

Tailored peptide hydrogel-based three-dimensional system improves stem cell maintenance,
therapeutic potential and differentiation

by

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B.S., Kansas State University, 2015
M.S., University of Kansas, 2017

AN ABSTRACT OF A DISSERTATION

submitted in partial fulfillment of the requirements for the degree

DOCTOR OF PHILOSOPHY

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Manhattan, Kansas

2024

Abstract

Stem cells are a type of cells capable of self-renewal and differentiation and exist in both fetal and adult tissues. Stem cells can be totipotent, pluripotent and multipotent with decreasing differentiation potentials. Multipotent stem cells like hematopoietic stem cells have already been used to treat some diseases as they can give rise to blood cells, but application of other stem cells is still limited. Pluripotent stem cells, i.e. embryonic stem cells (ESCs) are isolated from early-stage embryos and are thus limited by donor scarcity. Thanks to the development of human induced pluripotent stem cells (hiPSCs), cell source is no longer an issue, but the maintenance of iPSC still poses challenges. Previously we developed a three-dimensional (3D) method that produces large amount of high quality hiPSCs, but the mechanism for 3D iPSC maintenance is still unexplored. Based on the previous established 3D hiPSC culture methods using peptide hydrogel PGmatrix, we found that the better proliferation performance compared to other hydrogel-based 3D culture system was due to ability of the hiPSCs to modify its surrounding environment. During growth in PGmatrix, the hydrogel strength changed. The mechanical cues can be sensed by hiPSC, leading to modulation of proliferation.

The novel 3D method can help fulfill the need of high quality iPSCs for downstream applications like therapeutic treatments and differentiation. Liver hepatocytes, as an example, is in large demand not only for disease treatment but also for pharmaceutical development. However, hepatocytes derived from iPSCs are still functionally far from primary hepatocytes, there is a thus need to improve the differentiation method. Compared to differentiation in matrix-free non-adherent plate, the hepatocyte organoids obtained from PG-suspension had significantly higher expression of key hepatocytes genes, some gene expression and *in vitro* detoxification function were comparable to primary hepatocytes.

Besides modeling and treating diseases with cells or organoids differentiated from iPSC, there are also therapies that use stem cells directly. Currently mesenchymal stem cells (MSCs) are preferred for therapeutics because these cells can release more growth factors and signaling molecules than iPSCs, but 2D-culture MSCs showed varied level of treatment effects mainly because they were not adapted to the *in vivo* niche. Studies showed that MSC therapeutic potential improved after adopting 3D conformation, but there's yet a method that steady MSC 3D preparation and delivery for treatment. In therapeutic application, we encapsulated MSCs in PGmatrix overnight before treatment through injection. Compared to hyaluronic acid (HA) and Hystem hydrogel, PGmatrix preserved high cell viability, promoted pluripotency protein expression and improved skin wound healing at an early stage in mice. Together, these results demonstrated improvements in stem cell research through application of PG hydrogel 3D systems. By tailoring peptide hydrogel for different research fields, there may be more advancement in stem cell related applications.

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Acknowledgements

First and foremost, I would like to thank Dr. Xiuzhi Susan Sun for her guidance and support throughout my study. She has always been patient to address my confusions and correct my mistakes. Without her, this achievement would not have been possible. I would like to extend my gratitude to all my committee members, Dr. Jianfa Bai, Dr. Ping Li and Dr. Dylan Lutter for their valuable opinions and suggestions on my research. Dr. Dylan Lutter, together with Dr. Camila R. S. Souza have also helped with planning and conducting surgery on mice, I greatly appreciate their service. I would like to acknowledge Dr. Guangyan Qi for her assistance with lab equipment and experimental materials. She has been a good lab manager and friend; it was a pleasure to work with her. I would like to express my gratitude to Dr. Xuming Liu for his dedication and help with PCR experiments and analysis. Dr. Liu and Dr. Bai have helped me to better understand PCR experiments and results. I would also like to acknowledge Dr. Margaret A. Highland for her assistance in the pathological interpretation of histological samples. I would like to thank the confocal core lab and Joel Sanneman for assistance in confocal microscopy and sample sectioning with cryostat.

Dedication

This dissertation is dedicated to my mother, Mannan Li. Without your love and support, it would have been a difficult time for me, especially during the COVID pandemic. I will always remember your understanding and comfort when I'm depressed.

To Dr. Xiuzhi Susan Sun, Dr. Donghai Wang, Dr. Guangyan Qi and her family. Thank you all for welcoming me and being friends. Your support helped me survive my PhD program.

Chapter 1 - Introduction

Stem Cells and Their Categories

Stem cells are unspecified cells found in both fetal and adult tissues. The two distinctive properties shared by all stem cells are 1) they are capable of self-renewal; 2) they can differentiate into other somatic cell types. Different stem cells exist at different developmental stages, and their differentiation potential decreases at each step^{1,2}. Totipotent stem cells, i.e. zygote, form after fertilization. Totipotent stem cells can differentiate into all types of cells including extra-embryonic structures. That is to say, totipotent stem cells have the greatest differentiation potential, one cell is able to develop into a human being. Pluripotent stem cells (PSCs) possess reduced differentiation potential, they cannot differentiate into extra-embryonic structures but still retain the ability to form cells of all three germ layers. PSCs are widely used in developmental studies to mimic the process of organ development. One type of PSCs used for research is embryonic stem cells (ESCs), which are isolated from the inner mass of early stage embryos³. Multipotent stem cells have less potency and can differentiate into limited cell types. Unlike totipotent and pluripotent cells that exist solely in embryos, multipotent stem cells reside widely in adults where they are responsible for tissue repair and homeostasis¹. Hematopoietic stem cells (HSCs) and mesenchymal stem cells (MSCs) are representative multipotent stem cells. HSCs can give rise to all types of blood cells⁴, while MSCs can differentiate into osteocytes, chondrocytes and adipocytes⁵. Because of their limited potency, researchers can better predict their performance *in vivo*, HSCs and MSCs are thus explored as treatments for many different diseases. Unipotent stem cells have the least differentiation potential among all stem cells and give rise to only one type of somatic cells. They usually exist in specific regions of the human

body, i.e. epithelial stem cells in the bulge region of the hair follicle⁶. Similar to multipotent stem cells, unipotent stem cells can be found in adults and are important for tissue homeostasis⁷.

Pluripotent Stem cells and *In Vitro* Maintenance

Human PSCs are useful to study the development of human tissue and certain diseases. However, ESCs were the only available human PSCs in early studies. The scarcity of donor tissues has greatly limited PSC studies. A breakthrough in this field was achieved in 2006 when induced pluripotent stem cells (iPSCs) were invented⁸. This reprogramming technology was successfully applied to human cells the next year yielding human induced pluripotent stem cells (hiPSCs)⁹. Generation of hiPSCs boosted stem cell research and made it possible to study the development of specific disease or personalized therapies through production of hiPSCs from patient cells. There have been reports on clinical trials of cell therapies using ESC/iPSC-derived retinal pigment epithelium¹⁰ and cardiomyocytes¹¹. However, applications of hiPSCs in clinical research require large quantities of high-quality hiPSCs in limited time and in a culture environment that is fully defined, or better, xeno-free. Therefore, successful *in vitro* culture of human PSCs is of great significance for research in disease modeling, drug screening, and organ transplantation.

2D *In Vitro* Culture

Early research found that these cells are adherent and can be grown on a feeder layer that consists of mouse embryo fibroblasts (MEFs) inactivated by g-irradiation or mitomycin C¹². The MEFs secrete not only extracellular matrix (ECM) proteins for stem cell attachment but also various signaling proteins like growth factors that promote stem cell survival and growth. As live cells, MEFs vary from batch to batch and the substances they secrete to the culture environment

cannot be fully defined. Also, because of its xenogeneic origin, MEFs may trigger unusual immune responses or cause mycoplasma contamination¹³. Initial search for MEF alternatives focused on substrates from natural sources. The most successful one so far is Matrigel, which is an extract from Engelbreth-Holm-Swarm (EHS) mouse sarcomas¹⁴. This product is temperature sensitive and forms a hydrogel under room temperature. It can be coated on commercially available tissue culture polystyrene plates as a thin layer that supports hPSC survival and growth. Matrigel is compatible with a variety of human stem cell culture media, including those with defined compositions, making it a widely used MEF alternative. Unfortunately, Matrigel poses similar problems as MEFs because it is also derived from animals and does not have a consistent and defined composition.

Matrigel contains large protein components including laminin, collagen IV, and enactin as well as a large number of low-molecular weight proteins that are involved in cellular localization and various biological processes¹⁵. Based on these findings, researchers seek the use of a single or truncated ECM protein as the supportive layer for hiPSC culture in 2D. Among all ECM proteins tested, laminin^{16,17} and vitronectin¹⁸ had the best performance. Synthetic polymer coatings were also proposed^{19,20} but were not widely accepted.

3D In Vitro Culture

No matter how much improvements were made to the supportive coating, 2D culture fails to mimic the complex 3D microenvironment. In addition, 2D culture method cannot meet the need for large scale hiPSC production. 3D culture methods have been developed to overcome these limitations. There are two main types of 3D culture, matrix-free and matrix-dependent.

Matrix-free suspension culture can start with forming spheroids from single cell and grow them as cell aggregates or on microcarriers coated with Matrigel or ECM proteins²¹⁻²⁴. In these

cases, vulnerable hPSCs were exposed directly to shear stress, which would negatively affect cell viability. In some studies, signs of cell agglomeration were observed, which would lead to differentiation. In addition, it can be hard to closely monitor cell state for large scale production and the batch-to-batch variation remains to be examined²⁵.

Matrix-dependent 3D culture methods have been reported for various types of cells. The matrix consists of natural polymers like collagen²⁶, fibrin²⁷, gelatin²⁸, etc. or synthetic polymers like polylactic acid (PLA)²⁹, polyethylene glycol (PEG)³⁰, etc. However, hiPSC can be cultured in limited matrix-dependent 3D systems. Early studies reported hiPSCs maintained in hyaluronic acid (HA)-based hydrogel³¹ or chitosan-alginate scaffold³². These methods were not tested for long-term maintenance and the complicated method can greatly limit their use. A relatively successful 3D matrix was Mebiol gel, a hydrogel consisting of poly(N-isopropylacrylamide)-co-poly(ethylene glycol) (PNIPAAm-PEG) and was shown to maintain long-term culture of hiPSC³³. Previously, we developed a synthetic peptide hydrogel PGmatrix³⁴ and successfully maintained hiPSC in 3D with this hydrogel for over 50 passages³⁵. Comparison between PGmatrix and Mebiol gel not only helps to reveal the differences between the two hydrogels but also provides clues to understand the underlying mechanisms of 3D maintenance of hiPSCs.

3D Hydrogel Systems to Improve Stem Cell Differentiation

Compared to MSCs, PSCs are less stable and may form tumor *in vivo*. Instead of using PSCs directly for therapies, scientists tend to perform differentiation *in vitro* to produce functional tissues and organoids, then used these differentiated constructs for disease modeling and patient treatment. Human iPSCs can be derived from somatic cells of the patients, with advancement in reprogramming strategies, nonintegrative and nonviral systems have been

developed which increased the safety of hiPSCs³⁶. To create successful *in vitro* models, differentiation efficiency needs to be improved. We decided to study hepatic differentiation of hiPSCs as an example.

Liver Composition and Functions

Liver is the central organ for metabolism consisting of hexagonal lobules that surround a central vein³⁷. Within each lobule, hepatocytes connected via tight junctions were separated by numerous sinusoids. HSCs reside between hepatocytes and LSECs, while KCs exist in the sinusoids. Bile salts produced by hepatocytes are released into the bile canaliculi and eventually the bile duct, which is lined by cholangiocytes³⁸.

Hepatocytes are parenchymal cells that make up about 60% of the liver cells and are responsible for most liver functions³⁹. Some major functions performed by hepatocytes include: 1) synthesis and secretion of serum proteins like α -fetoprotein (AFP), albumin (ALB), transferrin, plasminogen, fibrinogen³⁷, 2) regulation of insulin and glucose, 3) lipid uptake and metabolism, 4) detoxification of general fat-soluble toxins by cytochrome P450 (CYP) enzymes, 5) nitrogen detoxification through urea cycle^{37,40}.

LSECs, HSCs and KCs are NPCs that make up about 40% of liver cells³⁹. LSECs consist of about 19% of liver cells, they form the sinusoidal endothelium that allows flow of molecules to the hepatocytes from the plasma. HSCs (about 6% of liver cells) are vitamin A storage cells, but also involve in liver metabolism, injury and regeneration. KCs (about 15% of liver cells) are specialized macrophages that take part in immunoregulation^{39,40}.

Hepatocyte Differentiation from hiPSCs

During embryonic development, some embryonic stem cells segregate to form definitive endoderm (DE). The DE then develops into three progenitor domains, in which the foregut

endoderm will lead to liver formation, giving rise to hepatocytes³⁹. In vitro differentiation process with hiPSCs mimics this developmental process.

In conventional 3-step differentiation methods, hPSCs are directed to form DE using low serum medium with activin A⁴¹ in 2D⁴² or in 3D by generating embryoid body (EB) spheroids then plate onto substrates to form 2D cell layer⁴³. The DE cells are then directed towards hepatoblasts usually with combination of FGFs and bone morphogenetic proteins (BMPs)⁴¹. Hepatoblasts are bipotent stem cells that can give rise to hepatocytes and cholangiocytes⁴⁴. To obtain functional hepatocytes, hepatocyte growth factor (HGF) and oncostatin M are usually used to promote hepatoblasts differentiation⁴¹. Though HLCs generated following conventional protocol did show specific hepatocyte functions including hepatic nuclear factor 4 alpha (HNF4 α), ALB and alpha1-antitrypsin (A1AT) production, urea secretion, and upregulation of Cytokeratin (KRT) 8 and KRT18, their metabolic phenotype largely overlaps with fetal hepatocytes⁴⁵. Other studies reported that urea production and CYP enzymes expression remained low compared to PHH^{46,47}.

To improve differentiation towards mature phenotype, researchers have tested different methods. Addition of cyclic adenosine 3', 5'-monophosphate (cAMP)⁴⁸, methoxamine hydrochloride⁴⁹, and insulin-like growth factor I⁵⁰ were reported to show promotional effects on hepatocyte maturation. On the other hand, genetic manipulation was explored to generate mature hepatocytes. Takayama et al. overexpressed FOXA2 and HNF1 α in hiPSC and obtained HLCs with CYP enzyme expression levels comparable to PHH, but drug metabolism capacity of these HLCs were still lower than PHH⁴⁷.

To address issues of low differentiation efficiency and heterogeneity of resulting HLCs, Pettinato et al. developed a 4-step protocol. Because hepatoblasts are bipotent, they add one

additional step that inhibited Wnt signaling to block undesired differentiation towards cholangiocytes. This protocol showed better yield of hepatocytes with good functionalities and was shown to be compatible with 3D culture⁴⁴.

3D Culture Improves Hepatocytes Maintenance and Differentiation

As described previously, hepatocytes exist in liver in a highly organized structure, it is impossible to represent such a complex configuration with 2D models. 2D culture and differentiation forced cells to adopt a flat, single cell layer organization with limited cell-cell interaction, restraining uniform nutrient transport and oxygen tension. In this non-physiological condition, hepatocytes act as single entities with little to no homo- and heterotypic interactions^{51,52}. In contrast, 3D culture allows for multi-cellular contact which helps hepatocytes to retain cell polarization and would promote signaling pathways to support proliferation and maintenance³⁹. In addition, it is possible to mimic physiological configurations of different cell types and ECM components in 3D systems for better in vitro models.

In its simplest form, 3D spheroid culture was tested on PHH, HepG2, HepRG by forcing spheroid formation in non-adherent plate. PHH spheroids showed improved phenotype stability compared to those in 2D culture⁵³. HepRG spheroids were reported to maintain physiologically relevant levels of CYP enzymes, though still lower than PHHs⁵⁴. Similarly, HepG2 spheroid culture had better culture stability with increased sensitivity to toxic compounds⁵⁵. Another study reported that HepaRG spheroids formed after embedded in Collagen hydrogel differentiated towards hepatocytes in the absence of DMSO, the resulting hepatocyte phenotype showed comparable levels of xenobiotic metabolism enzymes to HepaRG differentiated in 2D with DMSO⁵⁶.

Some studies harvested HLCs after 2D differentiation from hiPSCs and maintained these HLCs as spheroids in 3D suspension culture. All studies reported continued maturation of HLCs in 3D with increased albumin secretion, decreased AFP production, and elevated levels of some CYP enzymes^{48,57–59}. To improve differentiation efficiency from the beginning, some researchers developed 3D differentiation protocol with hiPSC spheroids. Rashidi et al. formed hiPSC spheroids in agarose microplate and induced hepatic differentiation with conventional 3-step protocol, following continued maturation in hepatocyte maintenance medium. After 20 days of differentiation and 10 days of maturation, the resulting HLCs showed hepatocyte phenotype with high AFP expression and low HNF4 α and albumin production compared to PHH⁶⁰. However, maturation was carried out for 365 days and showed that HLCs continued to mature with decreasing AFP and increasing albumin expression by day 90. Activities of CYP3A enzymes were maintained at comparable level after a year in culture⁶⁰. This result is promising, but the differentiation and maturation is overly time consuming for practical use. As mentioned in previous section, Pettinato et al. developed a 4-step differentiation protocol that improved hiPSC differentiation towards hepatocytes⁴⁴. This protocol was used on hiPSC spheroids formed in non-adherent microwells. After 16 days, the generated HLCs expressed greater levels of hepatocyte-specific makers and much less cholangiocyte-specific markers compared to HLCs generated with 3-step differentiation. In addition, HLCs from 4-step differentiation had better albumin and urea production⁴⁴.

However, most of these 3D studies discussed were performed with cells forced into spheroid form. These spheroids were usually dense cell clusters without organized structures, far from the physiological state in developing embryos, and are prone to necrosis in the core area. Moreover, the hiPSCs used for differentiation were proliferated in 2D culture, which may affect

their quality maintenance. In previous studies, we showed that hiPSCs can develop naturally into spheroids in 3D peptide hydrogel. These hiPSCs showed superior pluripotency maintenance based on results from both *in vitro* and *in vivo* assays³⁵. This result indicated that these naturally developed hiPSC spheroids can better mimic developing embryo and are better candidates for hepatic differentiation.

Three-dimensional spheroid culture demonstrated better differentiation efficiency, but the role of supporting scaffolds in 3D differentiation was underestimated in most of these studies. Extracellular matrix (ECM) exists naturally in the liver and is responsible for cell-matrix signaling in addition to supporting hepatic cells³⁹. HLCs derived from hiPSCs can be seeded into decellularized rat liver ECM and showed improved hepatocyte-specific functions⁶¹. Another study placed hiPSC-derived foregut cells in Matrigel, a hydrogel consists of ECM proteins derived from a mouse sarcoma⁶², for differentiation into hepatocytes. The resulted HLCs had albumin release levels and CYP2C9 induction capacity similar to that of PHHs⁶³. Despite these encouraging examples, scaffolds, especially hydrogel scaffold are more often used as 2D coating or in sandwich culture³⁹. Possible reasons for this condition are difficulty in material handling, limited nutrient flow into spheroids, and complications in analysis of 3D culture⁶⁴. The fully synthetic peptide-based hydrogel PGmatrix developed by our group has shown excellent handability³⁴ and successful application in 3D culture of hiPSCs³⁵. With possible modification, this hydrogel may be used for 3D hepatocyte differentiation of hiPSC spheroids. After differentiation, the shear thinning property of PGmatrix will allow the differentiated spheroids to be recovered easily with minimal damage. We believe this hydrogel system can provide better support for more efficient hiPSC differentiation towards hepatocytes.

Therapeutic Application of Stem Cells

An important application of stem cells is cell therapies. Regenerative medical research aims to repair or replace wounded or diseased tissues with healthy stem cells or tissue derived from stem cells to regain normal functions⁶⁵. Therapies using hiPSC-derived retinal cells and insulin-producing beta cells have been reported². To date, the majority of PSC based clinical studies are observational, because of possible tumor formation *in vivo*⁶⁶ and limited stability of iPSC genome⁶⁷. Multipotent stem cells like MSCs are thus more extensively studied for clinical applications.

Mesenchymal Stem Cells

MSCs exist in a variety of adult tissues including bone marrow, adipose tissue, umbilical cord, dental pulp⁶⁸, as well as lung⁶⁹ and hair⁷⁰. MSCs were first isolated from bone marrow in the 60s⁷¹. Bone marrow and adipose tissue are the sources for most MSCs used in research, the MSC yield from the two tissues differ greatly with adipose tissue yielding 500 times higher than bone marrow⁷². MSCs can attach to culture plastic, facilitating *in vitro* culture, but the proliferation varies by the source of MSCs. Umbilical cord-MSCs (UC-MSCs) had the shortest doubling time, followed by adipose tissue-MSCs (AD-MSCs), bone marrow-MSCs (BM-MSCs) proliferation is the slowest among the three⁷³. Though MSCs from different sources behave slightly differently, they all meet the minimal criteria defined by the Mesenchymal and Tissue Stem Cell Committee of the International Society for Cellular Therapy: plastic-adherent when in culture, express CD105, CD73 and CD90, but not CD45, CD34, CD14 or CD11b, CD79alpha or CD19 and HLA-DR on the surface, and can differentiate into osteoblasts, adipocytes and chondroblasts *in vitro*⁷⁴.

