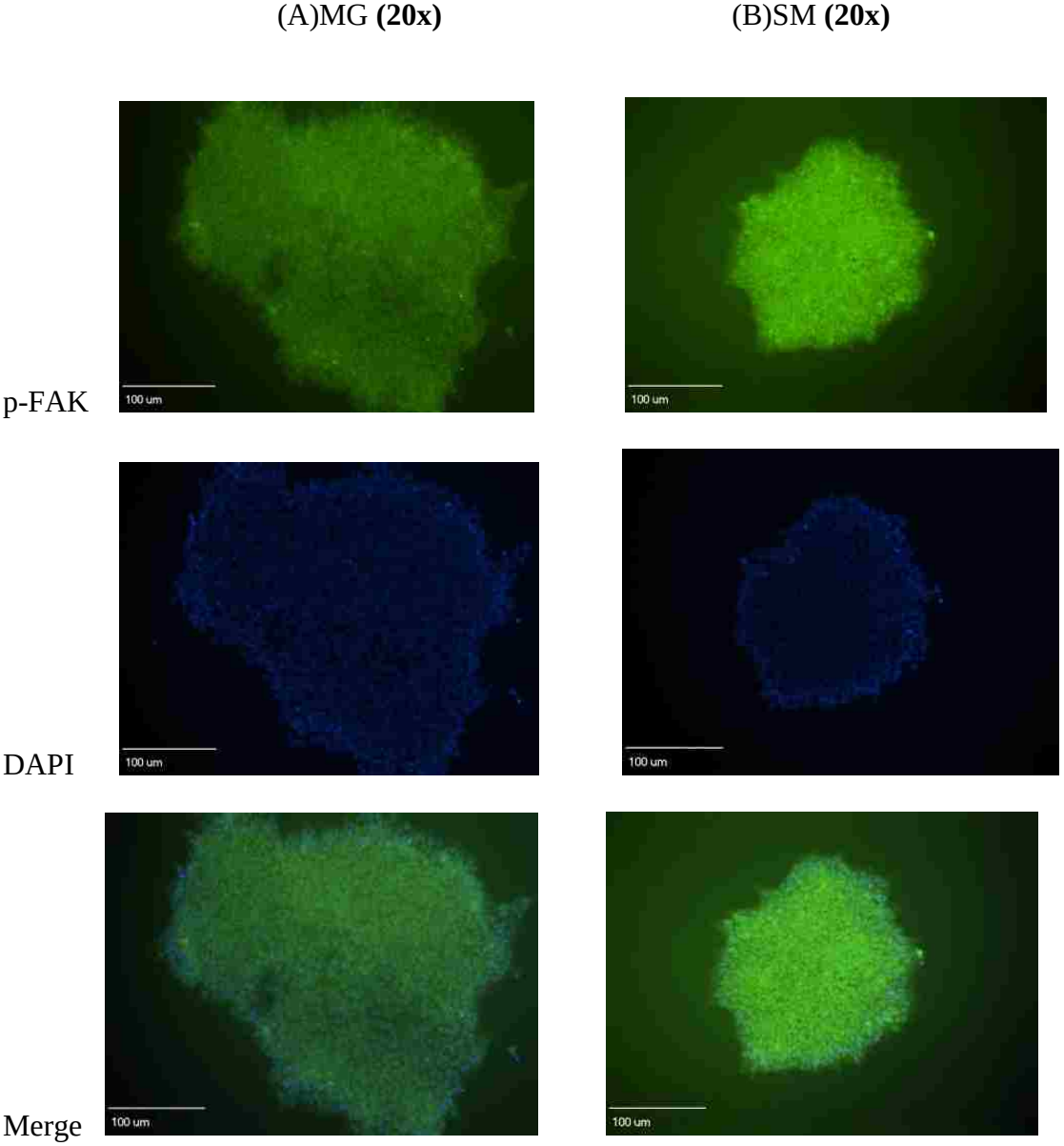

Figure 14.Micrographic images of p-FAK expression in cells grown on MG (A) and SM (B) surface. Scale bar: 100μm. Magnification: 20×. Rabbit anti-human p-FAK (1:50) and mouse anti-rabbit IgG –FITC (1:150) antibodies were used as primary and secondary antibodies.

