Culture of Human Umbilical Cord Mesenchymal Stromal Cells in a Three-dimensional Human Platelet Lysate Gel

by

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Abstract

The traditional cell culture method after isolation from the body involves growing cells in 2 dimensions on plastic culture plate. However, the natural structure and physiology is 3 dimensions. To mimic in vivo environment, there has an increasing interest to find the way to maintain physiological properties. Here, we describe culturing human umbilical cord mesenchymal stromal cells (HUC-MSCs in 3D setting using human platelet lysate gel. This gel is a fibrin-based structure like a blood clot. The preparation step of human platelet lysate (HPL) is by freeze-thaw cycles in order to release factors important for cells to grow and expand. Using of HPL to substitute for fetal bovine serum reduces potential cross contamination between species and xenogenicity. To maintain HPL media as a liquid, we add the anticoagulant heparin. Without adding anticoagulant, the gel will form. The aim of this study is to retrieve HUC-MSCs from HPL gel using Nattokinase, to characterize HUC-MSCs following the International Society for Cell Therapy’s MSC criteria, and to test a 3D invasion model with HPL-gel based structure. The result shows that using 1.75% Nattokinase at 60 minutes can recover the cells without reducing cell number and viability. After Nattokinase treatment, cells are able to attach to plastic and to increase in number. Moreover, they are able to differentiate into fat, bone, and cartilage no different from cells grown in 2D culture. However, to test surface markers by flow cytometry, all MSC markers are positive except CD 105. They are also positive of cell surface markers that should be negative. When seeded back to 2D culture for an additional passage, the MSCs meet ISCT criteria the same as control.
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Chapter 1 - Review of Literatures: Mesenchymal Stromal Cells and 3 Dimensional Cell Culture

Mesenchymal stromal cells (MSCs) are adult stem cells\(^1\). They were first described by Friedenstein and coworkers in 1976 and are typically defined as fibroblast-like structures. The term stromal refers to their supportive function: the cells serve physically as a scaffold and nutritively via blood supply\(^2\). The abbreviation MSCs can be confusing because it can stand for marrow stem cells, and multipotent mesenchymal stromal cells\(^2\). Therefore, the International Society of Cell Therapy (ISCT) defined the minimal criteria of MSCs as a mixed population of progenitors, stem cells, and differentiated cells that have ability to attach to plastic and self-renew, with positive cell surface markers, CD 105, CD 73, CD 90, more than 95%, and negative surface markers (less than 2%) for CD 45, CD 34, CD 14 or CD 11b, CD 79 alpha or CD 19, HLA-DR. Also, MSCs can differentiate to be osteocytes, chondrocytes, and adipocytes\(^3\).

Although stromal cells can be isolated from bone marrow (BM), the collection of BM-MSCs is a highly invasive and painful procedure that requires accessing the marrow cavity, and older age of the donor decreases \textit{in vitro} expansion potential and increases cell senescence\(^4\). For these reasons, there is an interest to find alternative sources of MSCs for use in regenerative medicine. MSCs isolated from many different tissues including amniotic fluid\(^5\), skeletal muscle\(^6\), adipose tissue\(^7\), dental pulp\(^8\), liver\(^9\), and umbilical cord\(^10\) have been evaluated as MSC sources.

### HUC-MSCs

Human umbilical cord-derived MSCs (HUC-MSCs) are isolated painlessly after birth. UC-MSCs are isolated from umbilical vein subendothelial layers, outer layers of umbilical vessels, intravascular space, and sub amnion region\(^2\). They are collected from donors who are all about the same age, and the procedure is not invasive when compared to bone marrow-derived
MSCs (BM-MSCs). HUC-MSCs have higher proliferation capacity both in terms of shorter population doubling time and more populations doubling before senescing compared with BM-MSCs\textsuperscript{11}. Also, HUC-MSCs may have higher osteogenic, adipogenic, and chondrogenic differentiation than BM-MSCs\textsuperscript{12}. Therefore, HUC-MSCs are a promising source of cells for clinical applications.

**Cell Expansion**

**Conventional 2D cell culture**

The conventional method of MSC culture is to expand them in tissue culture treated polystyrene plates in medium supplemented with fetal bovine serum (FBS) to provide nutrients and attachment factors. Cells grow after attaching to the surface in a 2 dimensional (2D) array; however, this can reduce important features and therapeutic effects when compared with 3D growth conditions. For example, Frith and colleagues (2009) reported that 3D culture reduced the viability of osteosarcoma *in vitro* by increasing IL-24 level\textsuperscript{13}. To support the attachment and growth of cells, serum as supplement must be added to the culture. Many laboratories use lot selected fetal calf serum; however, collecting and validating a lot of fetal bovine serum (FBS) requires significant labor, and animal serum causes contamination of xenogenic proteins when culturing human cells. Therefore, finding new sources of supplements that support attachment and growth is critical in order to prevent unknown and unanticipated variations in MSC expansion from occurring\textsuperscript{14}.