Therapeutic Potential of MSC

Because MSCs are easily grown in culture with intrinsic differentiation potential, early research tried to use MSCs as cell replacement therapy hoping for engraftment of the transplanted cells to differentiate and generate healthy tissues. Studies have shown that BM-MSCs tend to adopt chondrogenic and osteogenic lineages, while AD-MSCs had higher differentiation ability toward adipogenic lineage⁷⁵. Though this is likely linked to the epigenetic memories from their tissue sources⁷⁶, another study showed that UC-MSCs exhibited stronger osteogenic ability than BM-MSCs⁷⁷. In MSC transplantation study, research tried to trace MSCs after administration and observed that though there were no signs of malignant tissue formation, the engraftment of MSCs can be hardly observed⁷⁸. Studies have since shifted focus to its immunoregulatory abilities. MSCs can migrate to sites of injury or inflammation, and were reported to secrete plethora of growth factors, cytokines, immunomodulatory molecules and micro RNAs including vascular endothelial growth factor (VEGF), fibroblast growth factor 2 (FGF2), transforming growth factor (TGF), interleukin-6 (IL-6). These paracrine activities bring anti-inflammatory effects⁵. MSCs can regulate adaptive immune cells both directly and indirectly. When sensing inflammation, MSCs release large amounts of chemokine (C-X-C motif) ligand 9 (CXCL9) and other chemokines to recruit T cells, then suppress the activation and proliferation of T cells through secretion of indoleamine 2,3-dioxygenase (IDO). B cells can also be suppressed possibly via contact with membrane bound molecules on MSCs⁷⁹. Indirectly, paracrine factors released by MSCs can affect dendritic cells (DCs) maturation and monocytes differentiation thus impacting T cell proliferation⁷⁹. Even after apoptosis, MSCs still exhibited therapeutic effects⁸⁰. Studies reported that apoptotic MSCs can shift macrophages towards a M2 phenotype^{81,82}, other studies showed that therapeutic effects of MSC in some immune disorders were related to apoptosis of the transplanted cells^{83,84}.

With the massive amount of information obtained from numerous studies over the years, conflicting and confusing messages still possess questions on the mechanisms of MSC treatments as well as effects of conditioning and delivery methods for MSC transplantation.

3D Conditioning and Delivery of MSCs

When in tissue, MSCs reside in niches, that not only protect them from outside stress, but also generate signals that direct their growth and differentiation⁷². In stem cell niches, MSCs receive endocrine and paracrine signals from each other, from stromal supporting cells, neurons, and from extracellular matrix (ECM) proteins⁷. Researchers thus suggested that MSCs adopting 3D conformation or embedded in ECM mimicking materials may improve their therapeutic effects. Some studies showed that MSC differentiation in 3D environment can be improved by co-culture them with other supporting cells like human umbilical vein endothelial cells (HUVECs)⁸⁵, osteoblasts⁸⁶, and hematopoietic stem cells (HSPCs)⁸⁷. Other studies have tried to form 3D spheroids by forced aggregation on ultra-low attachment surfaces or contraction through reducing adhesion. Some reported rejuvenation of the 3D MSCs, with elevated stem cell gene expression and better proliferation^{88–90}. Others observed an increase in secretion of growth factors and immunomodulatory molecules, as well as improved treatment effects^{91–93}. However, impact from ECM proteins is largely underestimated by these studies. Previous research suggested that on site delivery of MSCs led to better treatment effects than intravenous methods⁷². But direct injection of MSCs to the injured site may lead to ossification and interfere with local host signaling⁹⁴. Applying biomaterials can not only provide support for MSCs to adopt 3D conformations, but also serve as delivery system that present direct contact with host tissues but still allows controlled release of MSC secretome.

MSCs can be encapsulated in hydrogel in different ways. Micromolding method uses specialized lithography equipment to encapsulate cells and allows precise control on MSC conformation and placement of different types of cells for co-culture^{95,96}. Electrostatic droplet extrusion method produces microbeads that have MSCs embedded. This process uses a needle to extrude liquid into an electrostatic field, forming hydrogel beads⁹⁷. Without the use of organic solvents, electrostatic extrusion is less stressful for MSCs to survive⁹⁸. A relatively new approach is to encapsulate cells with microfluidic devices. By manipulating fluids in microscopic environment, cells can be encapsulated in droplets and microfibers^{99,100}. Besides, there are also various bioprinting techniques that can be used to produce 3D MSC embedded constructs⁹⁸. However, these methods all require complex processes which may limit their application in clinical settings. Compared to the methods mentioned previously, injectable hydrogel is of special interest. This type of material is shear thinning, making it possible to load into a syringe for injection, minimizing patient pain. During injection, the hydrogel can protect embedded cells from mechanical stress. After encapsulation, the hydrogel can provide environmental cues to modulate cells, improve viability and secretion of therapeutic factors¹⁰¹. PGmatrix is a fully synthetic peptide hydrogel that is bio-compatible¹⁰². It was shown to improve pluripotency maintenance of hiPSCs in 3D³⁵, and can be used for the controlled release of certain drugs¹⁰³. These results suggested that PGmatrix can be a candidate material for 3D conditioning and delivery for MSC therapies.

Chapter 2 - Maintenance of Human iPSCs in Different 3D Systems

Revealed Role of Mechanical Cues

Abstract

Human induced pluripotent stem cells (hiPSCs) demonstrated great potential in basic research, disease modeling, drug development, and regenerative medicine. However, these applications, especially cell-based clinical therapy using hiPSCs, have been severely limited by various challenges, such as less-optimal traditional methods for culturing hiPSC, which often fail to maintain high-quality hiPSCs following multiple passages. For example, 2D culture for hiPSCs has been widely used for more than a decade; The 2D culture method is cost-effective and relatively easy to operate in a small scale; however, numerous passages usually result in unstable genetic integrity, reduced pluripotency, and spontaneous differentiation. As a result, several models using 3D culture for hiPSCs have been recently developed, yet the need to maintain clinical grade hiPSCs are still not completely fulfilled. In a previous study, a robust 3D culture system was developed using an innovative PGmatrix peptide hydrogel to examine hiPSC quality in comparison with 2D culture. Two hiPSC cell lines, ASC-hiPSC and TF-hiPSC were examined in parallel. It was found that 3D PGmatrix hydrogel not only significantly increased hiPSCs viability and proliferation, but also remarkably escalated expressions of some important pluripotency related genes compared to those in 2D culture on Matrigel or Vitronectin-XF. Furthermore, *in vivo* teratoma analysis suggested that hiPSCs cultured in 3D PGmatrix hydrogel resulted in greater pluripotency potential after long-term culture. To further evaluate this novel 3D system and find possible underlying mechanism, we compared hiPSC maintenance in 3D PGmatrix with another popular existing 3D culture with PEG based hydrogel (i.e.,

Mebiol). Compared to hiPSCs maintained in Mebiol gel, hiPSCs in PGmatrix showed much better growth performance in terms of viability ($p < 0.05$) and proliferation ($p < 0.01$) and expressed significantly higher levels of undifferentiated transcription factor 1 (UTF1) ($p < 0.05$) and human telomerase reverse transcriptase (hTERT) ($p < 0.01$). Since UTF1 is believed to be a more sensitive marker for pluripotent stem cells than the classical ones, (e.g., Oct4 and NANOG), higher expression of UTF1 may indicate high quality of undifferentiated status. Further analysis showed that the different growth performances in different hydrogels were mainly affected by degradability of the hydrogel. These signals can be transduced by mechanosensing genes Yes-associated protein 1 (YAP) and transcriptional coactivator with PDZ-binding motif (TAZ) at the center of the Hippo signaling pathway, eventually regulating cell growth and maintenance. Together, these findings demonstrated that 3D PGmatrix hydrogel is a unique 3D microenvironment to maintain hiPSCs at high quality during long-term culture. This innovative 3D culture method allows the generation of sufficient amounts of stable hiPSCs for basic research and future therapeutic application.

Introduction

Human pluripotent stem cells (hPSCs), including human embryonic stem cells (hESCs) and human induced pluripotent stem cells (hiPSCs), are able to self-renew indefinitely in theory and to differentiate into almost all somatic cell types; thus, they have drawn great attention in research community and hold great potential to improve human health¹⁰⁴. Unlike hESCs that are obtained from early-stage embryos, hiPSCs can be generated from somatic cells through ectopic expression of defined transcription factors⁹ and therefore avoid ethical problems related to the use of hESCs. In addition, the successful generation and specific differentiation of patient-

specific hiPSCs provide new approaches for disease modeling, drug screening/toxicity testing as well as personalized cell therapies. However, these applications have been hindered in part by the current less-optimal culture technologies that may reduce or mask some of the concerns related with tumorigenesis, low viability/retention, and uncontrolled *in vivo* differentiation. Commonly-used culture methods cannot completely fulfill the demand for hiPSCs in view of genetic quality, growth performance, stemness, functionality, and differentiation potentiality¹⁰⁵.

Traditionally, hiPSCs are seeded onto a thin layer of substrate or feeder and grow as colonies of monolayer. Mitotically inactivated mouse embryonic fibroblast (MEF)¹⁰⁶ and Matrigel extracted from mouse sarcoma are the most commonly-used feeder layer and substrates for hiPSC culture. In the past decades, researchers have put tremendous efforts into seeking alternative, well-defined xeno-free matrices, such as laminin^{16,17}, E-cadherin^{107,108}, vitronectin¹⁰⁹, synthetic polymers^{19,20,110}, and synthetic peptides^{111–113}. However, all these matrices are mainly limited to 2D culture, in which a monolayer of hiPSCs grows on top of matrix-coated flat or bead surfaces. Obviously, 2D culture is not representing the *in vivo* physiological environment. The side-to-side cell contact lack of appropriate stem cell niche often lead to unwanted gene expression, resulting in inefficiency of targeted differentiation¹¹⁴.

To overcome the above limitations, various 3D culture methods to better mimic *in vivo* conditions have been developed during the past decades. For example, suspension culture has been used for culturing non-adherent cells, but this method is not applicable to adherent cells, such as hiPSCs, for known issue of cell agglomeration^{115–117}. Other 3D cultures using scaffolds of natural polymers³² or hydrogel³¹ generated *in vivo*-like conditions for hiPSC culture to avoid cell agglomeration associated with the suspension system. Natural polymer-derived scaffolds generally require a relatively complicated process for cell encapsulation and harvesting, resulting

in significant cell loss that would hinder downstream analysis and applications. Hydrogel, on the other hand, is relatively easy to handle, but certain reagents used for gel formation or dissociation are harmful to hiPSCs³¹. Polyethylene glycol (PEG) tailored hydrogel (PNIPAAm-PEG or Mebiol Gel™), a temperature-sensitive hydrogel, was found to sustain a satisfactory growth rate for hESC and hiPSC lines³³.

Our group discovered and synthesized a new, tri-block amphiphilic peptide-based hydrogel termed as h9e³⁴. H9e is rationally designed from a group of selective amino acids that can self-assemble into nanofibers and then transform into a fast sol-gel reversible hydrogel through shearing force, such as pipetting or syringing, under neutral pH at room or body temperature^{118,119}. PGmatrix, a commercial product derived from h9e, has been used for 3D cultures of cancer cells^{120–123}. In a previous study, we optimized the PGmatrix-based 3D hydrogel for long-term culture and expansion of hiPSCs. Two hiPSC lines (ASC-hiPSC from foreskin fibroblasts and TF-hiPSC from CD34+ cord blood) were tested in this 3D system and in parallel in 2D Matrigel or Vitronectin-XF-coated plates. The results showed that hiPSCs maintained in 3D PGmatrix hydrogel had significantly high proliferation and viability, as well as notably higher expression of pluripotency marker genes in PGmatrix cultured hiPSCs than their 2D counterparts. Moreover, *in vivo* teratoma assay showed that hiPSCs maintained in PGmatrix for over 34 passages possessed excellent development potential³⁵. To test whether this is the result of adopting 3D culture, we compared growth performances of hiPSC cultured in 3D PGmatrix with a commercial PEG-based Mebiol Gel. This comparison suggested key role of mechanosensing genes YAP and TAZ in 3D maintenance of hiPSCs. Collectively, our data demonstrated that 3D PGmatrix hydrogel provides ideal environments suitable for maintaining

hiPSCs at high quality for hiPSC expansion and revealed a key factor for successful 3D hiPSC culture.

Materials and Methods

3D cell culture in PGmatrix

Frozen cells or hiPSCs dissociated from 2D culture, were encapsulated in 3D PGmatrix hydrogel at seeding densities of $1.8 - 2 \times 10^5$ cells/ml for ASC-hiPSC (Applied Stemcell Inc., Milpitas, CA, USA) and $2 - 3 \times 10^5$ cells/ml for TF-hiPSC (Thermo Fisher Scientific, Waltham, MA, USA). Specifically, cells suspended in 240µl mTeSR1 medium (Stemcell Technologies, Vancouver, Canada) containing 1% penicillin/streptomycin (Sigma-Aldrich, St. Louis, MO, USA) and 10µM rho-associated protein kinase ROCK inhibitor Y-27632 (Stemcell Technologies) were first mixed with 10µl PGworks, followed by adding 250µl PGmatrix nanofiber solution and mixed thoroughly without introducing air bubbles to reach 500µl of mixture with a 0.5% final concentration of peptide. The mixture was then plated into one well of a 24-well plate and incubated at 37 °C for 30 minutes to allow gel formation. At the end of incubation, mTeSR1 culture medium containing 1% penicillin/streptomycin and 10 µM ROCK inhibitor Y-27632 was added gently on top of the formed hydrogel and medium was changed daily after seeding. At least 4 wells of 3D culture for each cell line at each passage were prepared. Morphology images were acquired with a Zeiss Axio Vert A1 microscope (Carl Zeiss Microscopy, LLC., White Plains, NY, USA).

To release cells from the 3D PGmatrix hydrogel, the medium on top of the gel was replaced with Dulbecco's phosphate-buffered saline (DPBS) without $\text{Ca}^{2+}/\text{Mg}^{2+}$ (Thermo Fisher Scientific), followed by manual disruption (*e.g.*, pipetting). The mixture was then transferred to a

conical tube and additional DPBS was added to rinse the well to collect any remaining gel materials. The suspension cell-gel mixture was further diluted to more than 20 folds with DPBS. After centrifugation, the supernatant was removed and 1x TrypLE solution (Thermo Fisher Scientific) was added and gently pipetted to re-suspend cell pellet. Samples were then incubated at 37°C for 15 minutes to dissociate cell colonies and the reaction was stopped by adding mTeSR1 medium. The resulting cell suspension was centrifuged and re-suspended in mTeSR1 medium with 1% penicillin/streptomycin and 10 µM ROCK inhibitor Y-27632 for continuous culture in 3D PGmatrix hydrogel and further analysis. For cell number and viability measurements, cells collected from 4 wells of 3D PGmatrix culture were pooled, two samples were taken from the pooled cell suspension and were counted with a cellometer Auto2000 (Nexcelom Bioscience, Lawrence, Ma, USA) with acridine orange/propidium iodide (AO/PI) staining (Nexcelom Bioscience).

3D cell culture in Mebiol Gel

To compare PEG based hydrogel with PGmatrix, 3D hiPSC culture in commercially available PEG based hydrogel (Mebiol Gel from Cosmo Bio USA, Carlsbad, CA) was conducted according to the protocol published previously¹²⁴. Briefly, harvested ASC-hiPSC was resuspended in cold Essential 8 (E8) basal medium (Thermo Fisher Scientific)-dissolved Mebiol Gel and mixed gently on ice. The mixture was plated into 6 wells of a 12-well plate at 1×10^6 cells/ml and 100µl/well. After 10 minutes incubation at 37°C, 2ml pre-warmed E8 medium supplemented with ROCK inhibitor Y-27632 was added onto the top of Mebiol Gel. Media was changed daily for 4 days. On Day 5, medium on the top of gel was replaced with ice-cold DPBS (2ml/well). Mebiol gel was then disrupted by gentle pipetting on ice and transferred to 15ml conical tubes on ice (2 wells/tube, 3 tubes total), and the wells were washed twice with ice-cold

DPBS to collect remaining cells. The resulting mixture was then centrifuged at 200g for 5 minutes followed by removal of supernatant. Cell pellets were gently broken down into single cells or small clusters using the same procedures described above for PGmatrix cultured cells. Three samples (one from each tube) were taken for cell counting. Cell counting was performed with cellometer Auto2000 with AO/PI staining.

Reverse transcription quantitative PCR

Reverse transcription quantitative PCR (RT-qPCR) analysis was performed by the Kansas State Veterinary Diagnostic Laboratory to characterize gene expressions of commonly used stem cell markers in two hiPSC lines. Total RNA samples were extracted from each cell lines using the Direct-zol RNA MiniPrep kit (ZYMO Research Corp., Irvine, CA, USA) and diluted to 20 ng/μl. RT-qPCR reactions were conducted with Bio-Rad CFX96™ Touch™ Real-time PCR Detection System using iTaq Universal SYBR Green One-Step kit (Bio-Rad, Hercules, CA, USA). 20 μL reaction contains 10 μL of 2x iTaq universal SYBR Green reaction mix, 0.25 μL iScript reverse transcriptase, 1 μL of 10 μM forward and reverse primer mix solution (a final concentration of 500 nM) (primer sequences were listed in Table 2.1), 5 μL RNA sample, and 3.75 μL nuclease-free water. The first reverse transcription reaction was set at 50°C for 10 minutes, followed by polymerase activation and cDNA denaturation at 95°C for 1 minute. The samples then underwent 45 cycles of denaturation at 95°C for 10s and annealing and extension at 60°C for 40s for *hTERT*, *ESG-1*, *DNMT3B*, and 3 House Keeping genes (*EID2*, *ZNF324B*, and *CAPN10*)¹²⁵, or annealing at 52°C for 30s and extension at 72°C for 20s for *UTF1*, *NANOG*, *OCT4*, *REX1*, *SOX2*, *Myc*, *DPPA4*, *FGF4*, *GDF3*, *AFP*, *SOX1*, and *Brachyury*. Triplicate was conducted for each gene in all samples. Ct values were analyzed using Bio-Rad CFX Manager 3.0 software. Fold change of a target gene in a tested sample was compared with

that of a control sample (PGmatrix cultured hiPSCs) and normalized with average expression levels of 3 housekeeping genes.

Table 2.1 Primers used for RT-qPCR analysis

Gene	Sequence
<i>hTERT</i>	Forward (5' to 3'): GGAGCAAGTTGCAAAGCATTG Reverse (3' to 5'): TCCCACGACGTAGTCCATGTT
<i>ESG1</i>	Forward (5' to 3'): GCCGTGCGTGGTGGATAAGC Reverse (3' to 5'): GCCAAACAGATATTTCAGCACCAGC
<i>Myc</i>	Forward (5' to 3'): TCACACCCTTCTCCCTTC Reverse (3' to 5'): GGTTCAACCATGTCTCCTC
<i>NANOG</i>	Forward (5' to 3'): TGTGATTTGTGGGCCTGA Reverse (3' to 5'): GTGGGTGTTTGCCTTTG
<i>DPPA4</i>	Forward (5' to 3'): ACAGCAAAAGAGGCCA Reverse (3' to 5'): CACAGGAGGAAGAGCCA
<i>FGF4</i>	Forward (5' to 3'): CTCCTTCCCAACAACACTAC Reverse (3' to 5'): GTTCCCCTTCTTGGTCTT
<i>GDF3</i>	Forward (5' to 3'): GTTAATTCTGGCTTTGGG Reverse (3' to 5'): ATATAAGGCACAGGTTGG
<i>OCT4</i>	Forward (5' to 3'): AAAGAGAAAGCGAACCAG Reverse (3' to 5'): CCACATCCTTCTCGAGCC
<i>REX1</i>	Forward (5' to 3'): GTTTCGTGTGTCCCTTTC Reverse (3' to 5'): CTTTCCCTCTTGTTTCATTC
<i>SOX2</i>	Forward (5' to 3'): CAACCAGAAAAACAGCCC Reverse (3' to 5'): TCTCCGACAAAAGTTTCC
<i>UTF1</i>	Forward (5' to 3'): CTCCCAGCGAACCAG Reverse (3' to 5'): GCGTCCGCAGACTTC
<i>DNMT3B</i>	Forward (5' to 3'): GGAGCCACGACGTAACAA Reverse (3' to 5'): GGCATCCGTCATCTTTCA
<i>AFP</i>	Forward (5' to 3'): TACCTGCCTTTCTGGAAGAACT Reverse (3' to 5'): ATGTCTTCCCTCTTCACTTTGG
<i>SOX1</i>	Forward (5' to 3'): ACAGAGCCCATTTTCTCCATAA Reverse (3' to 5'): TTCCCTGTCAGATTTCTTCATT
<i>Brachyury</i>	Forward (5' to 3'): GAACGAGGAGATCACAGCTCTT Reverse (3' to 5'): TTCCTCCATCATCTCTTTGTGA

<i>hCAPN10</i>	Forward (5' to 3'): GGAGGTGACCACAGATGACC Reverse (3' to 5'): GTAAGGGGAGCCAGAACACA
<i>hZNF324B</i>	Forward (5' to 3'): GAGAATGGCCACGAGCTTT Reverse (3' to 5'): TTTACACTGTGGCAGGCATC
<i>hEID2</i>	Forward (5' to 3'): GAAGCCTGCAGAGCAAGG Reverse (3' to 5'): ATATCGAGGTCCACCCTGTG
<i>YAP</i> ¹²⁶	Forward (5' to 3'): CACAGCATGTTCGAGCTCAT Reverse (3' to 5'): GATGCTGAGCTGTGGGTGTA
<i>TAZ</i> ¹²⁶	Forward (5' to 3'): GGAGGACTTCCTCAGCAATG Reverse (3' to 5'): TCAAGGAAATCAGGGAAACG

Rheological test of PGmatrix hydrogel

PGmatrix hydrogel samples were prepared following the encapsulation process described previously by mixing the nanofiber solution with culture media/cell suspension and PGworks, then incubated at 37°C for 30 minutes for gelation. At different timepoints, a scoop of sample was taken from the culture well for measurement. The elastic modulus (G') of PGmatrix hydrogel was continuously measured during a 30-minute period using CVOR 150 rheometer system (Malvern Instruments, Malvern, Worcestershire, UK) with hydrogel-forming rate set under 1 Hz and 1% steady shear strain. A thin layer of silicone oil was spread over the circumference of the sample to prevent it from dehydration during the test.