CHAPTER 4 CONCULSION AND FUTURE WORKS

In this work, the attachment, proliferation, and induced differentiation of human iPS cells on Synthemax surface was evaluated and characterized. iPS cells colonies grown on Synthemax substrate exhibited less spreading and more compact morphology compared to colonies grown on Matrigel. We demonstrated that iPS cells retained stable proliferation and pluripotency marker protein expression after growing on the Synthemax substrate for ten consecutive passages. Further examination of cell-ECM interaction confirmed that iPS cells grown on the Synthemax surface primarily utilize $\alpha_V\beta_5$ integrin to mediate attachment to the substrate, since the Synthemax surface contains peptide sequences derived from vitronectin protein. Investigation of β-catenin revealed less activation of β-catenin-mediated Wnt signaling pathway on the Synthemax surface which might be the reason that the iPS cells colonies were more compact. The cytoskeleton characterization of iPS cells grown on the Synthemax surface revealed the formation of denser actin filaments in the cell-cell interface. The down-regulation of vinculin and up-regulation of zyxin expression were also observed in iPS cells grown on the Synthemax surface. Taken together, our experimental results suggest that Synthemax surface in combination with defined medium can provide a defined culture system for expansion of clinical grade human iPS cells for cell therapy applications.

In the future, further experiments such as teratomas forming from injection of long term cultured iPS cells on Synthemax surface to mice can be done to further confirm Synthemax surface performance on the maintenance of pluripotency of iPS cells. Importantly, as shown in Fig. 5,

we found that spontaneous differentiation of iPS cells became uncontrollable after more than 12 passaging on Synthemax surface. By contrast, iPS cells can be maintained in undifferentiated state for more than 40 passages on Matrigel-coated surface. The experimental result is consistence with other group's data (**45**). As Matrigel is a mixture of animal ECM proteins, it seems like multiple integrins expressed on the surface of iPS cell are able to bind to distinct ECM proteins of Matrigel, which provides to an iPS cell strong adhesion and spreading microenvironment. Therefore, as a future direction of developing chemically-defined synthetic peptide surface, multiple peptide sequences derived from various biological functional ECM proteins should be coated on a culture surface for long-term expansion and induced differentiation of hES/iPS cells. Moreover, to thoroughly investigate cellular cytoskeleton structure and reorganization on synthetic peptide substrate, 100x or 63x objective lens are essential for the study. Due to the pre-coating of the synthetic peptide on a six-well plate of Synthmax plate, we were unable to use a 100x objective lens to characterize the cytoskeleton structures. With the advent of self-coating peptide for HPSC self-renewal and induced differentiation, further investigation on the mechanism of cell-matrix interaction and cell fate affected by synthetic peptide substrates can be implemented.

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APPENDIX

5.1 Extraction of cytoplasmic and nuclear protein

1. Harvest with trypsin-EDTA and then centrifuge at 500 × *g* for 5 minutes
2. Wash cells by suspending the cell pellet with PBS.
3. Transfer 1-10 × 10^6 cells to a 1.5mL microcentrifuge tube and pellet by centrifugation at 500 × *g* for 2-3 minutes.
4. Use a pipette to carefully remove and discard the supernatant, leaving the cell pellet as dry as possible.
5. Add ice-cold CER I to the cell pellet (Table 1). Proceed to cytoplasmic and nuclear protein extraction, using the reagent volumes indicated in Table 1.

Table 1. Reagent volumes for different packed cell volumes.*

Packed Cell Volume (µL)	CER I (µL)	CER II (µL)	NER (µL)
10	100	5.5	50
20	200	11	100
50	500	27.5	250
100	1000	55	500

*For HeLa cells, 2 × 10^6 cells is equivalent to 20µL packed cell volume.

6. Vortex the tube vigorously on the highest setting for 15 seconds to fully suspend the cell pellet. Incubate the tube on ice for 10 minutes.
7. Add ice-cold CER II to the tube.
8. Vortex the tube for 5 seconds on the highest setting. Incubate tube on ice for 1 minute.
9. Vortex the tube for 5 seconds on the highest setting. Centrifuge the tube for 5 minutes at maximum speed in a microcentrifuge (~16,000 × *g*).
10. Immediately transfer the supernatant (cytoplasmic extract) to a clean pre-chilled tube.

11. Suspend the insoluble (pellet) fraction produced in Step 9, which contains nuclei, in ice-cold NER.
12. Vortex on the highest setting for 15 seconds. Place the sample on ice and continue vortexing for 15 seconds every 10 minutes, for a total of 40 minutes.
13. Centrifuge the tube at maximum speed (~16,000 × *g*) in a microcentrifuge for 10 minutes.
14. Immediately transfer the supernatant (nuclear extract) fraction to a clean pre-chilled tube.