Human Platelet Lysate (HPL) might be an alternative source for attachment and growth factors to support MSC culture. HPL may be made by pooling more than 25 donors of expired clinically screened platelets\textsuperscript{15}. MSCs grown in medium supplemented with HPL meet ISCT’s
MSC minimum definition no different from MSC grown in medium supplemented with FBS\textsuperscript{16}. When culturing adipose-derived MSCs (ASCs) in medium supplemented with HPL, there is a significantly lower population doubling time than when they are cultured in medium supplemented with FBS\textsuperscript{17,18}. The presence of HPL in MSCs culture medium increases the levels of IL-6, and IL-8\textsuperscript{19} and increases the migration rate of ASCs in transwell migration assay\textsuperscript{15}.

**3D culture**

Because common methods to expand cells *in vitro* are in a 2D setting, it is important to show whether 2D culture mimics the native condition, a highly complex 3D environment. Previous work suggests that culture in the 3D environment can affect MSC behavior\textsuperscript{20}. 3D cell culture opportunities have been increasing, aimed to provide the proper physiological environment and thus we can begin to study the effects of 3D microenvironment *in vitro*. There are many emerging techniques to create 3D environments, such as spheroid culture, 3D scaffolds\textsuperscript{21}.

**Spheroid culture**

In MSCs form spherical aggregates when grown in suspension, e.g., without attaching to a solid surface. Culturing MSCs at high density in spinning flasks ensures that the cells have uniform access to nutrients. Also, this method enhances cell-to-cell contact, which allows cells to form spheroids\textsuperscript{22}. This method is not limited to expanding MSCs and can be used to expand tumor cells\textsuperscript{23}. Culturing cells in bioreactors can be used to scale-up production and allows more cells to be produced for commercial and clinical applications\textsuperscript{24}. A bioreactor is a system that can control with gas exchange, nutrients, pH, and can dispose of waste. Another classical method that can produces spheroid culture is to place cells in non-adherent 96 microwells, coating plates with agarose gel, or a hydrophobic polymer\textsuperscript{25}. Using these methods, however, cell clusters may
develop a range of sizes and shapes. Micromodeling techniques can overcome this problem because they are well-designed for controlling cell size, but they require special facilities to generate the proper environment\textsuperscript{26}. Hanging drop is another model to produce MSC spheroids. MSCs aggregate in liquid media droplets hanging from the surface after inverting the tissue plate lid since gravity pulls them to the bottom of a hanging droplet\textsuperscript{27}. Cells attach to each other with extracellular matrix compositions. Hanging drop is easy to perform, MSC spheroids can be observed under light microscope, and it is inexpensive but it is labor-intensive\textsuperscript{25}. Hanging droplet method is commonly used to create embryoid bodies for embryonic stem cell culture, which imitates the inner cell mass in blastocysts\textsuperscript{28}. Also, co-cultures of different cell types can be applied to this method\textsuperscript{29}.

The safety and biodistribution of MSCs in the human body after intravenous injection is unclear. Unlike MSCs dissociated to single cells after being cultured in monolayer, injection of larger particles, e.g., 3D spheroids of MSCs, may cause embolism\textsuperscript{30}. On the other hand, dissociated, individual MSCs which had been cultured in spheroid condition are smaller and have more engraftment in brain stroke injury lesions in rat models compared to 2D expanded MSCs\textsuperscript{31}. This suggests that 3D culture MSCs may have an enhanced safety profile compared to 2D culture MSC.

**3D Scaffolds**

The definition of a scaffold is a 3D solid material with porous surface\textsuperscript{32}. The use of biocompatible scaffolds, e.g., those designed to be implanted into patients, aims to restore function through the delivery of living organic tissues\textsuperscript{33}. One important factor in the choice of scaffold materials is biocompatibility and the ability of cells to adhere and function normally within the scaffold. If scaffolds are intended to be used for clinical applications, we may require
only temporary support for cell growth and function. Then after new tissues restore function, the scaffolds should be degraded without causing toxic and immunological response. If scaffolds are used as *in vitro* models to study biological responses, they should have consistent native tissue structure. The need of degradation for this use is not the primary goal. Scaffolds might be derived from metals, glasses, polymers, biological materials or ceramics. Among those, polymers are the most widely used in the medical field and for tissue engineering-scaffold. They can be derived from either natural or synthetic sources. Natural polymers, such as collagen, fibrin, glycoaminoglycan (GAG), can be extracted from plants, animal, and humans. Synthetic-derived scaffold, such as poly(glycolic acid) (PGA) and poly (lactic acid), are widely studied.