Statistical analyses

Statistical analyses were conducted with SAS university edition (SAS Institute Inc., Cary, NC) and GraphPad Prism 8 (GraphPad Software, San Diego, CA). Student's t-test (two-tailed) was used to compare 2 groups with α level ≤ 0.05 as statistical significance.

Results and Discussion

Maintenance performance differ in different 3D hydrogel systems

We have observed rapid growth and high viability of hiPSCs cultured in 3D PGmatrix from previous study. Since this might be simply resulted from a 3D culture environment, we decided to compare our 3D PGmatrix with a commercial PEG-based 3D hydrogel Mebiol Gel³³ in parallel using the same stem cell line (ASC-hiPSC). Either mTeSR1 or E8 medium supplemented with ROCK inhibitor Y-27632 was used to yield four culture conditions (PG-mT, PG-E8, Mebiol-mT, and Mebiol-E8). We found that hiPSCs were able to form spherical colonies in all four conditions (Figure 2.1); however, the fold expansion (13.96 ± 1.87) and cell viability

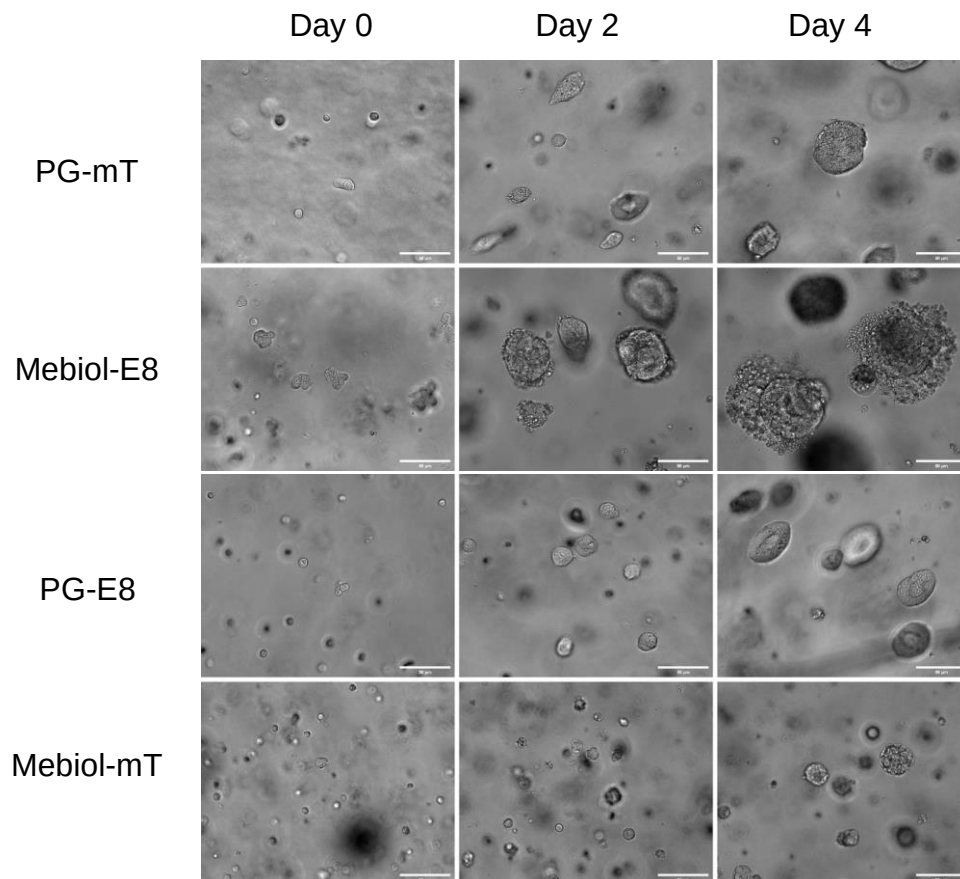


Figure 2.1 Morphology of hiPSCs grown in PG-mT, Mebiol-E8, PG-E8, and Mebiol-mT on days 0, 2, and 4 after encapsulation. Scale bar, 50 μ m.

($84.2 \pm 2.0\%$) of hiPSCs cultured in PGmatrix is significantly higher than those cultured in Mebiol gel (fold expansion of 9.69 ± 2.38 and viability of $13.0 \pm 1.6\%$, respectively) (Figure 2.2). In PGmatrix, the size and morphology of the spheres are more uniform in either mTeSR1 or E8

medium, although the fold expansion of hiPSCs in PGmatrix was significantly higher in mTeSR1 than in E8 medium (Figure 2.1). When the same medium (E8) was used, the fold expansion (10.48 ± 2.25) of hiPSCs is significantly higher in 0.3% PGmatrix than in 0.5% PGmatrix and the cell viability is close between 0.3% (>80%) and 0.5% PGmatrix (>72%) (Figure 2.2).

By contrast, when being cultured in Mebiol gel, hiPSCs formed a few large spheres in E8 medium and the cells on the edge of large spheres in Mebiol-E8 tend to die (Figure 2.1). Noteworthily, in mTeSR1 medium, much smaller spheres were observed in Mebiol Gel (Figure 2.1) and the fold expansion and cell viability are even lower than in E8 medium (data not shown).

Though the fold expansion was stable

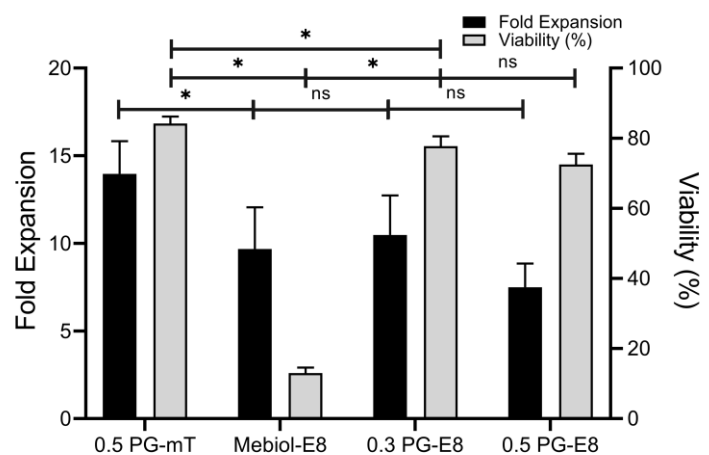


Figure 2.2 Fold expansion and viability of ASC-hiPSC grown in different 3D systems with different culture medium. Data are shown as means $\pm$ SDs. *: $p < 0.05$.

at around 10 folds during several hiPSC culture experiment carried out in Mebiol gel with E8 medium, which is in accordance with previous report³³, the viability was no more than 20% after 2 passages no matter which medium (E8 or mTeSR1) was used with this gel. As a result, it was impossible to compare 3D culture of Mebiol Gel with PGmatrix for long term hiPSC culture beyond passage 1.

Next, we examined the pluripotency of the hiPSC spheroids formed in 3D PGmatrix and 3D Mebiol gel using ASC-hiPSCs with the better culture conditions (Mebiol-E8 and PG-mT). As shown in Figure 2.3, hiPSC gene integrity was well-maintained in the 3D PGmatrix compared to

that in the 3D Mebiol gel. hiPSC spheroids grown in 3D PGmatrix expressed higher levels of pluripotent markers (*NANOG*, *OCT4*, *SOX2*, and *hTERT*) and lower or similar expressions of differentiation markers (*AFP*, *SOX1*, and *Brachyury*) than those grown in 3D Mebiol gel.

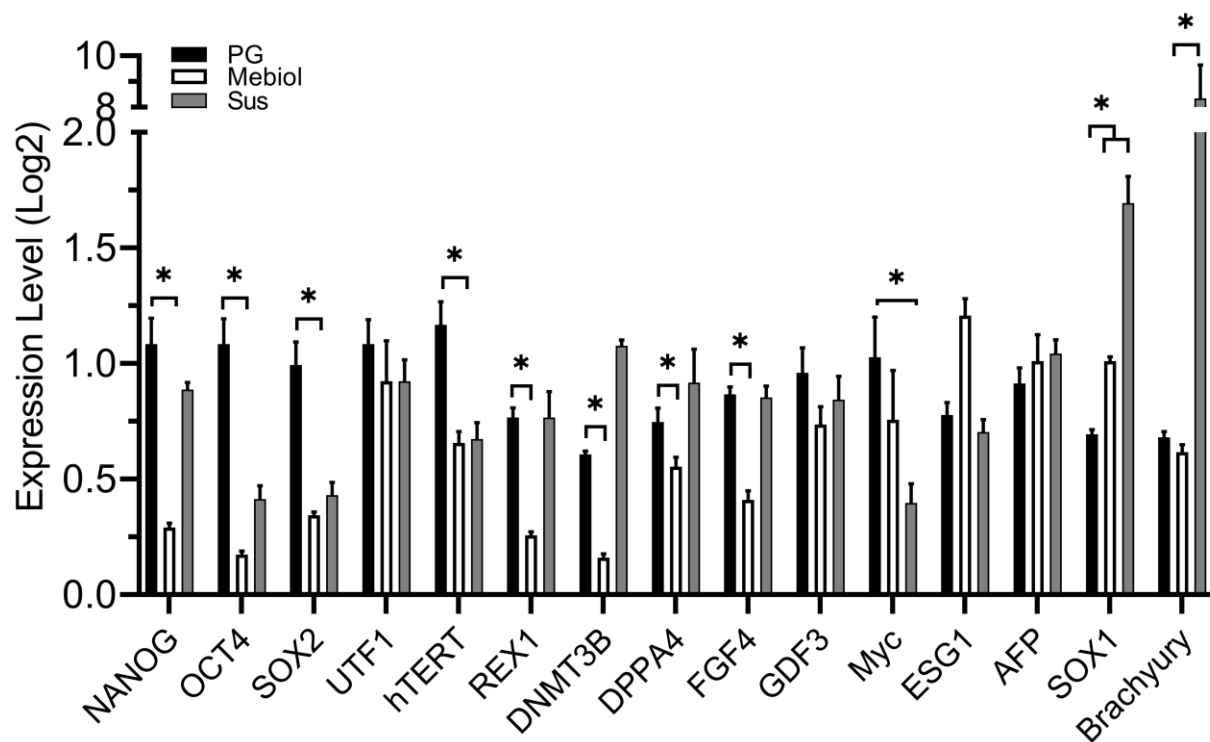


Figure 2.3 Pluripotency-related gene expression of ASC-hiPSCs from 3D PGmatrix with mTeSR1 (PG), 3D Mebiol gel with E8 (Mebiol), and suspension spheroids from nonadherent U-bottom plates (Sus). Data from each passage were obtained in triplicate and from three separate experiments. ASChiPSCs harvested immediately before parallel culture were used as controls. Data are shown as means $\pm$ SDs. *: $p < 0.05$.

Likewise, the expression levels of *REX1*, *DNMT3B*, and *FGF4* were also significantly reduced in the hiPSCs grown in Mebiol-E8. This implies that hiPSCs grown in Mebiol-E8 were more likely to unwanted differentiate since *DNMT3B* is responsible for de novo DNA methylation¹²⁷. These results indicated that spheroids formed in the 3D PEG-based hydrogel had lower pluripotency than those in the 3D PGmatrix. In addition, hiPSC spheroids formed in suspension with nonadherent U-bottom 96-well plates using 2D-cultured ASC-hiPSCs¹²⁸ were

also included for comparison (Figure 2.3). We observed strong upregulation of SOX1 and Brachyury and downregulation of NANOG, OCT4, SOX2, hTERT, and Myc, indicating initiation of unwanted differentiation in the hiPSC spheroids formed by the U-bottom suspension method. This finding suggested that the suspension method may be used to initiate differentiation but is not suited for the maintenance of hiPSC integrity.

Decrease in gel strength revealed role of mechanical cues

We suspected that the less satisfactory maintenance of hiPSCs in 3D Mebiol gel might relate to the low degree of gel degradation in culture, we thus monitored the gel degradation of PGmatrix during culture. It was found that the gel strength of PGmatrix rapidly decreased from

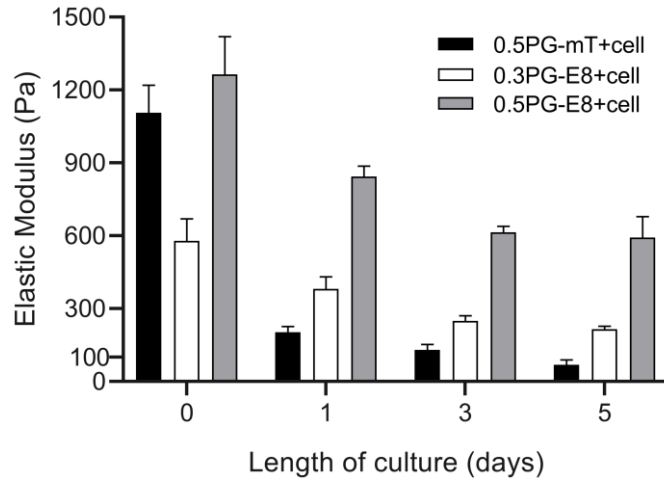


Figure 2.4 Changes in gel elastic modulus in PGmatrix during a 5-d culture period in culture media at varied peptide concentrations with ASC-hiPSC encapsulation. Data are shown as means $\pm$ SDs.

Day 1 after cell seeding in mTeSR1, while it was slowly reduced in culture with E8 medium (Figure 2.4). On Day 5, the final gel strength in mTeSR1 was about 39 Pa, whereas in E8 medium, it was about 215 Pa and 593 Pa for 0.3% and 0.5% PGmatrix gel, respectively (Figure 2.4). The gel strength results were clearly oppositely related to cell

growth results shown in Figure 2.2. The gel strength for 0.5% PGmatrix-mTeSR1 (0.5PG-mT) decreased the most rapidly, leading to the highest fold-expansion and viability. However, the 0.5% PGmatrix-E8 (0.5PG-E8) group had the lowest reduction in gel strength with the lowest

fold expansion and viability. While the gel strength, by soaking the gel alone without cells in these culture media, remained the same in E8 and decreased gradually in mTeSR1 (Figure 2.5). This finding suggested that gel degradability may affect 3D spheroid growth performance.

There are many cues to determine cell fate, and among those, physical cues, chemical cues, and biological cues have been highly recognized. Cell-environment

interactions have been proven to be an important physical cue for 3D culture. Stem cells secrete various substances during their growth, such as proteases and ECM proteins^{129–131}. The Hippo pathway has attracted increased attention as researchers attempt to elucidate the role of mechanical cues in stem cell niches. Mechanosensing genes YAP and TAZ¹³² are at the center of this signaling cascade. Therefore, we determined the expression levels of YAP and TAZ in the hiPSCs cultured in several 3D culture conditions (Figure 2.6). We found that the YAP/TAZ expression levels did not differ between 0.5% and 1% PGmatrix with mTeSR1 but decreased significantly in the hiPSCs grown in 0.5% PGmatrix with E8 and in Mebiol gel with E8 medium (Figure 2.6). The different expression levels were inversely related to the degradability of the

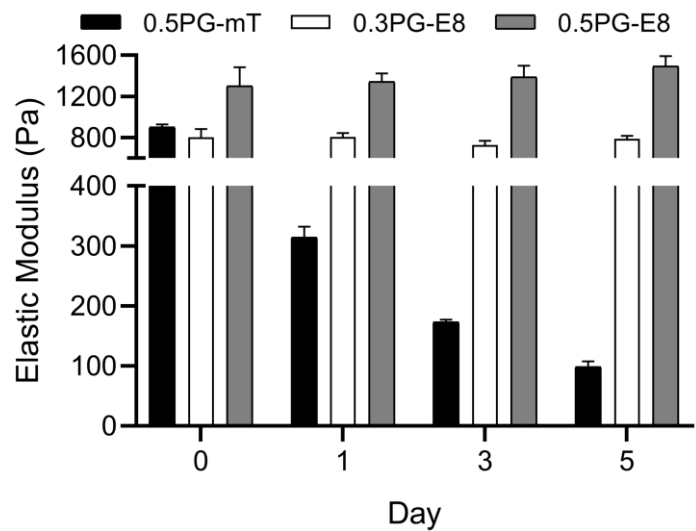


Figure 2.5 Changes in gel elastic modulus in PGmatrix during a 5-d culture period in culture media at varied peptide concentrations with ASC-hiPSC encapsulation. Data are shown as means $\pm$ SDs.

gel, thus suggesting that the expression of YAP/TAZ was linked to gel degradability. Thus, the low fold expansion and viability observed with the hiPSCs grown in Mebiol gel is likely the

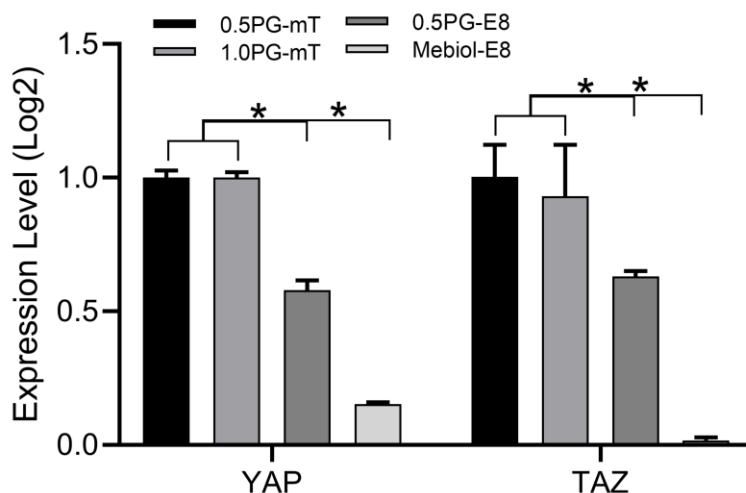


Figure 2.6 Expression of YAP/TAZ in the hiPSCs grown under different 3D culture conditions. Data are shown as means $\pm$ SDs. *: $p < 0.05$.

result of a nondegradable microenvironment (Figure 2.2, 2.6). Previously, we did not find clues of hiPSC adhesion directly to PGmatrix nanofibers (data not shown), which suggests that PGmatrix would not directly bind to hiPSCs to support their growth.

Proposed mechanism for 3D maintenance of hiPSC

We speculated that when encapsulated in a 3D matrix, factors from the culture medium and secreted by hiPSCs may continually modify the surrounding environment to favor cell proliferation and pluripotency maintenance. As illustrated in Figure 2.7, initially following the encapsulation of sufficient number of hiPSCs, some soluble factors like insulin, bFGF, TGF β , and Nodal from medium supplement would bind to surface receptors such as GPCRs (G protein-coupled receptors) and RTKs (receptor tyrosine kinases) to activate essential pathways including phosphoinositide-3-kinase–protein kinase B/Akt (PI3K/Akt) for survival¹³³. PI3K/Akt responding to signaling from adhesion and binding of various surface receptors with soluble

factors has been proven essential for pluripotent stem cell maintenance^{134–137}. During this initial

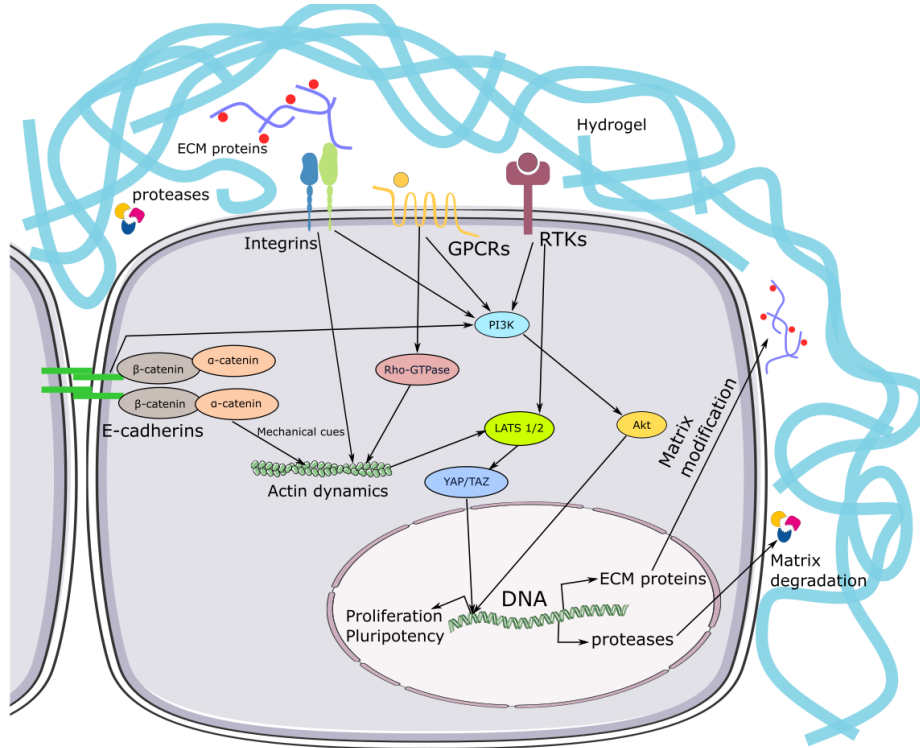


Figure 2.7 Proposed mechanism for hiPSC growth and maintenance of pluripotency in 3D PGmatrix hydrogel.

survival and adaptation period, hiPSCs would initiate their secretion of ECM proteins and some proteases that would start remodeling the surrounding PGmatrix environment through matrix degradation and modification. Meanwhile, the secreted ECM proteins such as laminin and vitronectin would bind to integrin that would trigger various related signaling pathways to promote proliferation and pluripotency maintenance^{130,138,139}. The microenvironment of ECM-protein-modified-PGmatrix would facilitate cell migration, leading to increased cell-cell contact via E-cadherin^{140–142}. Cell-ECM protein signaling via integrin and cell-cell signaling via E-cadherin are closely linked to cell cytoskeleton, these binding events occurred at cell surface would affect actin dynamics and relay the signals through Hippo pathway. With appropriate mechanical signals, the signaling cascade including LATS 1/2 would cause upregulation of the

mechanosensitive YAP and TAZ proteins, as observed in the RT-qPCR analysis (Figure 2.6). These YAP/TAZ proteins would then relocate into the nucleus to affect gene expression related to pluripotency maintenance^{143–147}. Critical maintenance pathways including PI3K/Akt can be enhanced by cells through “remodeling” of their microenvironment. Soluble signaling factors like bFGF and TGF β are also part of hiPSC secretome, that would contribute to hiPSC maintenance through paracrine signaling. Therefore, the degradability or modifiability of a 3D matrix should be a critical property influencing hiPSC growth performance and maintenance of pluripotency, which has been proven true for neural progenitor cells¹³². It would be integrating to further confirm the role of PGmatrix in Hippo signaling pathway.