5.2 Western blot

1. Cells were cultured for 48 hours on Matrigel coated plate and Synthemax plate and detached by Typsin EDTA
2. Cells were collected by centrifuged at 300 ×g for 10 min and washed by Dulbecco's Phosphate buffered Saline
3. All cells were lysed with lysis buffer by using a 1 ml syringe with 20G$1^{1}/_{2}$ needle up and down 20 times.
4. Cell lysates were centrifuged with 21,000 ×g at 4 ^{o}C for 15 min.
5. Equal amount of cellular protein with 2×Laemmli loading buffer containing 5% of β-mercaptoethanol were heated at 98°C for 5 minutes.
6. the samples were centrifuged at 21,000 ×g for 5 min. Proteins were loaded into a 4-20% Mini-Protean® Precast gel.
7. Start the electrophoresis at 200 V for 35min.
8. Pre-wet membrane in transfer buffer 10 minutes before use at room temperature.
9. Cut the top right corner of a membrane and label the top left corner with the blot number.

10. Prepare the transfer apparatus: fill the box half full with pre-cold transfer buffer. Wet sponges and filter paper in transfer buffer.
11. Carefully transfer the gel to the filter paper, such that the top right corner is on the right and faces away from the hinge.
12. Transfer the membrane on top of the gel to match the orientation of the gel (nicked corner of gel to nicked corner of membrane). This ensures transfer of protein from left to right on the membrane, with marker on the left and samples numbering up.
13. Ensure that the membrane and gel remain wet, and remove any bubbles in between them. Complete the transfer sandwich with filter paper and sponge, then clamp the tray closed. Close the transfer box, place it in a box filled with an ice.
14. Transfer 1 hr at 100V.
15. Perform blocking with PBST/5% non-fat dry milk and incubate for 2h, shaking at room temperature.
16. Incubate with primary antibody overnight
17. . Wash the membrane 3 times with 1×PBST, 5 min each time
18. Incubate the membrane scond Antibody Peroxidase Conjugated (1:2000 in PBST/5% non-fat dry milk, v/v) for 1 hour
19. Wash the cells 3 times with PBST, 5 min each.
20. Mix the two substrate components at a 1:1 ratio to prepare the substrate Working Solution and incubate membrane 1 minute in the prepared Super Signal West Substrate Working Solution.
21. Analyze the membrane and take images.

5.3 Immunofluorescent staining

1. Rinse cells briefly twice in 0.5ml/well ice-cold PBS w/o Ca^{2+}/Mg^{2+} at room temperature.
2. Fix the samples in freshly made 0.5ml/well 4% paraformaldehyde in PBS pH 7.4 for 15 min at room temperature with shaking.
3. Wash the samples three times with 0.5ml/well ice-cold PBS.

   Note: The cells can be stored in 0.02% (w/v) sodium azide in PBS at 4°C for several days.
4. Incubate the samples for 10 min with 0.5ml/well PBS w/o Ca^{2+}/Mg^{2+} containing 0.5% Triton X-100 (in room temperature) with shaking.
5. Wash cells in 0.5ml/well PBS three times, each for 5 min with shaking.
6. Block: incubate cells with 0.5ml/well blocking buffer (5% sheep serum, 5% donkey serum, 0.05% Tween-20, 0.1% Triton X-100 in PBS) for 1 hour to block nonspecific binding of the antibodies (10% serum from the species that the secondary antibody was raised in) with shaking.
7. Incubate cells in 150µl/well mixture of two primary antibodies in blocking buffer overnight at 4^{o}C with shaking.
8. Decant the mixture solution and wash the cells three times in 0.5ml/well wash buffer, each for 5 min with shaking.
9. Dilute the fluorophore-conjugated secondary antibody/antibodies, away from light, in blocking buffer. Be sure that the correct isotype-specific secondary antibody for each primary antibody is used.

10. Incubate cells with 150µl/well mixture of two secondary antibodies which are raised in different species with two different fluorochromes (FITC-conjugated sheep against mouse and TRITC-conjugated donkey against rabbit) in blocking buffer for 1 hr at room temperature in dark with shaking.
11. Decant the mixture of the secondary antibody solution and wash three times with PBS each for 5 min in dark with shaking.
12. 4 drops of VECTASHIELD Mounting Medium with DAPI were added to each well and incubate for 1 minute.
13. Visualize the cells using a fluorescence microscope equipped with the appropriate filters for different dyes and take images.