Another type of 3D scaffold that has high water content is called hydrogel. This will help cells to be encapsulated. The most popular hydrogel is derived from animal sources because they have the same biological components as the native condition. The preparation step is not complicated. That is, cell suspensions are mixed with hydrogel and then they gel via polymerization. The benefit of 3D matrix materials is that cells are surrounded in 3D environment, in contrast to the 3D scaffold where cells migrate into the construct. Additionally, due to high water component (more than 95%), cells are able to access sufficient nutrients, and a wide range of the pore size imitates the *in vivo* setting. However, there is a limitation due to weak mechanical properties but the stiffness might be adjustable by altering the concentration of the solid components of the hydrogel.

**From 2D to 3D culture: Human Platelet Lysate Gel (HPL gel)**

Media supplemented with HPL has been used commonly in 2D conventional cell culture. In the step of preparation of HPL enriched media, it is important to add heparin, an anticoagulant, to prevent gel formation. Without adding heparin into HPL media, a hydrogel
structure may form by crosslinking of fibrin triggered by the calcium in the medium\textsuperscript{37}. The main component of the HPL gel is a fibrin scaffold, which provides growth factors to support growth and a physiological environment important for expanding cells and for studying biological responses. This provides two advantages for MSC culture. First, medium with added heparin reduces the effects of cellular proliferation and differentiation\textsuperscript{38}. Second, since HPL gel occurs naturally in wound beds \textit{in vivo}, this hydrogel imitates a native 3D structure for MSC expansion\textsuperscript{37}. Therefore, HPL gel can be used as 3D matrix to culture MSCs.

Walenda and colleagues cultured BM-MSCs in HPL gel\textsuperscript{39}. After filling a tissue culture plate with HPL and then allowing the gel to form, MSCs were seeded on top with HPL-medium supplemented with heparin. Cells grow in the layer between HPL gel and HPL medium without contacting the plastic surface. Methyl thiazolyl tetrazolium (MTT) assay, a metabolic assay that can be used to determine cell proliferation indirectly, and Fibroblastoid colony-forming unit (CFU-F) assay was performed. The results showed that HPL gel groups had higher proliferation in both experiments than in 2D culture. Also, there were no differences in cellular morphology and population doubling time between 2D conventional method and 3D HPL gel. Flow cytometry revealed that both experimental groups express CD 29, CD 73, CD 90, CD 105 and are negative for CD14, CD 31, CD 34, CD 45. MSCs cultured in 3D HPL gel had trilineage differentiation capability. Therefore, HPL gel provides a new setting for MSCs expansion as a 3D matrix.

\textbf{Cell Retrieval from HPL with Nattokinase (NK)}

NK is a serine protease enzyme that is extracted from natto, a fermented soybean product with \textit{Bacillus subtilis}, that are commonly consumed in Japan. Hiroyuki Sumi, a Japanese
researcher in Chicago University, was the first person to discover that an enzyme extracted from natto can dissolve fibrin and he named this enzyme “Nattokinase”\textsuperscript{40}.

NK can lyse fibrin gel directly by hydrolysis, and can convert pro-urokinase to urokinase. Also, NK is able to degrade plasminogen activator inhibitor 1 resulting in inhibition of platelet aggregation and to increase tissue plasminogen activator, which then enhances fibrinolysis\textsuperscript{41}. In addition to NK use \textit{in vitro}, NK can be used to treat cardiovascular diseases in clinical settings\textsuperscript{42}. This enzyme is able to decrease blood pressure in pre-hypertension and stage 1 hypertension in double-blinded study in humans after oral intake\textsuperscript{40}. In summary, NK is considered to be extremely useful because of its effectiveness, low cost, preventive use, and easy administration by oral application\textsuperscript{43}.

In 2D cell culture, trypsin and EDTA are used commonly to lift MSCs off the plate for passage. However, in 3D HPL gel, in my pilot studies (data not shown) trypsin-EDTA was not effective to dissolve the hydrogel for cell retrieval. In contrast, Carrion and colleagues reported using NK to recover human BM-MSCs from fibrin gel\textsuperscript{44}. After retrieving MSCs from 3D fibrin gel with NK, they maintain the potential for adipogenic and osteogenic differentiation and can express genes related to adipogenic and osteogenic differentiation including PPAR gamma, CEBP alpha, Runx2, and BGLAP. Therefore, NK is useful compliment to trypsin-EDTA for passaging cells grown in HPL gel.

\textbf{Cell migration and invasion model}

\textbf{Definition}

Migration, in the context of cell biology, refers to the cell’s movement in the body from one to another location; whereas, invasion, in terms of pathology, is where cells pass through
barriers. However, the clear difference between migration and invasion is not easy to discern in various fields\textsuperscript{45}.