Conclusion

By comparing two 3D maintenance hydrogel systems for hiPSC, we found that PGmatrix outperformed Mebiol Gel, promoting rapid and consistent cell growth with high cell viability. By examination of changes in hydrogel mechanical properties during culture, it was shown that the gel degradability by cells strongly favors hiPSC growth performance, and this relationship is speculated to link to the Hippo signaling pathway and the mechanosensitive YAP/TAZ pathway. We believe that usage of this 3D platform in stem cell research will not only promote the production of high-quality hiPSCs, which could lead to improvement of differentiation efficiency in generations of various types of somatic cells including hepatocytes, but also provides a new tool to manufacture hiPSC at an industrial scale for downstream applications.

Acknowledgements

We sincerely thank Dr. Xuming Liu, Dr. Jianfa Bai and the Kansas State Veterinary Diagnostic Laboratory for their help in conducting PCR experiments and result analysis. We acknowledge Dr. Guangyan Qi for her help in PGmatrix preparation and hydrogel rheological analysis.

Chapter 3 - Physiological 3D Differentiation of hiPSCs Towards Hepatocyte Organoids in Tailored Peptide Hydrogel

Abstract

Liver is the key organ for metabolism and detoxification, failure in liver function is among most common causes of death worldwide. Pairing of donor and recipient have greatly limited treatment availability of liver transplant. As an alternative, hepatic cell transplantation (HCT) can take advantage of donor liver that was not suitable for liver transplantation or unused. However, this new treatment is still largely restricted by donor availability. To solve this problem, hepatocytes derived from human induced pluripotent stem cells (hiPSCs) were developed. Compared to earlier 2D methods, more recent hepatic differentiation studies tend to adopt 3D protocols that improved differentiation efficiency. To date, all published 3D methods have started with hiPSC single cells and resulted in dense hepatocyte-like cell spheroids that do not well resemble real liver organization and showed limited hepatocyte functions. In this study, we developed a novel 3D hepatic differentiation protocol based on synthetic peptide hydrogel Peggel. We formulated hydrogel so that Peggel provides nanofiber network matrix support for differentiating spheroids yet functions as suspension culture. Using previously published culture method, hiPSCs were physiologically grown into spheroids in 3D culture and the hiPSC spheroids were put directly into Peggel for 3D differentiation towards hepatocyte organoids without dissociation into hiPSC single cells. With elimination of enzymatic dissociation step, physiological properties of the highly organized hiPSC spheroids can be preserved, better mimicking liver development process. As a result, we obtained human induced hepatocyte (hiH) organoids that developed complex structures and polarity. These organoids not only expressed

hepatocyte characteristic genes *HNF4A* and *TTR* similar to human primary hepatocytes (hPH), but also showed significantly superior CYP enzymatic functions *in vitro* compared hepatocyte organoids differentiated in non-adherent suspension. These promising results indicated the superior differentiation efficiency of this novel 3D hydrogel differentiation method and suggested the importance of using physiological hiPSC spheroids instead of hiPSC single cells as starting materials. HiH organoids generated with our novel methods may serve as potential candidates for HCT.

Introduction

Liver is the central organ for metabolism³⁷. Hepatocytes are parenchymal cells that make up about 60% of the liver cells and are responsible for most liver functions³⁹. Some major functions performed by hepatocytes include: 1) synthesis and secretion of serum proteins like α -fetoprotein, albumin, transferrin, plasminogen, fibrinogen³⁷, 2) regulation of insulin and glucose, 3) lipid uptake and metabolism, 4) detoxification of general fat-soluble toxins by cytochrome P450 (CYP) enzymes, 5) nitrogen detoxification through urea cycle^{37,40}. Because of its critical functional role, liver failure is one of the most common cause of death worldwide¹⁴⁸. While whole organ transplant cannot fulfill treatment needs, hepatic cell transplantation (HCT) has been proposed as an alternative therapy. However, research on HCT is greatly hindered by the scarcity of primary hepatocytes (PHs). This shortage in research materials also affected other studies on liver toxicity^{149,150}, liver disease modeling¹⁵¹ and liver tissue engineering^{152–154}.

To solve this problem, *in vitro* differentiation towards hepatocytes using human pluripotent stem cells has been explored. Human embryonic stem cell were initially used to generate hepatocyte-like cells (HLCs)^{43,155,156}. With the emergence of human induced pluripotent

stem cells (hiPSCs)⁹, hiPSCs has become the major and highly promising source for *in vitro* hepatic differentiation, as it allows generation of patient-specific cells for disease modeling and therapeutic applications.

The more common approach for hepatic differentiation has been to culture hiPSCs with 2D method as monolayers on coated surfaces, which has often led to poor pluripotency maintenance in hiPSCs and inefficient differentiation results¹¹⁴. Recently, more researchers has focused on 3D methods that has been shown to improve differentiation efficiency and generated HLCs with better functional perperties⁴⁴, as 3D spheroids can better recapitulate the *in vivo* hepatogenesis process^{44,157,158}. However, all 3D hiPSC spheroids used in these studies were generated by forcing single cells to aggregate in non-adherent environments^{128,157}. Spheroids formed in suspension is nowhere near that during embryogenesis and the resulting HLCs were still far from PHs in terms of *in vitro* and *in vivo* functions^{61,157}. In this study, we demonstrate generation of functional human induced hepatocyte (hiH) organoids from 3D grown hiPSC spheroids. These physiologically relevant hiPSC spheroids can greatly improve hepatic differentiation results.

Another problem with current 3D hepatocyte differentiation research is underestimating the role of extracellular matrix (ECM)⁵¹. ECM proteins not only provide anchoring sites for cells, but also provide biochemical and mechanical signals that modulate cell performances. In this study, we adopted a fully synthetic peptide hydrogel (PepGel) 3D suspension matrix, which is further tailored to provide optimal conditions for 3D hiPSC differentiation towards hiH organoids. This technology can serve as a potential solution for production of *in vitro* hiH organoids, which will benefit clinical research and development in liver tissue engineering and therapeutic applications.

Materials and Methods

3D Culture of hiPSC

hiPSC 3D encapsulation and culture hiPSCs derived from human foreskin fibroblast (Applied Stemcell Inc., Milpitas, CA, USA) were maintained in 0.5% PGmatrix 3D culture with mTeSR1 medium (Stemcell Technologies, Cambridge, MA, USA) supplemented with ROCK inhibitor Y27632 (Stemcell Technologies) following steps described by Li et al¹⁵⁹. Briefly, hiPSCs suspended in mTeSR1 medium as single cells or small clusters were mixed with PGworks and PGmatrix hiPSC matrix (PepGel LLC, Manhattan, KS) following manufacturer's instructions and plated into 24-well plate. After incubated at 37°C for 30 minutes to complete gelation, mTeSR1 medium (Stemcell Technologies) supplemented with 10μM ROCK inhibitor Y27632 were added gently on top of the hydrogel. Medium was changed every day since day 3 and cultured up to day 5.

hiPSC 3D colonies (spheroids) retrieving and differentiation preparation Cells were harvested according to growth performance, usually on day 5 of each passage. At the end of each passage, hydrogel was disrupted by thorough pipetting. The mixture was collected and transferred into a 50ml centrifuge tube. The wells were washed with Dulbecco's Phosphate Buffered Saline (DPBS) without Ca²⁺/Mg²⁺ (Sigma-Aldrich Inc., St. Louis, MO, USA) and collected into a 50ml tube. More DPBS was then added to dilute the mixture more than 20 folds, followed by centrifugation at 200 g for 5 minutes.

To prepare differentiation, for 3D system, after removing the supernatant, hiPSC spheroids were directly resuspended in embryoid body (EB) medium for hepatic differentiation in PGmatrix 3D Suspension kit (PePGel LLC). For the U-shape Ultra-low attachment 96-well

plate (U96) (Corning, Glendale, AZ, USA), the hiPSC spheroids retrieved from 3D culture was dissociated into single cells by resuspending in TrypLE (Thermo Fisher Scientific, Waltham, MA, USA), incubated at 37°C for 30 minutes. After 30 minutes, mTeSR1 medium was added then centrifuged at 200 g for 5 minutes. Following removal of supernatant, the hiPSC single cells were resuspended in EB medium for U96 plating for differentiation.

Hepatic differentiation

The differentiation process followed Pettinato *et al.*¹⁵⁷ 4-stage protocol with modifications. The overall process is shown in Figure 3.1. The day of plating is noted as day -1, hiPSC spheroids or single cells were resuspended in EB medium (Table 3.1), encapsulated in PGmatrix 3D Suspension kit or plate into U96 plate and incubated at 37°C overnight. On day 0, differentiation started towards definitive endoderm by adding Activin-A, basic fibroblast growth factor (bFGF) and Transforming Growth Factor-beta (TGF- β) (PeproTech, Cranbury, NJ, USA). All differentiation supplements were purchased from PeproTech unless otherwise specified. Day 4, medium was switched to EB medium supplemented with bone morphogenetic protein 4 (BMP-4) and fibroblast growth factor 4 (FGF4) for differentiation toward foregut endoderm. Stage 3 started on day 8 by switching to EB medium with Wnt inhibitory factor 1 (WIF-1) (R&D systems, Minneapolis, MN, USA) and dickkopf-related protein 1 (DKK-1) for hepatoblast differentiation. The final stage of hepatocyte maturation started on day 12 with supplementation of oncostatin M (OSM) and hepatic growth factor (HGF) and continued until day 16 (Figure 3.1). Medium was changed every other day during each stage.

Plating method differs for different treatment groups. For differentiation in PG-suspension (PG-sus), hiPSC spheroids were resuspended in EB medium, then mixed with PG-

suspension (Peggel) and PGworks at a seeding density of 1.5×10^4 spheroids/ml (7500 spheroid per well for 24-well plate). The volume ratio of spheroid suspension: PG-suspension: PGworks is 5.78:1:0.07 on Day -1. The plate was then incubated at 37°C. For medium switching at the beginning of each stage (Day 0, 4, 8, and 12), spheroids were recovered by diluting hydrogel with DPBS (no $\text{Ca}^{2+}/\text{Mg}^{2+}$) (Sigma) and centrifuged at 700g for 5min. The spheroids were then resuspended in fresh medium for the next differentiation stage and mixed with PG-suspension and PGworks at a ratio of 3.05:1:0.04. Every other day, fresh medium was added followed by gentle mixing to ensure even dispersion (Figure 3.1). For differentiation in PGmatrix hydrogel (PG-embedded), hiPSC spheroids resuspended in EB medium were mixed with PGworks and PGmatrix at the same ratio used for hiPSC 3D culture. After 30 minutes of gelation at 37°C, medium was added on top of the gel. To switch to the next stage, the medium on top of the gel was replaced by medium for next stage. This medium change was performed 3 times on the day of switching with 3 hours in-between changes. For differentiation in suspension (U96-sus), hiPSC single cells were resuspended in EB medium and plated into U-shape Ultra-low attachment 96-well plate at 3.5×10^4 cells/well. Spheroid morphology was monitored with an Axio Vert A1 microscope (Carl Zeiss Microscopy, Germany).

Table 3.1 Differentiation media formulation

Embryoid Body (EB) Medium			
Reagent	Ratio (%)	Supplier	
DMEM/F12	93	Thermo Fisher	
Embryonic stem-cell qualified fetal bovine serum (ES-FBS)	5	Thermo Fisher	
Insulin-Transferrin-Selenium-Sodium Pyruvate (ITS-A) (100x)	1	Thermo Fisher	
55mM 1-Thioglycerol	0.1	Sigma-Aldrich	
Antibiotic-Antimycotic solution	1	Thermo Fisher	
Differentiation Supplements			

Day	Reagent	Final conc. (ng/ml)	Stock conc. (µg/ml)
0 2	FGF-2/bFGF	10	100
	Activin A	100	100
	TGFβ	10	100
4 6	FGF-4	10	100
	BMP-4	10	100
8 10	WIF-1	1000	200
	DKK-1	100	100
12 14	HGF	50	100
	OSM	30	100

Reverse transcription quantitative polymerase chain reaction (RT-qPCR)

Expression of hepatocyte marker and functional genes were tested using RT-qPCR. Upon completion of the differentiation process, organoids were retrieved, and total RNA samples were extracted with Direct-zol RNA MiniPrep kit (Zymo Research Corp., Irvine, CA, USA) and diluted to 20ng/µl. RT-qPCR reactions were conducted with a Bio-Rad CFX96™ Touch™ Real-time PCR Detection System using iTaq Universal SYBR Green One-Step kit (Bio-Rad, Hercules, CA, USA). The first reverse transcription reaction was set at 50 °C for 10 min, then polymerase activation and cDNA denaturation were set at 95°C for 1 min. Next, the samples went through 45 cycles of denaturation at 95°C for 10 s and annealing at 58°C for 30 s and extension at 72°C for 20 seconds. Primer sequences are available in Table 3.2.

Table 3.2 Gene and primers for RT-qPCR

Gene		Sequence	T _m
<i>ALB</i>	Forward	GATCTGCTTGAATGTGCTGATG	60
	Reverse	ATGCAGTGGGATTTTCCAA	
<i>HNF4A</i>	Forward	GTGGACAAAGACAAGAGGAACC	60
	Reverse	GCTGTCCTCATAGCTTGACCTT	
<i>A1AT</i>	Forward	GGTGCTGCTGATGAAATACCTG	60
	Reverse	TTTCCAGGTGCTGTAGTTTCC	
<i>AFP</i>	Forward	CTGCACTTCTTCATATGCCAAC	60

	Reverse	GAGGGACATATGTTTCATCCAC	
<i>CYP3A7</i>	Forward	TGACTAGAGCAAGTTTCATGTTCAC	60
	Reverse	CCTCCCTGAAAGGTTTCAGTAAA	
<i>ASGR1</i>	Forward	GAGACGTTTCAGCAACTTCACAG	60
	Reverse	CTCAGGTCCTTCTGCTGTTTCT	
<i>CYP3A4</i>	Forward	GTATGGAAAAGTGTGGGGCCTTT	60
	Reverse	CATTCTTTCAGTAGCACTGTTTTG	
<i>TDO2</i>	Forward	GCAAAAAGAGGTGCTACTGTCC	60
	Reverse	CTCCCTGAAGTGCTCTGTATGA	
<i>TTR</i>	Forward	AATCCAAGTGTCTCTGATGGT	60
	Reverse	GCTCTCCAGACTCACTGGTTTT	
<i>PSMB6</i> ¹⁶⁰	Forward	GAAGACCTGATGGCGGGA	60
	Reverse	CGGAGCCTCCAATGGC	

Immunofluorescence

HLC organoids were collected and fixed with 10% neutral buffered formalin (NBF) (Sigma-Aldrich). After fixation, the organoids were washed with wash buffer (DPBS with $\text{Ca}^{2+}/\text{Mg}^{2+}$ supplemented with 0.2% Triton-X 100 and 0.1% gelatin from cold water fish) (all from Sigma-Aldrich) and blocked with 10% normal goat serum (Thermo Fisher) in wash buffer overnight. Samples were then incubated with primary antibodies (Table 4.3) in blocking solution at 4°C overnight. After washing 3 times with wash buffer, the samples were incubated with corresponding secondary antibodies (Table 3.3) at 4°C overnight followed by nuclei staining with 4',6-diamidino-2-phenylindole (DAPI) solution (Abcam, Cambridge, UK) for 1 hour. The samples were then washed 3 times with wash buffer to remove excess reagent and mounted onto glass slides for fluorescence imaging with a Zeiss LSM 700 confocal microscope (Carl Zeiss Microscopy).

For staining of polarity markers, the samples were fixed with 10% NBF then embedded in Tissue-Tek® O.C.T. Compound (Sakura Finetek USA, Inc., Torrance, CA, USA), frozen and sliced with a Leica CM3050S cryostat (Leica Biosystems, Deer Park, IL, USA) into 14 µm slices and mounted onto glass slides. The slides were dried at 37°C for 2 hours before the immunostaining process.

Table 3.3 Antibodies for Immunofluorescence

Antibody	Supplier	Dilution Ratio
Phalloidin-iFluor 488	Abcam	1:1000
alpha Tubulin Monoclonal Antibody (DM1A)	Thermo Fisher	1:400
Rabbit AFP Polyclonal Antibody	Thermo Fisher	1:200
Rabbit Albumin Polyclonal Antibody	Thermo Fisher	1:200
Mouse HNF4A Monoclonal Antibody (K9218)	Thermo Fisher	1:1000
Rabbit ABCB11 Polyclonal Antibody	Thermo Fisher	1:500
Mouse Anti-alpha 1 Sodium Potassium ATPase antibody	Abcam	1:500
Goat anti-Rabbit IgG (H+L) Cross-Adsorbed Secondary Antibody, Alexa Fluor™ 488	Thermo Fisher	1:500
Goat anti-Mouse IgG (H+L) Highly Cross-Adsorbed Secondary Antibody, Alexa Fluor™ Plus 647	Thermo Fisher	1:500

Oil Red O staining

Lipid storage was detected with an Oil Red O stain kit (Abcam) following manufacturer's protocol with modifications. Organoids were fixed with 10% NBF for 1 hour, then washed with DPBS with $\text{Ca}^{2+}/\text{Mg}^{2+}$. The fixed organoids were then resuspended in propylene glycol, plated into clear well plate and incubated at room temperature (RT) for 2 minutes, followed by 6-minute incubation with Oil Red O solution. The organoids were then incubated with 85% propylene glycol for 1 min and washed twice with deionized water. Staining was observed with an Axio Vert A1 microscope.

Periodic Acid-Schiff (PAS) staining

Glycogen storage was detected by periodic acid-Schiff (PAS) staining system (Sigma) following manufacturer's protocol with modifications. Organoids were fixed with 10% NBF for 1 hour followed by washing with DPBS with $\text{Ca}^{2+}/\text{Mg}^{2+}$. The samples were suspended in periodic acid solution and plated into clear well plate for 5-minute oxidization at RT, then washed 3 times with deionized water. Schiff reagent was then added followed by 15 minutes incubation at RT. After incubation, deionized water was added for 5 minutes for color development. Samples were observed with an Axio Vert A1 microscope.

Indocyanine Green (ICG) uptake

This assay showed general metabolic activities of organoids before and after hepatic differentiation. ICG powder (Sigma-Aldrich) was dissolved in dimethyl sulfoxide (DMSO) (Sigma-Aldrich) at 5 mg/ml. The stock was aliquoted and stored at -80°C . The stock solution was thawed and diluted with EB medium to reach a final concentration of 1mg/ml right before use. Organoids retrieved were washed with DPBS, resuspended in 1mg/ml ICG solution, and incubated at 37°C for 1 hour. The organoids were then washed with DPBS before imaged with an Axio Vert A1 microscope.

Intracellular urea content

Intracellular urea content was measured with a colorimetric kit (Abcam) following manufacturer's protocol. Samples containing about 2×10^6 cells were washed with cold PBS and resuspended in assay buffer, then homogenized by passing through a 31-gauge needle (Air-Tite Products Co., Inc., Virginia Beach, VA, USA). The samples were centrifuged for 5 minutes at 3000 g to remove insoluble material. The supernatant was collected and kept on ice. Standards,

background, and test samples were prepared following manufacturer's instructions in a 96-well plate and incubated at 37°C in dark for 1 hour before measured at 570 nm with SpectraMax Plus Absorbance Microplate Reader (Molecular Devices, LLC., San Jose, CA, USA).

Cytochrome P450 activity

Activities of cytochrome P450 (CYP) enzymes were measured to evaluate hepatocyte detoxification functions. Promega P45-Glo kits (Promega, Madison, WI, USA) were used to assess the functions of CYP3A4, and CYP1A2 following manufacturer's instructions. After completion of hepatic differentiation, organoids were retrieved and plated in triplicates into a U96 plate at about equal cell numbers. The samples were incubated with EB medium supplemented with 0.1% DMSO (for control), or 50µM Omeprazole (for CYP1A2), or 20µM Rifampicin (for CYP 3A4) at 37°C for 72 hours with medium change every 24 hours. After drug induction, the samples were washed 3 times with DPBS and incubated with corresponding CYP substrates for 1 hour. Then, 25µl/well of the incubated solution was taken and mixed with Luciferin Detection Reagent, the sample organoids left were mixed with CellTiter-Glo Cell Viability Assay reagent (Promega). The plate was then placed on a Fisherbrand microplate shaker (Fisher Scientific) for 5-minute mixing at 1000 rpm, incubated in dark at room temperature for 20 minutes before measuring luminescence with a GloMax Navigator luminometer (Promega). Enzyme activity level = P45-Glo reading / CellTiter-Glo reading $\times 10^6$.

Statistical Analyses

Statistical analysis was performed with JASP Ver 0.18.1 (The JASP Team). Comparison between groups were carried out using ANOVA, followed by post hoc tests with Tukey correction with 95% confidence interval.