5.4 Integrin blocking assay

1. Detach IMR 90 cells by dispase and collect them in the 1.5 mL tube.
2. Wash the cells by CMRL-BSA medium.
3. Count cell numbers and add 70,000 cells in each tube with 1 ml CMRL-BSA medium.
4. Add 10 µl integrin antibodies to each tube.
5. Seeding on the plates and incubate at 37 ^{o}C in CO_2 incubator. After incubation until the cells attach to the plates.
6. Wash cells by CMRL-BSA medium for 3 times
7. Fix by 0.5 ml/well100% ethanol for 5 minutes.
8. Stain the cells by 0.5 ml/well 0.4% crystal violet in methanol for 5 minutes
9. Wash the wells by dd H_2O twice.
10. Take Images count the colony numbers.

5.5 Purification of total RNA from animal cells using spin technology

1. Carefully remove all medium by aspiration and wash twice by DPBS.
2. Cells lysed directly by adding 600 µL Buffer RLT.
3. Use pipet to mix and detach the cells and transfer to a new tube.
4. Pass the lysate at least 5 times through a blunt $20G1^{1}/_{2}$ needle fitted to an RNase-free syringe.
5. Add 1 volume of 70% ethanol to the homogenized lysate, and mix well by pipetting.
6. Transfer up to 700 µl of the sample, including any precipitate that may have formed, to an RNeasy spin column placed in a 2 ml collection tube (supplied). Close the lid gently, and centrifuge for 15 s at ≥8000 x g (10,000 rpm). Discard the flow-through.
7. Add 700 µl Buffer RW1 to the RNeasy spin column. Close the lid gently, and centrifuge for 15 s at ≥8000 x g (10,000 rpm) to wash the spin column membrane. Discard the flow-through
8. Add 500 µl Buffer RPE to the RNeasy spin column. Close the lid gently, and centrifuge for 15 s at ≥8000 x g (10,000 rpm) to wash the spin column membrane. Discard the flow-through
9. Add 500 µl Buffer RPE to the RNeasy spin column. Close the lid gently, and centrifuge for 2 min at ≥8000 x g (10,000 rpm) to wash the spin column membrane.
10. Place the RNeasy spin column in a new 1.5 ml collection tube. Add 30–50 µl RNase-free water directly to the spin column membrane. Close the lid gently, and centrifuge for 1 min at ≥8000 x g (10,000 rpm) to elute the RNA.

5.6 Quantitative real time–polymerase chain reaction

1. Thaw 2x QuantiTect Multiplex RT-PCR NoROX Master Mix, template RNA, primer and probe solutions, and RNase-free water. Mix the individual solutions, and place them on ice. QuantiTect Multiplex RT Mix should be taken from –20 °C immediately before use, always kept on ice, and returned to storage at –20 °C immediately after use.
2. Prepare a reaction mix according to Table A1 (multiplex RT-PCR using the LightCycler 2.0)

Table A1 Reaction setup for duplex on RT-PCR for other cyclers

Component	Volume/reaction
Reaction mix	
2x QuantiTect Multiplex RT-PCR NoROX Master Mix	25 µl
20x primer–probe mix 1*	2.5 µl
20x primer–probe mix 2*	2.5 µl
QuantiTect Multiplex RT Mix	0.5 µl
RNase-free water	Variable
Optional: Uracil-N-glycosylase, heat-labile	Variable (2 units/reaction)
Template RNA (added at step 4)	Variable (≤250 ng/reaction)
Total reaction volume	50 µl

* **IMPORTANT:** For duplex RT-PCR using cyclers other than LightCycler 2.0 that do not require ROX passive reference dye, a 20x primer–probe mix consists of 8 µM forward primer, 8 µM reverse primer, and 4 µM probe in TE buffer, resulting in a final concentration of 0.4 µM forward and reverse primer and 0.2 µM probe.

3. Mix the reaction mix thoroughly, and dispense appropriate volumes into PCR tubes, PCR capillaries, or the wells of a PCR plate.
4. Add template RNA to the individual PCR tubes, capillaries, or wells.
5. Program the real-time cycler according to Table A2.

6. Place the PCR tubes, plates, or capillaries in the real-time cycler, and start the cycling program.
7. Perform data analysis.

Table A2 PCR cycling conditions

Temperature	**Time**	**Cycle step**
50 °C	20 minutes	1cycle
95 °C	15 minutes	1cycle
94 °C 60 °C	45 seconds 45 seconds	40 cycles