Results

Scalable 3D suspension peptide hydrogel for robust hiH organoid differentiation

Previously, we developed a 3D hydrogel system that maintains hiPSCs with fast proliferation and good pluripotency¹⁵⁹. We hypothesized that these hiPSCs naturally grown in 3D hydrogel will be better qualified for improved differentiation results compared to 2D-cultured hiPSCs. Though the original PGmatrix had sufficient performance in maintaining hiPSC, the optimal condition for 3D hepatic differentiation remains to be developed. Since 3D differentiation in non-adherent suspension was reported to yield HLCs with improved hepatocyte properties¹⁵⁷, we adopted PepGel 3D suspension matrix (PG-sus), a peptide hydrogel that provides nanofiber network matrix support for 3D embedded hiPSC spheroids yet functions as suspension culture. When encapsulated in PG-sus, cells are surrounded by organized peptide fibers that provide weak support. After addition of fresh medium, the well contents were gently mixed by pipetting, disrupting the peptide fiber network, leading to a liquid-like state. During subsequent incubation, peptide fibers reorganize to recover to organized state, returning to hydrogel. The PG-sus hydrogel can thus provide weak support during hepatocyte differentiation with easy medium supplement and spheroids retrieval. Because of the difference in hydrogel properties between PGmatrix and PG-sus, we adopted the original 4-stage differentiation protocol for use with PG-sus (Figure 3.1). During this process, hiPSC spheroids were harvested

from 3D culture in PG-embedded and then embedded directly into PG-sus loaded with differentiation culture medium without any enzymatic digestion and re-clustering (Figure 3.1).

This practice helped to avoid enzymatic damage and preserved the naturally developed characteristics of hiPSC spheroids. At the end of each differentiation stage, spheroids were retrieved by simple centrifugation and then embedded in PG-sus again loaded with the next stage differentiation medium. The unique self-healing feature of the peptide hydrogel (PG-sus) allows

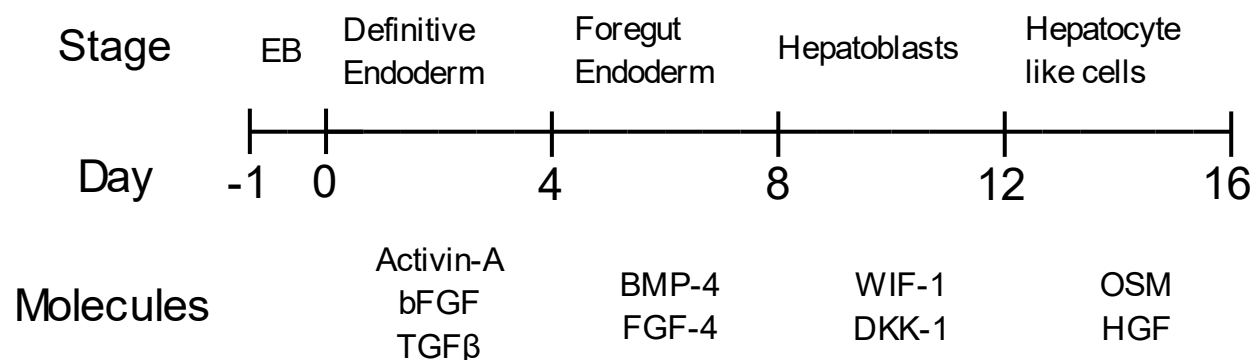


Figure 3.1 Schematic illustration of hiPSC maintenance and differentiation process in 3D PG hydrogel systems.

adding medium into the PG-sus matrix at any time during culture as needed. In addition, the PG-sus system can be easily scaled up using culture flasks up to liter scales and potentially further expanded in scales using stirring tank bioreactor with optimization.

hiH organoids possess superb gene marker expressions and polarized morphologic structures

The hiPSC spheroids grew more uniformly in PG-sus throughout the 4-stage differentiation process, developing into hiH organoids with complex hollow chamber structures (Figure 3.2a). hiH in PG-embedded system also generated structures similar to those observed in PG-sus. In contrast, in non-adherent U-shape plate (U96-sus) the aggregated hiPSC spheroids also started growing but remained as a single aggregate and finally differentiated into a dense

and large hiH spheroid with no apparent structural heterogeneity (Figure 3.2a). RT-qPCR analysis showed that hiH organoids in PG-sus not only had significantly better expressions of hepatocyte marker genes than those with U96-sus, but also outperformed PG-embedded organoids in all the genes tested. Specifically, Expression of *AFP*, *ALAT*, *CYP3A7* and *TDO2* were significantly elevated in PG-sus hiH organoids, which suggested much better maturation than U96-sus organoids. Moreover, the hiH organoids in PG-sus were even comparable to PHs in expression of *HNF4A* ($p=0.615$) and *TTR* ($p=0.813$) (Figure 3.2b). This indicated that hepatocyte differentiation favors 3D environment with weak support. The *HNF4A* gene encodes for hepatocyte nuclear factor 4 α , which is an important transcription factor enriched in liver and is critical to hepatocyte differentiation and maintenance^{161,162}. The expression of *HNF4A* increases when hiPSC-derived definitive endoderm differentiates towards the hepatic fate^{161,162}. The *TTR* gene encodes transthyretin, which is a transporter for thyroxine and vitamin A primarily produced in the liver¹⁶³. Elevated *TTR* expression was observed from stage 2 (data not shown), indicating differentiation towards hepatic lineage. However, the hiH organoids in PG-sus produced high levels of *AFP* and relatively low levels of *ALB* and *CYP3A4* compared to PHs, indicating their premature state. This is an understandable situation as hepatocytes in the newborn can take up to two years to achieve full maturity¹⁶⁴.

hiH organoids in PG-sus demonstrated higher differentiation efficiency and multi chamber structures. Upon completion of hepatic differentiation, we stained the hiH organoids generated in PG-sus and U96-sus for AFP, ALB, and HNF4A proteins and found that both types of organoids expressed these characteristic proteins (Figure 3.2c). Nonetheless, these proteins were evenly distributed in hiH organoids in PG-sus, but for U96-sus they mostly existed on the organoid surfaces observed from Z-stack images (Figure 3.2d), indicating better differentiation

efficiency of hiH organoids in PG-sus. Furthermore, multi chamber structures were observed in hiH organoids in PG-sus (Figures 3.2c, d), which can be caused by hepatocyte polarity¹⁶⁵. One key polarity mechanism of hepatocytes is to induce morphologic integrity of tight junctions and apical plasma membranes that allow the formation of microvilli and bile canaliculi networks to support important liver functions¹⁶⁵. Polarization requires energy, as well as carefully orchestrated cooperation between cell adhesion molecules, cell junctions, cytoskeleton, extracellular matrix and intracellular trafficking machinery¹⁶⁵. Hence, we cut the hiH organoids with a cryostat into 14- μ m-thick slices and stained them with apical membrane protein ATP-binding cassette, subfamily B, member 11 (ABCB11) and basolateral protein $\alpha 1$ Na⁺/K⁺-ATPase (NKA α 1). Examination with confocal microscopy indicated that hiH organoids in U96-sus had ABCB11 production on most cells but barely expressed NKA α 1 (Figure 3.2e). On the other hand, the hiH organoids in PG-sus expressed elevated levels of NKA α 1 in addition to ABCB11, where the merged image showed co-localization of the two proteins on different sides of some cells (Figure 3.2e arrows), suggesting that polarity was developed in the hiH organoids in PG-sus and attributed to the multi chamber morphologic structures within the organoids. This observation coincides with our PCR result, PG-sus hiH organoids had higher level of maturation than U96-sus, but they were still premature compared to PHs. Taken together, the 3D PGmatrix systems provided desirable microenvironment to support the formation of physiologically

relevant hiPSC spheroids that were then further transformed into polarized multi-chamber morphologic structured hepatocyte organoids.

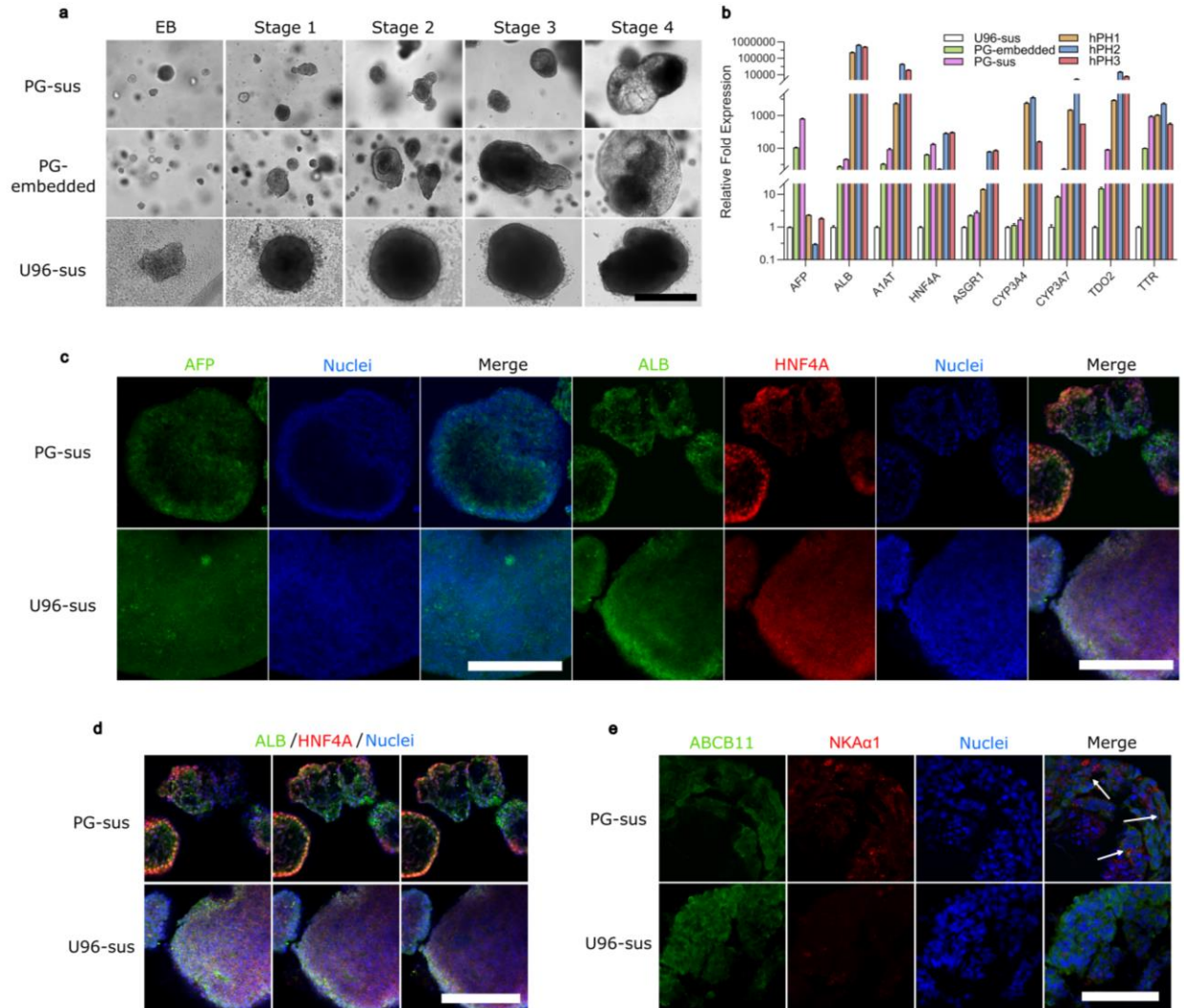


Figure 3.2 hiH organoids in PG-sus showed superior morphological and hepatocyte characteristics compared to U96-sus spheroids. **a**, Morphology of organoids in different 3D systems during hepatic differentiation. Scale bar 300μm; **b**, Expression of hepatocyte marker genes in organoids generated from different 3D systems in comparison to 3 primary hepatocytes; **c**, Hepatocyte marker protein expression in PG-sus and U96-sus organoids. Scale bar 200μm; **d**, hiH organoids from PG-sus and U96-sus stained for ALB and HNF4A viewed at different Z plane. Scale bar 200μm; **e**, hiH organoids from PG-sus and U96-sus stained for polarity markers ABCB11 and NKAα1. Arrows showed existence of both markers on different side of cells. Scale bar 100μm.

hiH organoids showed intriguing functional properties *in vitro*

The hiH organoids were characterized *in vitro* for nutrients storage, metabolic properties, and cytochrome P450 (CYP) enzyme activities. The hiH organoids in PG-sus were able to store neutral lipids and glycogen and showed metabolic capacity. hiH organoids differentiated in PG-sus were stained with Oil Red O and revealed the ability to store neutral lipids¹⁶⁶ (Figure 3.3a). hiPSC spheroids before differentiation were used as the negative control. Periodic acid–Schiff (PAS) staining resulted in dark purple color, indicating storage of glycogen in differentiated cells¹⁶⁷. Uptake of indocyanine green (ICG) by hiH organoids demonstrated the existence of hepatocyte specific basic metabolic functions¹⁶⁸. PG-sus hiH organoids were able to adsorb ICG in the surrounding environment. Ammonium metabolism is often assessed by measuring urea secretion into the medium when hepatocytes are incubated with ammonium chloride¹⁶⁹. However, this method requires accurate cell viability measurements for the final calculation. Despite starting with similar cell numbers and viability of the tested hiH organoids, viability changes during the necessary 24-hour incubation introduces variability to the final calculation. Therefore, we measured the intracellular urea content as an indicator for ammonium metabolism and urea production with a colorimetric assay for hiH organoids and primary hepatocytes. The results showed no significant differences between hiH organoids from U96-sus, PG-sus, and PHs (Figure 3.3b), suggesting similar levels of urea generation capacity in the three groups.

To assess one of the most important hepatocyte function, detoxification capacity, we measured the activities of two representative P450 enzymes in liver, CYP1A2 and CYP3A4^{44,157}. CYP1A2 is a major form of human CYP1A subfamily and the last major CYP enzyme to mature after birth¹⁷⁰. Activity of CYP1A2 is important as it is the main clearance mechanism for about 10% of medications such as clozapine, duloxetine, and propranolol, as well as caffeine and

endogenous compounds including melatonin¹⁷¹. CYP3A4, on the other hand, is the most abundant and important CYP enzyme involved in over 40% of phase I metabolism¹⁷². Not only does CYP3A4 account for about 20% liver CYPs, it is also responsible for metabolism of close to 50% of drugs¹⁷³. Therefore, measurements of CYP1A2 and CYP3A4 activities can represent a large part of hepatocyte CYP activities, providing information on detoxification capacity of hiH organoids. Basal activity levels of these two enzymes were measured using DMSO as the inducer. hiH organoids in PG-sus had significantly higher basal activities than U96-sus hiH organoids in both CYP1A2 ($p=0.009$) and CYP3A4 ($p=0.005$) (Figure 3.3c, d). When induced, CYP1A2 activity increased for all three groups, but U96-sus organoids had significantly lower induced activity level than that in PG-sus ($p=0.022$). PG-sus hiH organoids had similar CYP1A2 activity level as those of PH whether induced or not ($p=0.926$, 0.333 respectively). Though PG-sus hiH organoids had lower CYP3A4 activity than PH ($p<0.001$), their basal activity level was notably higher than that of U96-sus organoids ($p=0.005$). For U96-sus, these CYP activities of the hiH organoids were significantly lower than those of PH with and without induction ($p<0.001$). These results suggested that hiH organoids in PG-sus had much higher level of maturation than those in U96-sus.

Discussion

In this research, we adapted a 4-stage hepatic differentiation protocol⁴⁴ for use with the PGmatrix peptide hydrogel so that growth and differentiation of hiPSCs can be carried out entirely in 3D environment. Upon development completion, mature hepatocytes in human liver are not directly densely packed clusters but separated by sinusoids and bile canaliculi¹⁷⁴. In many

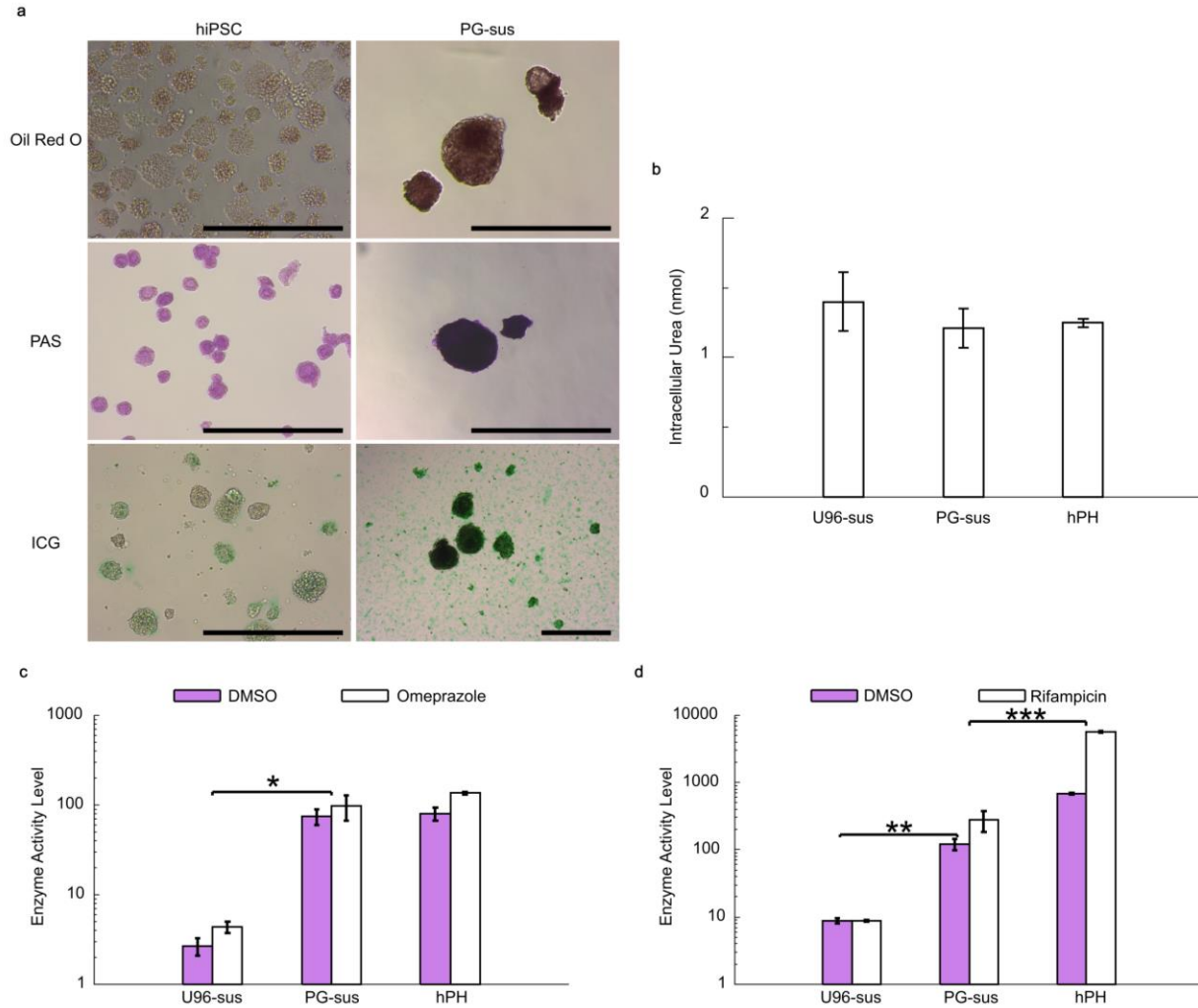


Figure 3.3 PG-sus hiH organoids showed major hepatocyte functional properties. a. PG-sus showed ability to store neutral lipids (Oil Red O) and glycogen (PAS) as well as basic metabolism (ICG) compared to undifferentiated hiPSC spheroids. Scale bar 300µm; b. U96-sus and PG-sus hiH organoids showed similar intracellular urea content to PH; c & d. Activity levels of CYP1A2 (c) and CYP3A4 in different hiH organoids and PH under control (DMSO) and induced conditions. Activity level = CYP assay luminescent intensity / viability luminescent intensity $\times 10^6$. Results are shown as mean $\pm$ SEM, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

studies, HLC organoids obtained from matrix-free suspension system were dense and compact cell clusters^{157,174,175}, as observed with U96-sus in this study (Figure 3.2a). These studies cells are aggregated into spheroids with limited cell performance leading to limited differentiation potential. In more recent publications, researchers were able to generate organoids that possessed some liver structural characteristics^{176,177}. However, these organoids were still generated from

dense cell clusters formed in suspension. The resulting organoids went through limited *in vitro* functional analyses and the *in vivo* hepatocyte function data of these organoids was not available. In terms of hepatocyte function, these organoids do not seem superior to U96-sus organoids generated in this study, indicating the limitation of conventional Matrigel or matrix-free suspension system. Some studies showed that mechanical cues do have an effect on differentiation^{178,179}, which is in agreement with our findings, hepatocyte marker gene expression was different when the differentiation was carried out in different 3D systems (Figure 3.2b). To date, all hepatocyte organoids derived from hiPSCs have included dissociation and aggregation steps¹⁸⁰. Though it is understandable since 3D culture systems for hiPSC are not widely used, the dissociation step completely interrupted normal development and forced hiPSCs to adopt unnatural 3D conformations, leading to very limited maturation and functions compared to PHs^{175,180,181}. In contrast, hiPSCs used in our study were maintained in 3D following previously published methods, which allowed spheroids to physiologically develop as the cells proliferated¹⁵⁹. By transferring these hiPSC spheroids directly into 3D PG matrices with differentiation medium, we eliminated the need to break down hiPSC colonies into single cells and preserved the structural characteristics of these hiPSC spheroids, mimicking the natural development process^{38,182}. In fact, this method has greatly improved differentiation efficiency towards hepatocytes. Structural heterogeneity can be visually observed in developing spheroids in both PG-sus and PG-embedded conditions (Figure 3.2a). When examining stained hiH organoids, the existence of hollow chamber structures can be clearly identified in PG-sus organoids (Figure 3.2c, d). By preserving physiological characteristics of the starting hiPSC spheroids, the resulting PG-sus hiH organoids showed much better maturation and hepatocyte functions than U96-sus counterparts, supported by comparison of gene expression (Figure 3.2b),

development of cell polarity (Figure 3.2e), as well as *in vitro* CYP enzyme activity assays (Figure 3.3c, d). Polarity is an important functional characteristic of mature hepatocytes^{165,183,184}. Though complete polarity shown in mature hepatocytes^{165,184} was not observed in PG-sus hiH organoids, we found clues of emerging polarization with apical and lateral membrane proteins co-existing on different sides of cells in PG-sus organoids (Figure 3.2e), indicating higher level of maturation than U96-sus organoids.

In addition, the easy handling feature made it possible to scale up production of hiH organoids with this 3D PG-sus systems, which would greatly benefit research requiring large amounts of organoid models like drug testing and development as well as cell therapies. However, it will require additional studies to elucidate the mechanisms of 3D hepatic differentiation and improve the hydrogel system to prepare for large scale production. Compared to liver organoids derived from adult or fetal cells¹⁸⁵, liver organoids generated with our 3D system are more accessible and easier to handle and will meet the huge demands for downstream applications including but not limited to *in vivo* translational research, tissue engineering via 3D bioprinting, drug screening, *in vitro* liver disease modeling, and future liver cell therapies.

Conclusions

In this study, we successfully differentiated hiHs in a PGmatrix 3D hydrogel system through 4-stage differentiation protocol using physiologically grown hiPSC spheroids. By carrying out maintenance and differentiation process entirely in 3D PG hydrogel, hiH organoids resulted from hiPSCs presented significantly better hepatocyte gene expression, complex structural properties with emerging cell polarity. *In vitro* analyses showed superior hepatocyte functions of these 3D PG-sus hiH organoids, compared to those differentiated in non-adherent

suspensions. The PG hydrogel systems not only improved differentiation efficiency for hiH organoids generation, but it can also be further improved for large scale production of hiH organoids, providing support for drug testing and clinical HCT therapies.

Acknowledgement

We sincerely thank Dr. Xuming Liu for his help on RT-qPCR experiments and Dr. Jianfa Bai for providing discussion on PCR data analysis. We would like to acknowledge Confocal Core Lab for their support on cryosectioning and confocal microscopy.

Chapter 4 - Injectable Peptide Hydrogel Encapsulation of Mesenchymal Stem Cell Improved Viability, Stemness, Anti-Inflammatory Effects, and Early Stage Wound Healing

Abstract

Human adipose-derived mesenchymal stem cells (hADMSCs) are adult stem cells and are relatively easy to access compared to other sources for mesenchymal stem cells (MSCs). They have shown immunomodulation properties as well as effects on improving tissue regeneration. To better stimulate and preserve the therapeutic properties of hADMSC, biomaterials for cell delivery have been studied extensively. To date, hyaluronic acid (HA)-based materials have been most widely adopted by researchers around the world. PGmatrix is a new peptide-based hydrogel that has shown superior functional properties in 3D cell cultures. Here we reported the *in vitro* and *in vivo* functional effects of PGmatrix on hADMSC in comparison with HA and HA-based Hystem hydrogels. Our results showed that PGmatrix was far superior in maintaining hADMSC viability during prolonged incubation and stimulated expression of SSEA4 (stage-specific embryonic antigen-4) in hADMSC. hADMSCs encapsulated in PGmatrix secreted more immune responsive proteins than those in HA or Hystem, though similar VEGF-A and TGFβ1 release levels were observed in all three hydrogels. *In vivo* studies revealed that hADMSCs encapsulated with PGmatrix showed improved healing of skin wound in diabetic-induced mice at early stage, suggesting possible anti-inflammatory effects, though similar re-epithelialization and collagen density were observed among PGmatrix and HA or Hystem hydrogels by day 21.

Introduction

Mesenchymal stem cells (MSCs) are adult stem cells found in bone marrow, adipose tissue, synovial fluid, and other human tissues¹⁸⁶. MSCs can generate and secrete a large variety of bioactive factors that can modulate immune response^{187–189}, promote angiogenesis¹⁹⁰, stimulate cell survival and proliferation, and affect differentiation of specific cells for tissue repair¹⁹¹. Studies have demonstrated great potential of MSCs and their secretome in alleviation or treatment of diseases, such as osteoarthritis^{192–196}, type 2 diabetes mellitus^{197,198}, obesity induced metabolic dysregulation¹⁹⁹, cardiovascular diseases¹⁹⁰, and graft-versus-host disease (GVHD)¹⁸⁹. Allogeneic MSC transplants were proven safe by multiple clinical studies^{200–202}, which expands therapeutic potentials of MSCs. MSCs can be isolated from a variety of tissue sources including bone marrow, adipose tissue, and cord blood²⁰³. Human adipose-derived MSCs (hADMSCs) are more easily accessible than MSCs from other sources, exhibit comparable therapeutic effects²⁰³, and have been widely adopted for cell therapy research^{193,194,199}. MSCs can be used in suspension directly after harvesting, or after preconditioning methods that alter cell properties^{189,204–207} or promote secretion of therapeutic factors^{208–211}. Existing preconditioning methods commonly used are hypoxia^{212–214}, stimulation with inflammatory factors^{215,216}, and 3D configuration²¹⁷. Various studies have investigated the formation of MSC aggregates that occur in a suspension setting and have found that such aggregate formation can negatively alter MSC functionalities, such as stemness and therapeutic factor secretion^{204,217,218}. For this reason, researchers have focused on 3D hydrogel encapsulation, as it is not only relatively easy to operate, but also serves as a delivery system^{219,220}. Shear-thinning hydrogels are usually preferred as they allow for injection, which simplifies transplantation and minimizes patient pain. In addition, hydrogel reduces mechanical stress on cells during injection, offers environmental cues

to improve cell viability, and promotes secretion of therapeutic factors²²¹. Both non-crosslinked hyaluronic acid (HA)²²² and hyaluronan-based hydrogel (Hystem)²²³ have shown positive effects on MSCs and have been widely adopted by researchers as promising delivery materials in cell-based therapies^{222–225}. However, the non-crosslinked HA gel and Hystem hydrogel are still relatively hard to handle, HA gel is sticky, while Hystem is temperature sensitive, which hinders their application in clinical settings. It is necessary to identify potential alternatives to advance clinical applications.

In addition to HA based hydrogels, peptide hydrogels have gained attention in recent years. Peptides can be designed from scratch to accommodate specific needs and are fully synthetic which ensures consistency across batches. Rationally designed peptide hydrogels can self-assemble to form nanoweb structures mimicking the natural ECM, thus supporting cell performance in a 3D space^{226–229}. Several peptide hydrogels have been utilized in life science research, including EAK16, RADA16 (commercial name PuraMatrix, BD Bioscience), Fmoc-FF, Fmoc-RGD, and h9e (commercial name PGmatrix, PepGel LLC). Among these peptide hydrogels, PGmatrix has shown significant advantages in 3D cell cultures^{227,230–232} and *in vivo* virus delivery by injection^{233,234}. PGmatrix possesses tunable nanoweb pore size, gel strength and indefinite sol-gel recovery properties²³², and superior biocompatibility without causing inflammatory effects in subcutaneous injection²³⁵. We hypothesized that PGmatrix would provide improved functional cyto-properties of MSCs in 3D encapsulation, as well as *in vivo* delivery.

The present study was aimed to compare the *in vitro* and *in vivo* properties of human adipose-derived mesenchymal stem cell (hADMSC) in 3D encapsulation within non-crosslinked HA, Hystem, and PGmatrix hydrogels. PGmatrix supported prolonged viability maintenance led

to high expression of the stem cell marker stage-specific embryonic antigen-4 (SSEA4) and elevated release of immune response related factors in hADMSC compared to HA and Hystem. In the diabetic mouse skin wound healing model, PGmatrix-encapsulated hADMSC showed better healing at an early stage, suggesting possible improvement in anti-inflammatory and immunosuppressive effects. Hypertrophic epithelium re-epithelialization was observed among all three hydrogel treated groups compared to the control group, but no significant difference in collagen density was found between the groups.

Materials and Methods

Culture of hADMSCs

2D cell culture method was used for hADMSC population expansion. To ensure quality of hADMSCs (ATCC, Manassas, VA, USA) for subsequent experiments, all hADMSCs used were cultured for less than 5 passages. After thawing, hADMSCs were cultured in T150 flasks at 3000 cells/cm² with MEM-alpha (low glucose, with Glutamax) (Thermo Fisher Scientific, Grand Island, NY, USA) containing 10% human Platelet Lysate (Stemcell Technologies, Vancouver, BC, Canada), heparin (Sigma-Aldrich), and 1% penicillin-streptomycin (Sigma-Aldrich); culture media was changed every other day. Cells were harvested when reaching 80% confluency. Culture media was removed from the flask and cells were then rinsed with Dulbecco's phosphate-buffered saline (DPBS). To detach the hADMSCs from the culture flask, 0.05% trypsin-EDTA (Sigma-Aldrich) was added after removing DPBS, and cells were incubated at 37°C for 3-5 min followed by gentle tapping. Culture media, equal to the volume of trypsin solution, was then added to stop enzymatic reaction. The hADMSC suspension was transferred

to 50 ml centrifuge tubes and centrifuged at 200 x g for 5 min. The supernatant was discarded, and cells were resuspended in fresh culture media or DPBS for downstream experiments.

Hydrogel and Cell Encapsulation

The cell density for each hydrogel encapsulation and the non-cross linked HA gel was 6×10^6 cells/ml. PGmatrix hydrogel (PepGel LLC., Manhattan, KS, USA) was prepared at 0.3% gel concentration (based on preliminary experiments; data not shown), following the manufacturer's recommended method, by mixing the cell suspension in PGmatrix stock solution, and PGwork, then incubating at 37°C for gelation. For non-crosslinked HA gel²²², sodium hyaluronate powder (Sigma-Aldrich, St. Louis, MO, USA) was weighed and mixed with hADMSC suspension to reach a final 3% HA concentration, which is optimal according to Feng *et al.*²²². For Hystem, the cell suspension was mixed with the Hystem hydrogel kit (Sigma Aldrich) components, Gelin-S, Glycosil, and Extralink, following the manufacturer's instruction, and incubated at 37°C for 1 hour for gelation.

Cell Counting Kit-8 (CCK8)

Cell counting kit-8 (CCK8) assay is a colorimetric assay similar to MTT assay but has no cytotoxicity. The resulting optical density (OD) reflects cell metabolic activity level and cell viability. Wells in a 96-well plate were coated with 1% PGmatrix, followed by 30 min gelation at 37°C, to prevent cell adhesion to plastic. hADMSC –encapsulated in 3% Hystem and 0.3% PGmatrix were plated into 3 wells for each treatment. Twenty microliters/well hADMSC-encapsulated 3% HA gel and hADMSC suspension were plated into 18 wells for each treatment. All plates were incubated at 37°C for 1 hour to allow complete gelation of PGmatrix and

Hystem. CCK8 solution (Dojindo Molecular Technologies, Inc., Rockville, MD, USA) was mixed with DPBS at 1:9 ratio, 100 μ l mixture/well (10 μ l CCK8 solution for suspension wells) was added at 0, 3, 6, 12, 24, 48 hours after gelation and incubated for 1 hour at 37°C. Absorbance at 450nm was measured with SpectraMax Plus Absorbance Microplate Reader (Molecular Devices, LLC., San Jose, CA, USA) right after CCK8 addition and after 1 hour incubation. The final optical density (OD) of each well = OD after 1 hour incubation – OD right after CCK8 addition. After measurement, the CCK8 mixture in Hystem and PGmatrix wells was replaced with DPBS. Wells containing 3% HA and suspension were discarded after measurement.

Cell Viability Assay

Acridine orange (AO) and propidium iodide (PI) dyes stain all cells and dead cells, respectively. Suspension of hADMSCs and hADMSCs encapsulated with 0.3% PGmatrix, 3% HA, and Hystem were prepared as described previously and loaded into 1ml syringes. The syringes were incubated at 37°C. To measure viability, a droplet of the sample was taken from each of the 4 groups. The droplets were added to 50 μ l DPBS and pipetted to ensure even cell distribution. Forty microliters of the diluted samples were mixed with 40 μ l AO/PI staining solution (Nexcelom Bioscience LLC., Lawrence, MA, USA). 20 μ l mixture (containing > 200 cells) was then loaded into a counting chamber and counted with a Cellometer Auto2000 (Nexcelom Bioscience, Lawrence, MA, USA). Viability = live cell number / total cell number. Counting was performed in triplicates on each 80 μ l cell-dye mixture.

Immunostaining

Immunostaining was performed to assess expression level of pluripotency marker stage-specific embryonic antigen-4 (SSEA4), and organization of actin cytoskeleton. Hydrogel encapsulated hADMSCs were prepared as described earlier and loaded into 1 ml syringes, 2D samples were prepared by plating hADMSCs suspended in media onto gelatin-coated glass coverslips. All samples were incubated at 37°C overnight. Hydrogel encapsulated hADMSCs were transferred into 1.5 ml microtubes before overnight fixation of all samples using 10% neutral buffered formalin. After fixation, samples were washed with wash buffer (DPBS without $\text{Ca}^{2+}/\text{Mg}^{2+}$ + Triton X-100 + gelatin from cold water fish) (Sigma-Aldrich) and incubated with glutamine solution (Sigma-Aldrich) for 15 minutes to remove free aldehydes. The staining process was carried out using Invitrogen™ Pluripotent Stem Cell 4-Marker Immunocytochemistry Kit (Thermo Fisher) following manufacturer's instruction. Primary antibodies were diluted with wash buffer, added to each sample, and incubated overnight at 4°C. Samples were then rinsed with wash buffer to remove excess antibodies. Secondary antibodies were diluted, added to each sample, incubated for 3 hours, and rinsed with wash buffer. Samples in microtubes were moved onto glass coverslips.

To stain actin, Phalloidin-iFluor 594 (Abcam, Boston, MA, USA) and Deoxyribonuclease (DNase) I Alexa Fluor 488 Conjugate (Thermo Fisher) stock solutions were prepared according to manufacturers' protocols. After removing free aldehydes, fixed samples were incubated with wash buffer for 5 minutes to increase permeability. Samples were then incubated with Phalloidin and DNase I working solution for 30 minutes, followed by rinsing with wash buffer to remove excess staining conjugates. One drop of 4',6-diamidino-2-phenylindole (DAPI) solution (Abcam) was added to each sample and incubated for 10 minutes at room temperature. Glycerol was added to each sample before mounting the cell suspension. Images were acquired with a Zeiss

700 microscope (Carl Zeiss Microscopy, LLC., White Plains, NY, USA) and analyzed with Fiji-ImageJ²³⁶.

Mass Spectrometry

Mass spectrometry (MS) was used to detect total proteins released by hADMSC. To collect conditioned medium (CM) samples, an equal number of harvested hADMSCs were either plated into a T75 flask for 2D method or encapsulated in Hystem or 0.3% PGmatrix hydrogel then transferred into 50 ml centrifuge tubes. Cells in T75 flask were incubated at 37°C overnight for attachment, while hydrogel encapsulated samples in 50 ml tubes were incubated at 37°C for 1 hour to complete gelation. After incubation, hADMSC culture medium in T75 flask was removed and the cells were washed twice with DPBS, and MEM-alpha medium was added to the flask. For hydrogel encapsulated samples in 50 ml centrifuge tubes, the same amount of MEM-alpha medium was added into the two tubes. The samples with MEM-alpha medium were then incubated at 37°C for 48 hours before CM collection. Collected CM samples were filtered through 0.22 µm syringe filters (Corning) to remove cell debris.

The resulting filtrates were concentrated using 3 kDa Amicon Ultra-4 centrifugal filter units (MilliporeSigma, Burlington, MA, USA), washed with 1 x TBS (50 mM Tris-Cl, pH 7.6; 150 mM NaCl), and reconcentrated to remove phenol red. Total protein concentration of the concentrated samples was determined with a BCA protein assay kit (Thermo Fisher). Protein digestion, peptide separation, and MS were performed as follows: 50 µg total proteins were digested with sequencing grade modified trypsin (Promega Corporation, Madison, WI, USA) at a ratio of 10:1 on S-TrapTM column (ProtiFi, Farmingdale, NY, USA). After incubation overnight at 37°C, proteins were eluted from the column sequentially with 50 mM ammonium bicarbonate,

0.2% formic acid in water, and 50% acetonitrile in water. Eluents were pooled, dried with speed-vac, and resuspended in 50% acetonitrile in water supplemented with 0.1% formic acid. Peptide separation was performed with ACQUITY M-Class UPLC (Waters Corporation, Milford, MA, USA) on a HSS T3 Column (Waters, 100 Å, 1.8 µm, 300 µm x 150 mm). Elution was performed at 2 µL/min with a gradient of acetonitrile (containing 0.1% formic acid) from 5% to 50% in water (containing 0.1% formic acid) over 120 min, followed by an isocratic gradient of 50% acetonitrile in water for an additional 10 min. Detection was performed with Xevo G2-XS Q-Tof mass spectrometer (Waters) coupled with NanoLockSpray ionization source, operated under MS^E mode for data-independent acquisition.

Raw data were processed using Progenesis QI for Proteomics (Waters) by manually specifying the threshold intensities at 250 and 150 counts for low and elevated energies, respectively. Peptides containing more than 6 residues were selected and identified using UniProt proteome (ID: UP000005640) as the search database. Criteria for protein identification search were set as follows: two possible missed cleavages using trypsin, and two modifications including one fixed cysteine carbamidomethylation and one variable methionine oxidation. Ion matching requirements were set at fragments/peptides ≥ 3 , fragments/protein ≥ 7 , and peptides/protein ≥ 1 . Fold changes of protein expression levels from different CM samples were calculated using the Experiment Design Function in Progenesis QI for Proteomics.

Enzyme-linked immunosorbent assay (ELISA) for Human Vascular Endothelial Growth Factor A (VEGF-A)

Nine transwells in a 24-well plate (Corning Inc., Corning, NY, USA) were coated with 50 µl 0.5% PGmatrix or Hystem hydrogels and incubated at 37°C for 1 hour for gelation. Three

wells of approximately 1.5×10^4 hADMSCs/well were encapsulated in 3% HA, Hystem, and 0.3% PGmatrix following methods described previously. The samples were then incubated at 37°C for 1 hour to allow gelation. Another 3 wells of suspended hADMSCs were plated directly onto transwells and 1 ml of MEM-alpha medium was added to each well. One hundred fifty microliters of CM were collected from each well at 6, 24, 48, 72 hours after gelation. The CM samples were centrifuged at 350 x g for 10 min to remove cell debris, then stored at -20°C until the ELISA was performed. To detect VEGF-A, a precoated VEGF-A ELISA kit (Thermo Fisher) was used following manufacturer's instructions. Briefly, the plate was washed with wash buffer and dried before adding standard diluents and samples in duplicate. The plate was then covered and placed on a microplate shaker (400 rpm) at room temperature for 2 hours. After shaking, the plate was emptied and washed 6 times with wash buffers. Then 100 µl Biotin-Conjugate was added to each well, the plate was covered and placed on the shaker at room temperature for 1 hour, followed by another set of 6 washes. 100 µl diluted Streptavidin-HRP was then added to all wells, including blanks, and the plate was placed on the shaker for 1 hour, at room temperature. The plate was emptied and washed 6 times before adding 100 µl TMB Substrate Solution. The plate was then incubated at room temperature in the dark for 30 min. 100 µl stop solution was quickly added to all wells at the end of the incubation and absorbance was measured at 450nm with SpectraMax Plus Absorbance Microplate Reader (Molecular Devices, LLC.).

Diabetic mice wound healing model

All animal procedures were conducted in accordance with institutional guidelines using a protocol approved by the Institutional Animal Care and Use Committee (IACUC) at Kansas State University (Protocol #4332, Date of approval 8/21/2020). Thirty-two male CD-1 mice aged

7-8 weeks were obtained from Charles River Laboratory and housed individually at Kansas State University. Mice were housed individually with free access to food and water and under 12 h-light/12-h dark cycles for one week before diabetes induction. Streptozotocin (STZ) (Sigma-Aldrich) was dissolved in physiological saline at 4 mg/ml (Baxter, Deerfield, IL, USA), sterile filtered with 0.22 μ m syringe filter (Corning) into sterile glass vials and kept in dark on ice. STZ solution was made right before each injection. The mice received 5 daily intraperitoneal injections of STZ solution at 40 mg/kg body weight, with 10% sucrose (Sigma-Aldrich) added in drinking water for 5 days to induce diabetes. Blood glucose was monitored with Contour Next One whole blood glucose meter (Ascensia Diabetes Care, Parsippany, NJ, USA), a mouse with a blood glucose > 250 mg/dL was considered diabetic. Ten days after the last STZ injection, mice were anesthetized with isoflurane inhalation. An 8 mm round full thickness excisional wound was created by sharp dissection on the dorsolateral thoracolumbar region of each mouse. A sterilized stainless steel wound splint (Menards, Eau Claire, WI, USA) was placed on each wound and secured with 4-0 nylon sutures (MWI, Boise, ID, USA) to the skin edges to minimize wound contraction. An injection of either DPBS control, or one of the encapsulated hydrogels was applied directly into the skin edges immediately after splint suturing was completed. Mice were randomly assigned to one of the following treatment groups: 100 μ l DPBS (Sigma-Aldrich); 6×10^5 hADMSCs encapsulated in 100 μ l 0.3% PGmatrix; 6×10^5 hADMSCs encapsulated in 100 μ l Hystem hydrogel; 6×10^5 hADMSCs encapsulated in 100 μ l 3% HA. Each encapsulation was incubated at 37°C overnight. Mice were monitored for 21 days after the surgery with photos taken immediately following surgery (day 0) and on days 4, 7, 10, 14, 21. Photos of the wound were analyzed with Fiji-ImageJ. Wound outlines were carefully traced, and the surface area within the outlines was measured to track wound area change during healing.

Histological Analysis

All mice were euthanized in a CO₂ chamber 21 days post-surgery, and tissue from the wound area was excised with scissors and fixed in 10% neutral buffered formalin (Sigma-Aldrich). Tissue samples were processed into paraffin, sectioned at 4 μ m, and stained with hematoxylin and eosin (H&E) and Masson's trichrome, following standard procedures, by the histology laboratory at the Kansas State Veterinary Diagnostic Laboratory. Healing was assessed by a boarded anatomic pathologist, in a single blind study design, and a subjective scoring system was used to assess the skin sections. Score 1 represents re-epithelialization with relatively "thin" epithelium (<6 cell layers thick); Score 2 represents re-epithelialization with hypertrophic epithelium; Score 3 represents thick re-epithelialization with basement membrane (dermal-epidermal junction) clefting and/or ulceration. Collagen deposition was evaluated in Masson's trichrome stained sections using Fiji-ImageJ, following the method described by Chen *et al.*²³⁷. Collagen intensity was converted to optical density (OD) using formula $OD = \log_{10}(\text{Max Intensity}/\text{Mean Intensity})$, in which Max Intensity = 255, Mean Intensity = Total integrated collagen density/total area measured. The formula was derived from a method described by Wieck *et al.*²³⁸

Statistical Analyses

Statistical analysis was performed with JASP²³⁹. Data measured at multiple timepoints, including CCK8, viability, ELISA, and wound healing, were analyzed with repeated measure ANOVA with α level ≤ 0.05 as statistical significance. Fluorescence intensity of SSEA4 was compared using Student's t-test (two-tailed) with α level ≤ 0.05 as statistical significance. All

data are shown as the means $\pm$ standard error of the mean (SEM). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Results

PGmatrix significantly extended hADMSC survival after encapsulation

The effect of using different hydrogels on hADMSC viability and metabolism over 48 hours after encapsulation was measured with a CCK8 assay (Figure 4.1). Cells had very limited nutrient supply since the only medium added was that used during encapsulation. Results showed that the optical density (OD) values of hADMSCs encapsulated in 0.3% PGmatrix and Hystem remained high in the first 12 hours and then slowly decreased up to 48 hours, but the OD with Hystem was much lower right after encapsulation (0 hour) than with the 0.3% PGmatrix and suspension groups (Figure 4.1). While the OD values of hADMSCs encapsulated in 3%

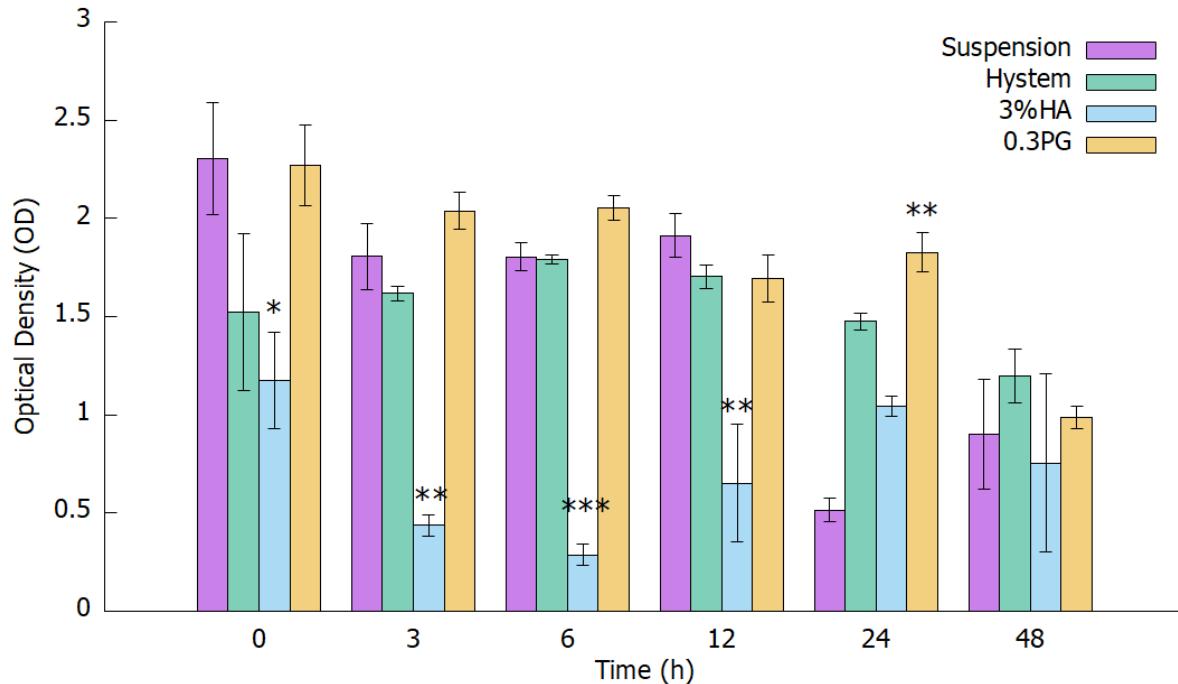


Figure 4.1 After encapsulation with no culture medium provided, CCK8 results were recorded as increase in optical density (OD) at each time point (OD after 1 h CCK8 incubation—OD right after CCK8 addition). Results are shown as mean $\pm$ standard error of the mean (SEM), * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

hyaluronic acid (HA) remained significantly lower than the other 2 hydrogel groups and the control group during the first 12 hours ($p < 0.05$) and reached a similar level as other groups by 48 hours. Suspension of hADMSCs showed high OD readings comparable to PGmatrix and Hystem groups during the first 12 hours but dropped to a level significantly lower than PGmatrix group ($p = 0.003$) 24 hours after encapsulation (Figure 4.1).

According to the principle of the CCK8 assay, the OD readings obtained reflected combined results from cell viability and metabolism. To better estimate the cell viability, change after encapsulation, we took a droplet from each test sample at each time point and counted live/dead cells with a cellometer (Figure 4.2). During the first 6 hours after encapsulation, viability of the Hystem group decreased the most rapidly, significantly lower than other groups ($p < 0.001$). After 12 hours, only PGmatrix group maintained high viability above 80%, significantly higher than the suspension group ($p = 0.048$) and the Hystem and 3% HA groups ($p < 0.001$). All groups dropped to 50-60% viability after 24 hours. The 0.3% PGmatrix group

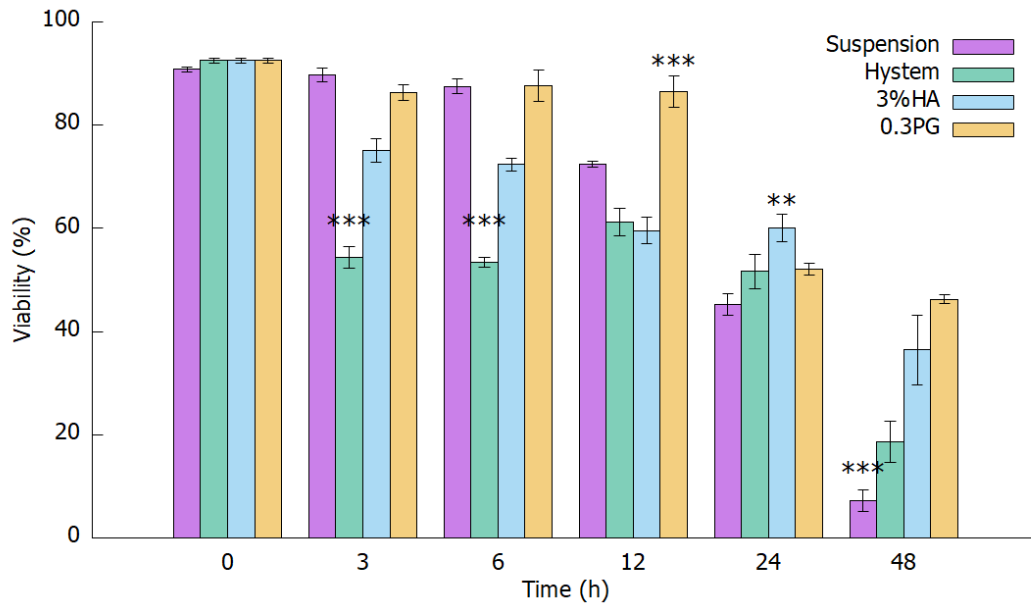


Figure 4.2 Percent viability at each time point after encapsulation with no culture medium, measured with cellometer. Results are shown as mean $\pm$ SEM, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

maintained viability at 46.3% at 48 hours after encapsulation, while the other two encapsulation groups decreased to below 40%. The suspension group had the lowest viability at 7.3%, followed by Hystem group at 18.6%. The 0.3% PGmatrix hydrogel demonstrated superior protective effects on hADMSCs. Although the Hystem group had a lower percent viability, cell metabolism was enhanced, with the Hystem group having OD readings comparable to the PGmatrix group during the first 12 hours.

PGmatrix was superior to maintain SSEA4 surface antigen in hADMSCs

A subpopulation of MSCs express pluripotency markers like SSEA4, these SSEA⁺ cells were shown to have higher differentiation potential²⁴⁰. To examine SSEA4 production, immunostaining was performed on hADMSCs encapsulated in different hydrogels after overnight incubation at 37°C overnight to detect for possible upregulation of stem cell markers;

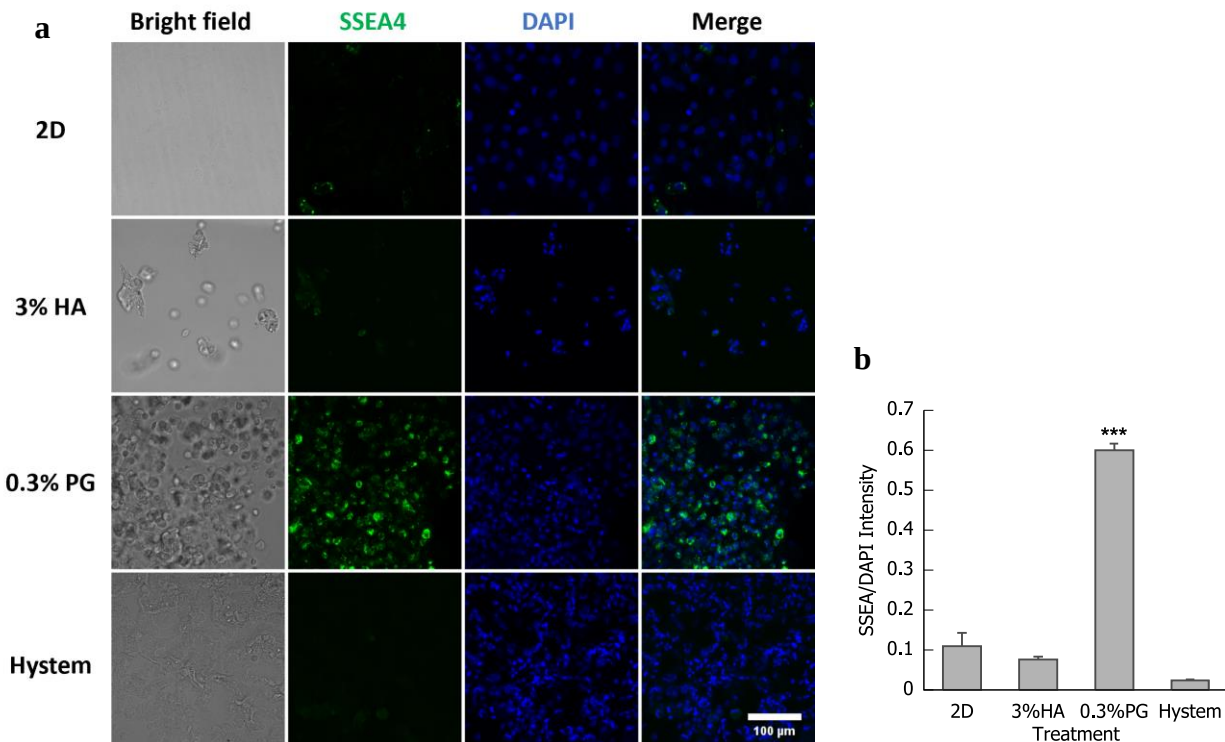


Figure 4.3 Encapsulated and 2D-plated hADMSCs with stage-specific embryonic antigen-4 (SSEA4) immunostaining and nuclear staining (DAPI). (A) Confocal images of cells, scale bar = 100 μm; (B) Fluorescence intensity, shown as SSEA4/DAPI. Results are shown as mean ± SEM, *** $p < 0.001$.

2D plated hADMSCs were used as control. The 0.3% hADMSCs encapsulated in PGmatrix showed a high level of SSEA4 fluorescent immunolabeling (Figure 4.3a). The quantified SSEA4 intensity of 0.3% PGmatrix group was significantly higher than all other groups (Figure 4.3b).

In addition, immunolabeling for cytoskeleton filaments, F-actin and G-actin, was

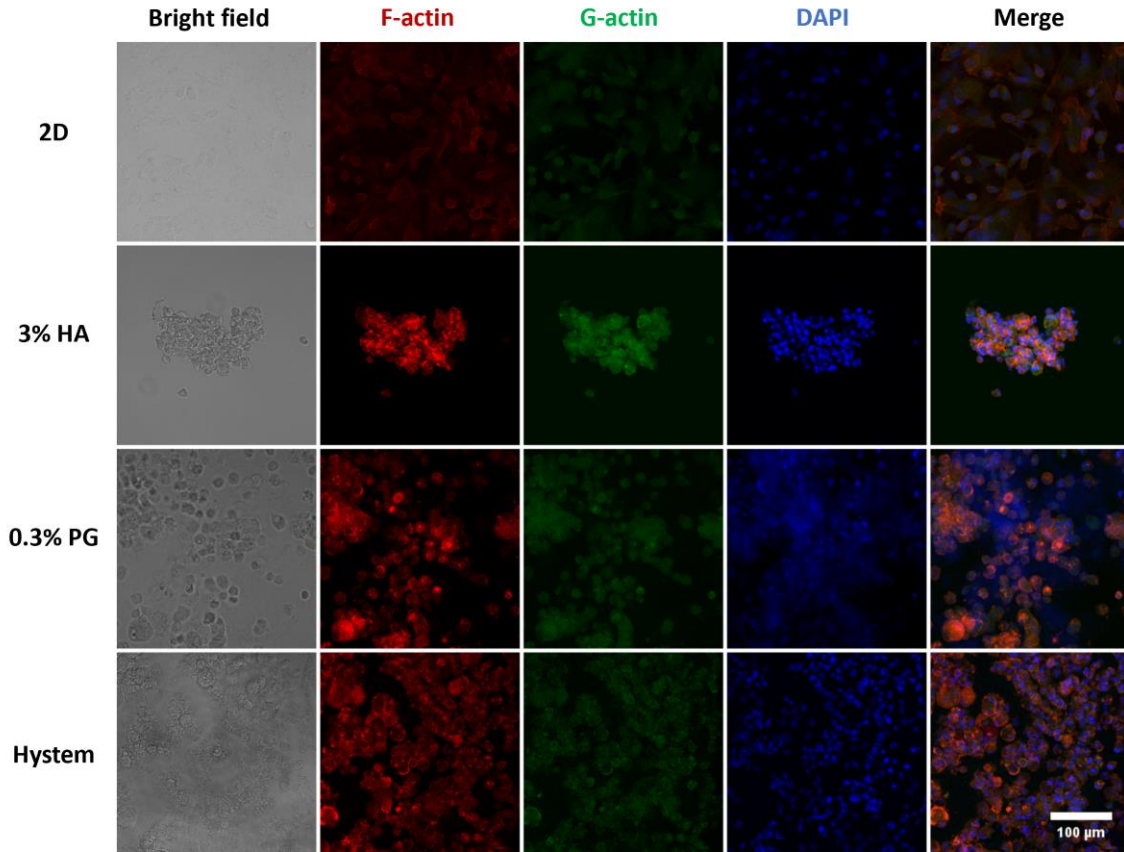


Figure 4.4 Immunostaining of F- and G-actin in hADMSCs encapsulated in different materials and incubated at 37 °C overnight. Nucleus was labeled with DAPI. Scale bar = 100μm.

performed on these hADMSCs. We found that though release of actin tension, reflected by reduction in F-actin stress fibre, existed in all 3D encapsulated hADMSCs, actin organization is slightly different in hADMSCs in different hydrogels (Figure 4.4). In 3% HA, F-actin formed various small clusters dispersing within and on the edge of hADMSCs. Similar F-actin distribution was observed in hADMSCs treated with 0.3% PGmatrix as 3% HA, except F-actin clusters were larger in PGmatrix encapsulated hADMSC. In contrast, F-actin in Hystem

encapsulated hADMSCs formed bright cortical filaments to support cell shape, with the rest relatively evenly distributed inside the cells. G-actin in these groups followed similar patterns as F-actin, with PGmatrix group having the highest level of actin agglomeration. Different from Zhou et al.²⁰⁴, we observed bright F-actin clusters when F-actin stress fibre reduced in 3D. Assessment of F-actin fluorescence intensity showed no difference between the groups (Figure 4.5) indicated changes in F-actin organization, not F-actin protein production.

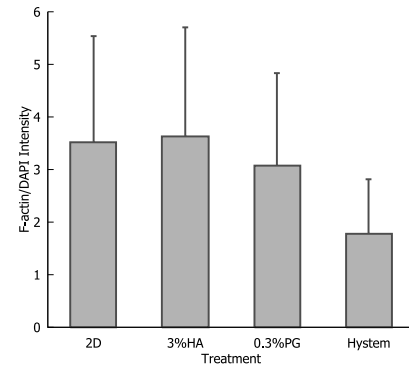


Figure 4.5 F-actin signal intensity shown as F-actin/DAPI. Results are shown as mean ± SEM.

hADMSCs in PGmatrix secreted high immune responsive proteins and comparable levels of VEGF-A and TGFβ1 factors as other hydrogels

To obtain comprehensive understanding of hADMSC secretome in 2D and 3D PGmatrix and HA based hydrogel Hystem, mass spectrometry (MS) was performed on conditioned medium (CM) collected after incubating encapsulated hADMSCs for 48 hours. Out of the 1013 proteins identified with MS, 99 proteins were detected with high confidence (≥ 10 unique peptides identified). The 0.3% PGmatrix and Hystem samples had the highest abundance in 32 and 23 of the 99 proteins, respectively. The remaining 44 proteins were the most abundant in the 2D sample. Interestingly, we found that most extracellular matrix (ECM) and adhesion related proteins, including collagen alpha-1(VI) chain (COL6A1), fibronectin (FN1), Laminin beta-1 (LAMβ1), secreted protein acidic and rich in cysteine (SPARC), and insulin-like growth factor-binding protein 7 (IGFBP7), were high in 2D and Hystem samples and low in the 0.3% PGmatrix sample (Figure 4.6a). While immune response related proteins, including semaphoring-7A (SEMA7A), alpha-1-antitrypsin (SERPINA1), apolipoprotein A-I (APOA1).

and galectin-3-binding protein (LGALS3BP) were often the highest in 0.3% PGmatrix sample and least abundant in 2D samples (Figure 4.6b). Intracellular proteins, including elongation factor 1-gamma (EEF1G), heat shock protein 90-beta (HSP90AB1), plastin-3 (PLS3), and annexin (ANXA2) were generally more abundant in Hystem samples (Figure 4.6c), suggesting loss of cell membrane integrity and cell death in Hystem samples.

In addition to cell properties, factors secreted by hADMSCs have been shown to play important roles in their therapeutic potentials^{241,242}. The MS data also showed vascular endothelial growth factor receptor 1 (VEGFR-1/FLT1) and transforming growth factor beta-1

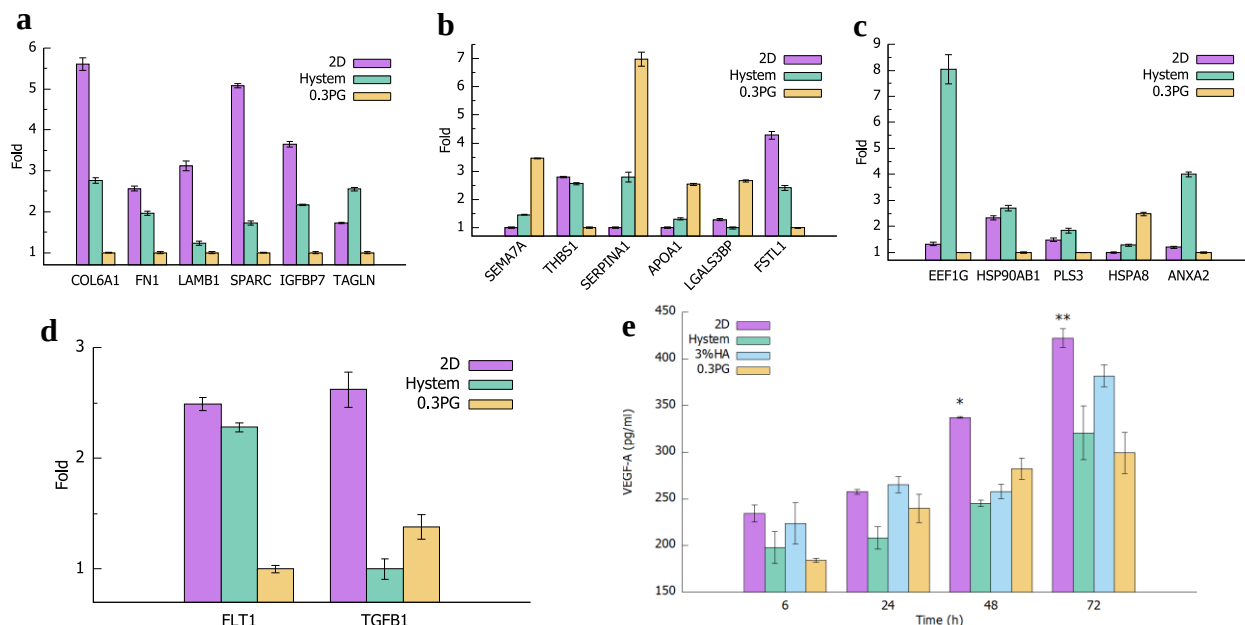


Figure 4.6 Analysis of the secretome of hADMSCs in Hystem and 0.3%PG encapsulations and 2D matrix. (A–D) Mass Spectrometry results of protein abundance expressed as fold change using the sample with lowest abundance of each protein as the reference. Representative proteins identified with high confidence (≥ 10 unique peptides) are grouped into 3 categories: extracellular matrix (ECM) and ECM binding proteins (A); immune-response-related proteins (B); intracellular proteins (C) Folds of Vascular endothelial growth factor receptor-1 (VEGFR1/FLT1) and transforming growth factor beta-1 (TGFβ1) are shown in (D,E) Levels of VEGF-A released by hADMSCs in 2D or 3D encapsulation measured with ELISA. Results are shown as mean $\pm$ SEM, * $p < 0.05$, ** $p < 0.01$.

(TGFβ1). Hystem-encapsulated hADMSCs released more VEGFR-1, while PGmatrix-encapsulated hADMSCs secreted slightly more TGFβ1 (Figure 4.6d). As VEGFR-1 involves in

regulating vascular endothelial growth factor (VEGF) level, its production and secretion depends on the extent of VEGF signaling needed²⁴³. To confirm if VEGFR-1 would reflect a similar level of VEGF, an ELISA assay was used to determine VEGF-A released by hADMSC under 2D matrix or 3D encapsulation with different hydrogels at different culture time points up to 72 hours (Figure 4.6e). Hystem and 0.3% PGmatrix groups had no significant differences in VEGF-A release; in contrast, the 2D group released significantly more VEGF-A after 48-hour incubation. However, 3% HA showed a slight increase in VEGF-A release level after 72-hour incubation.

PGmatrix encapsulation promoted healing at early stage

A diabetic mice wound skin model was used to assess the effect of different hydrogels on the therapeutic effect of hADMSC 3D encapsulations. Diabetes was induced in CD-1 male mice by 5 daily injections of streptozotocin (STZ). 10 days after the last STZ injection, 8 mm round full thickness excisional wound was created on the dorsum of each mouse with a sterilized steel

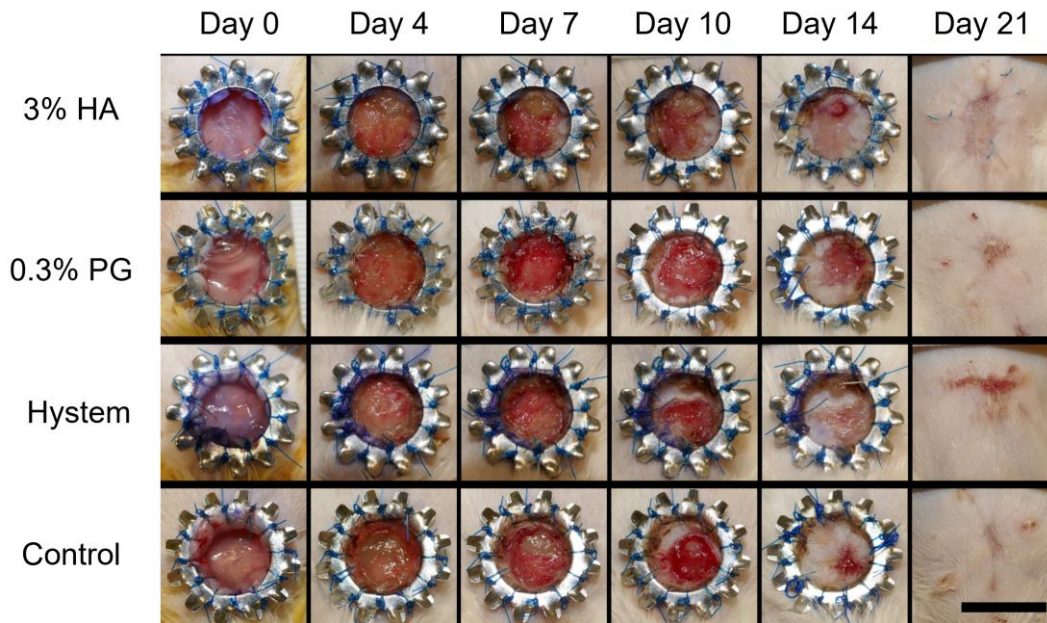


Figure 4.7 Skin wound healing in diabetic mice. Photos of the wound area taken after surgery (day 0) and 4, 7, 10, 14, and 21 days after surgery. Scale bar = 10 mm.

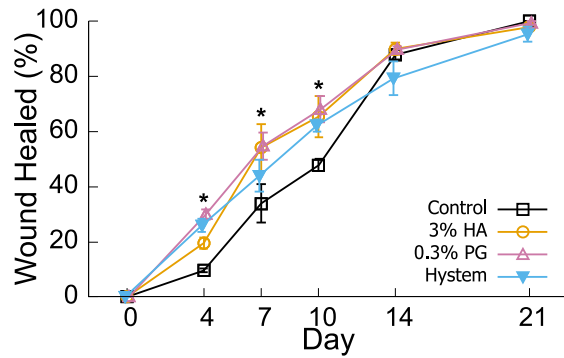


Figure 4.8 Quantification of wound surface healing. Wound healed (%) = $100 - \text{wound area} / \text{Day 0 wound area} \times 100$. Results are shown as mean $\pm$ SEM, * $p < 0.05$.

wound splint attached to minimize contraction (Figure 4.7). After splint attachment, mice were randomly assigned to receive injection of DPBS as control, hADMSCs encapsulated in 3% HA, 0.3% PGmatrix or Hystem hydrogels incubated at 37°C overnight. The healing process was tracked for 21 days. The 0.3% PGmatrix group had significantly better healing than the HA and

control group in terms of wound area by day 4 after the surgery (Figure 4.7, 4.8). Then no significant difference was seen among the three hydrogels by day 7 and 10 though all of them showed better healing than control. However, all groups reached similar healing levels after 14 days, as the effect of healing contracture was observed to become prominent after day 14. and no difference was identified between groups by 21 days (Figure 4.7, 4.8).

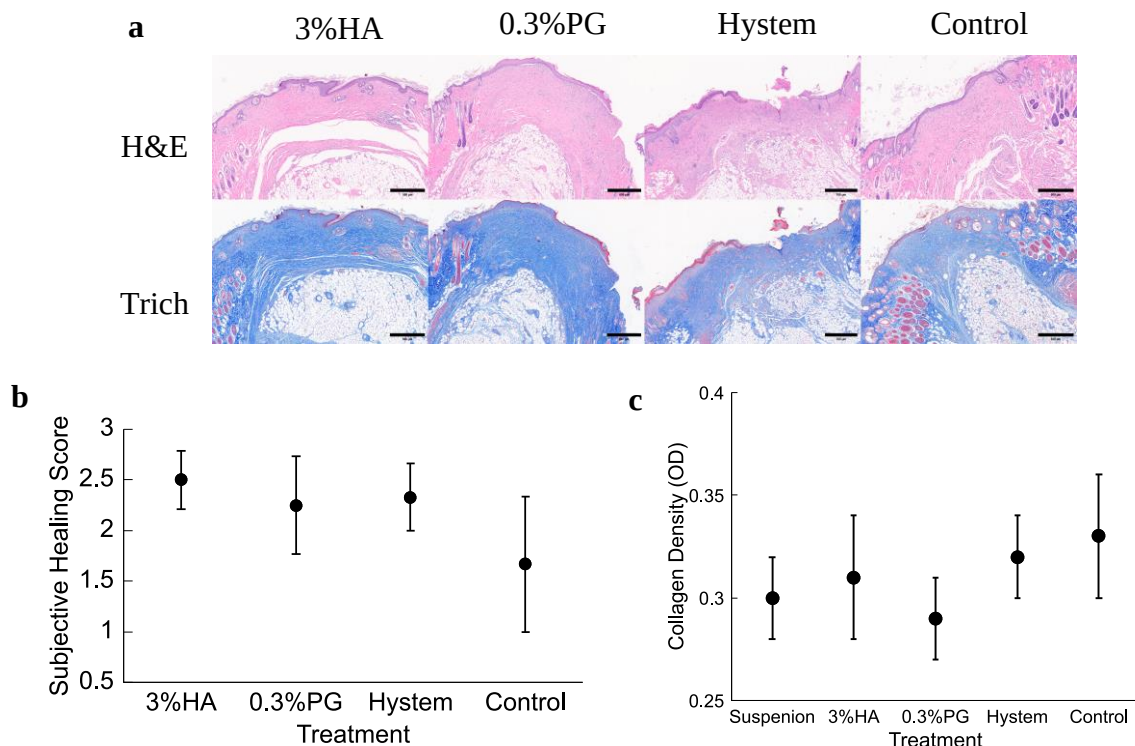


Figure 4.9 Histologic analysis of mouse cutaneous wound samples. (A) Hematoxylin and eosin (H&E) and Masson's Trichrome (Trich)-stained samples from each treatment group. Scale bars were shown on the bottom right of every image. Scale bar for 3% HA and Control = 500 μm , scale bar for 0.3% PG = 600 μm , scale bar for Hystem = 700 μm . (B) Re-epithelialization scores for each treatment group. Results are shown as mean $\pm$ SEM. (C) Collagen density from each treatment group expressed in optic density (OD). Results are shown as mean $\pm$ SEM.

All mice were euthanized for histologic analysis on day 21. The excised wound samples were fixed and stained with hematoxylin and eosin (H&E) and Masson's Trichrome (Trich) (Figure 4.9a). The images were assessed by a boarded anatomic pathologist, in a single blind study design, and scored for re-epithelialization from 1 to 3, with 1 being the closest to healed epithelium. Of all samples evaluated, all hydrogel groups had a similar level of re-epithelialization (Figure 4.9b). Although some studies have shown that MSCs would promote collagen expression and deposition during wound healing^{222,244,245}, a significant difference among the hydrogel groups was not detected in this study (Figure 4.9c).

Discussion

Mesenchymal stem cells and their secretome have been gathering attention for various therapeutic effects^{246,247}. Some studies intended to use the MSC secretome only to avoid risks related to cell transplant^{208,219}, but the use of cells may be more beneficial than the secretome alone, as MSCs might be able to reside and differentiate into target tissue²⁴⁷. In clinical settings, it is difficult to ensure transplant with freshly harvested cells. A benefit of MSCs is that they can be prepared either with frozen stock or with biomaterials as a delivery carrier.

Hyaluronic acid (HA) and HA-based hydrogels like Hystem have been used extensively in MSC delivery studies and have shown varied levels of beneficial effects^{219,222,248,249}. But these hydrogels are still relatively hard to handle, which hindered their application in clinical settings. We developed a shear-thinning peptide hydrogel (PGmatrix)^{233,250} and have used it successfully

for virus delivery²³⁴ and 3D culture of hiPSCs²³². The main peptide of PGmatrix was made of 19 amino acids rationally designed by utilizing human muscle and spider silk protein domains²³², and it provided mainly mechanical support for cells without significant chemical or biological cues²³². PGmatrix was also identified to have minimal cell damage from mechanical stress through pipetting or extrusion printing nozzle²³², and high *in vivo* injection biocompatibility²³⁵. Thus, PGmatrix may be an alternative to HA or HA derived hydrogels for *in vivo* delivery of MSCs.

We tried to mimic real world setting in this study. Syringes loaded with encapsulated hADMSCs were usually prepared hours before treatment with no access to fresh culture media during this process. Based on all the data collected in this study, PGmatrix significantly extended hADMSC survival after encapsulation, while HA-based Hystem seemed to stimulate cell metabolic activity. These results are probably linked to different ways the hydrogels interact with hADMSCs after encapsulation. SSEA4 is commonly used as marker protein for undifferentiated human pluripotent stem cells, but is also synthesized by a subpopulation of MSCs from several tissue sources²⁵¹. SSEA4⁺ hADMSCs were reported to have better endothelial and osteogenic differentiation potential²⁴⁰. Although we have observed elevated expression of SSEA4 in hiPSCs cultured in PGmatrix²³², which was difficult to interpret due to the high inherent SSEA4 level in hiPSC, the results from this study showed that PGmatrix upregulated SSEA4 in hADMSC, as compared to HA hydrogels (Figure 4.3). Since PGmatrix does not contain cell adhesion sites, the SSEA4 upregulation effect might relate to mechanical cues provided by the PGmatrix environment. According to actin staining images (Figure 4.4) and intensity quantification (Figure 4.5), hADMSCs had very bright actin agglomeration and much dimmer actin in other parts of the cells when encapsulated in PGmatrix than in HA or Hystem. Researchers have found that when

in 3D configuration, MSCs can cluster together to form spheroids^{218,222,252}. According to Zhou et al.²⁰⁴, MSCs in 2D culture exhibited high amount of F-actin stress bundles across the cell body, after moved into 3D configuration, most intracellular stress fibre was replaced with thin filaments and spots of actin clusters. Expression level of β -actin reduced during this transition, releasing actin cytoskeleton tension. Relaxation of actin tension would improve MSC stemness and therapeutic effects^{204,222,253,254}. Though we did not detect change in F-actin intensity between 2D and 3D, formation of more compact actin clusters in PGmatrix-encapsulated hADMSC may explain the SSEA4 upregulation effect and suggested better stemness of these hADMSCs compared to those in suspension or encapsulated in HA hydrogels.

Besides improved viability and stemness, cell secretome is also considered the key to hADMSC therapeutic effects. According to MS analysis, hADMSCs encapsulated in PGmatrix had higher release levels than Hystem encapsulated hADMSCs for most of the immune responsive proteins detected, not limited to SEMA7A^{255,256}, SERPINA1²⁵⁷, APOA1²⁵⁸, LGALS3BP²⁵⁹ (Figure 4.6c), as well as Alpha-2-macroglobulin²⁶⁰, plastin-2²⁶¹ and immunoglobulins (IGHM, IGHG1, IGKC) (data not shown). This showed that PGmatrix had a stronger stimulation effect on the release of immune responsive proteins. In addition, hADMSCs in all three hydrogels had comparable VEGF-A release according to ELISA assay, and similar TGF β 1 release was found in the medium according to MS analysis (Figure 4.6d, 4.6e). Similar release of VEGFR-1 was observed from MS analysis. Because VEGFR-1 is involved in regulating VEGF levels, its production and secretion depends on the extent of VEGF signaling needed²⁴³. TGF β 1 was reported to have pro-inflammatory effects^{262,263}, but 3D encapsulated MSCs were found to have better anti-inflammation effects^{218,220,222}, which are in good agreement

with lower TGF β 1 release in Hystem and PGmatrix encapsulated hADMSCs than in the 2D matrix.

MS analysis also revealed that hADMSCs in Hystem, as compared to PGmatrix, showed higher abundance of most ECM proteins, including collagen, fibronectin, and laminin, as well as proteins related to cell adhesion and motility, including SPARC²⁶⁴, IGFBP7²⁶⁵ (Figure 4.6a). Furthermore, abundant intracellular proteins that are not expected to be secreted into the CM, like EEF1G²⁶⁶, PLS3²⁶⁷ and HSP90AB1²⁶⁸, were found in the CM of the Hystem hADMSCs (Figure 4.6c). Together with the high level of membrane protein annexin (ANXA2) detected in the CM of Hystem hADMSCs, suggests that a large number of cells from hADMSCs in Hystem might have experienced loss of cell membrane integrity or cell death that caused release of intracellular and membrane materials. This is consistent with the results from CCK8 and viability assay, in which cell viability could not be well maintained after encapsulation with Hystem with limited medium. This would limit the practical use of Hystem when high cell viability is paramount.

With *in vitro* analysis, PGmatrix is superior to HA or HA based hydrogels in terms of maintaining cell viability in limited culture medium and releasing more immune responsive proteins. In our *in vivo* study, PGmatrix improved wound healing in early stage starting at day 4, and then became similar to HA and Hystem by day 7 up to day 10 (Figure 4.7, 4.8). All hydrogel groups accelerated wound healing before day 10 compared to the control, however, no significant difference was observed among all groups by day 14, and all achieved complete healing, by gross examination, by day 21 (Figure 4.7, 4.8). According to literature^{222,244,245}, re-epithelialization and collagen deposition would favor wound healing. However, no significant differences in re-epithelialization or collagen density were observed among the treated or control

groups (Figure 4.9b, 4.9c). The healthy outbred mice were chosen with the purpose of better representing a heterogeneous population compared to inbred diabetic strains, however, the diabetes situation induced with STZ in the healthy outbred mice did not provide significant effects of slower healing complexity as expected by day 21 although all mice exhibited diabetic symptoms before wound creation. Nevertheless, healing differences was obviously observed at earlier timepoint such as day 4 (Figure 4.8), and our MS analysis data (Figure 4.6b) suggested that PGmatrix encapsulation promoted release of immune responsive proteins, which would help reduce inflammation and contribute to faster wound healing as observed in early stage of the healing process. For more complex and larger wounds, the promotional healing effects of encapsulated hADMSC might be more observable, as reported in some chronic, nonhealing wounds²⁴⁴. In summary, pretreatment of MSC with hydrogel encapsulation may be better suited for use in promoting anti-inflammatory and immunosuppressed environments, and thus suitable for use in inflammatory and autoimmune diseases. PGmatrix encapsulation would be a better alternative to HA hydrogels as it improves maintenance of cell viability. Nonetheless, choosing optimal MSC delivery hydrogels merits further exploration.

Conclusion

PGmatrix encapsulated hADMSCs showed superior cell viability maintenance, higher SSEA4 expression, and increased release of factors related to the immune response. All three hydrogels encapsulated hADMSCs had similar secretion levels of VEGF-A and TGF β 1. Beneficial effects of PGmatrix encapsulation on diabetic skin wounds were observed during the early stage of healing. Together, our research suggested that encapsulation with PGmatrix or HA-based hydrogels as pretreatment and delivery method for MSC may be suitable for

inflammatory or autoimmune disease models, while PGmatrix encapsulation would be better than HA-based hydrogels for tissue regeneration, as MSC viability may be more important. Peptide hydrogel PGmatrix performed equally well or better than HA-based Hystem in all tests, rising as a new candidate material for therapeutic cell delivery.

Acknowledgements

We are grateful to Dr. Dylan Lutter and Dr. Camila R. S. Souza for their help in conducting mice surgeries. We acknowledge Dr. Margaret A. Highland for her help in histological analysis. We sincerely thank Wei Wu and Dr. Ping Li for their help in conducting Mass Spectrometry experiments and help with result analysis. We also acknowledge Confocal Core supported by the College of Veterinary Medicine at Kansas State University for their help in confocal imaging.

Chapter 5 - Conclusion

This dissertation explored applications of a fully synthetic, peptide hydrogel which can be tailored for use in different aspects of stem cell research. The basis of any stem cell research is cell culture, which lays the foundation by providing high quality research materials. For pluripotent stem cells like hiPSCs, *in vitro* culture has long been an issue as spontaneous differentiation is almost unavoidable in traditional 2D culture. Previously we developed a novel 3D culture system that allows hiPSCs to proliferate steadily while maintaining excellent pluripotency. In this dissertation, we explored the difference between this PGmatrix and a PEG-based, temperature sensitive Mebiol gel in their ability to support hiPSC maintenance. Our results showed that Mebiol gel cannot stably support hiPSC proliferation and cell recovery is difficult with great cell loss. Further analysis showed that hiPSCs grew better in a 3D hydrogel that can be modified by the cells. This led to the discovery of a key mechanism which centered around mechanosensing genes YAP and TAZ for hiPSCs 3D culture in PGmatrix hydrogel. In the proposed mechanism, when hydrogel and the surrounding 3D environment (i.e., PGmatrix hydrogel) was modified by hiPSC during growth, the change in mechanical strength can be sensed by the cells through activation of YAP and TAZ, eventually leading to modulation of proliferation and pluripotency maintenance. This mechanism is very different from those discovered for 2D maintenance and suggested that maintaining hiPSCs in 3D culture might be more physiologically relevant.

Next, we seek to improve stem cell differentiation with 3D PG hydrogel system and chose hepatic differentiation. We used a modified PGmatrix hydrogel (PGmatrix 3D Suspension (PG-sus), which can provide scaffolding support necessary to the hepatocyte differentiation but functions as if it is a suspension culture system. With our previous study on hiPSC 3D culture

and the PG-sus system, we were able to carry out hiPSC maintenance and differentiation all in 3D system without introducing colony dissociation steps. The physiologically relevant hiPSC spheroids can yield final hiH organoids that were superior to those generated in non-adherent suspensions, not only in terms of hepatocyte marker gene expression, but also in terms of *in vitro* hepatocyte functions. These results are encouraging and indicate the possibility of producing large amounts of functional hiH organoids for drug testing and cell therapy development.

Last, we explored the use of PGmatrix peptide hydrogel as cell delivery agent in stem cell therapeutic studies using MSCs as an example. In this section, we showed that conditioning of MSCs by encapsulating the cells in hydrogel and incubating overnight can improve their therapeutic performance in wound healing. Different from HA and HA-based hydrogel, MSCs retained better cell viability, released more proteins related to ECM and ECM binding proteins, and immune response proteins. This difference might relate to greater remodeling of the cell cytoskeleton when encapsulated in PGmatrix. Though in the *in vivo* wound model tested, the improvement effect from PGmatrix preconditioning is seen mostly at the early stage of wound healing, this suggested that the beneficial effect may be significant for chronic, hard to heal wounds, making PGmatrix a good candidate material for therapeutic cell delivery.

Overall, conducting stem cell research in 3D system is an important direction for this field as it better mimics the *in vivo* environment, making results more meaningful to real world applications. The PGmatrix shown in this dissertation is a new material that has great potential to build 3D systems for various biological studies, contributing to advancement in research for human health.

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