In experimental biology, there is clear discrimination between migration and invasion. When using the word “migration”, it aims to focus on cells moving in 2D condition, while “invasion” is used for cell movement in a 3D matrix. Invasion needs an extracellular matrix to encase cells, then the matrix is lysed and the cells migrate. So, cells can migrate without invasion but cannot invade without migration\textsuperscript{45}. There are a lot of different methods for studying cell migration and invasion in the field of experimental biology. These methods are summarized below.

**Cell migration models**

Wound healing assay (Scratch assay) is the most common method to study migration in 2D surfaces because it is cheap and easy to perform and it does not require special equipment. After cells grow and cover all areas of a plate in monolayer, a pipette tip is used to scratch the cell layer to create a cell-free area (e.g., wound). The movement of cells into the wound is timed until the cell-free area or “wound” is closed. Importantly, attention is paid to the differences between cell migration and cell proliferation, especially when longer culture times are used\textsuperscript{46}. One limitation of this method is that it is difficult to create an even scratch line.

The transwell migration assay (Boyden chamber assay) is another popular assay to measure migration changes. This method is based on cells moving through a porous surface that separate two compartments. Cells will be placed in one compartment and a chemotaxis factor will be added to another compartment to see whether cells cross through the pores. After an incubation period, cells that are attracted by chemotaxis factor will be fixed, stained, and counted. Drawbacks of this method are that cells are not easily tracked in real time, and one
cannot control the rate of cell migration. Also, by adding the chemotaxis factor to one compartment, the gradient of the chemotaxic stimulus will decrease over time, reducing the signal to migrate. However, this assay can observe whether cells can attach to the plate or not\textsuperscript{47}.

There is still a need to have assays to track cell migration. Cell exclusion zone assay can be done by putting a stopper on plate at the time of seeding cells to create a cell-free area. The advantage is one can create an exact width zone with a sharp clear boundary each time. Companies such as Platypus Technologies (http://www.platypustech.com) sell the stopper.

Fence assay (Ring assay) has the same principle as cell exclusion zone assay, but instead cells are seeded in the center area encircled inside by a stopper with a ring shape\textsuperscript{45}. After removing the ring, cells that are not attached to the plate will be removed by rinsing gently. Cell in the circle area will migrate in a radial direction\textsuperscript{45}.

Microcarrier bead assay is the method to put a bead covered with cells in a tissue plate and later cells on the bead will demonstrate movement to the plate. Beads will be remove by suction and then cells that migrate into the plate can be fixed and observed. The benefit of this method is that cells are in close-contact, mimicking the body’s environment. The limitation of this method is there has not much space on beads that cells can expand. This method is not broadly used in the experiment\textsuperscript{45}.

Spheroid migration assay uses the combination techniques between 2D and 3D culture but shares the same principles with Microcarrier bead assay\textsuperscript{45}. That is, after culturing cells in spheroid shape, cell spheroids are placed on a tissue culture plate. The advantage of the spheroid migration assay is the spheroid formation of cells ar closely mimics \textit{in vivo} conditions due to the tight contact of cells.
Capillary chamber migration assay was created for studying chemotaxis. This assay requires two chambers connected by a capillary tube. One chamber is plated with cells in medium; whereas, another chamber is added with chemotaxis agent. Both chambers are covered with a glass slide and then this system creates a stable concentration gradient, which is the advantage of this method.

There are other assays to measure leukocyte migration. For example, capillary tube migration assay is designed to test leukocyte migration. In this method, capillary tubes are filled separately with blood, buffy coat, and plasma after centrifugation. Leukocytes will migrate into serum and can be monitored under a microscope. Leukocyte migration agarose technique assay is done by filling a tissue culture plate with agarose and using a mold to create circular wells in order to put cells in one hole and to add chemotaxis factor in another hole. Cells can migrate below agarose gel toward stimulants. Another method is called single cell motility assay, which tracks individual cells. Gold colloidal particles are added to a special plate to coat it in a homogeneous layer seen as dark dots. If there is migration of a cell, it will remove particles from the plate and the “trail” will be easily seen under the microscope by the disruption of the gold particles.

**Cell Invasion Models**

The transwell invasion assay is the most common one used to track cell invasion. The principle is the same as the transwell migration assay but the filter will be coated with layer of extracellular matrix. Cells will invade from the top through the bottom. Once cells invade to another side, a cotton swab will be used for removing the remaining cells before staining of the invasive cells.
Platypus invasion assay is an assay that can create cell free area by a stopper as same as the stopper described previously in cell exclusion migration assay\textsuperscript{45}. This method is done by adding extracellular matrix first and then the cells are added on top with the stopper that creates the cell-free area. Once, the stopper is removed, another layer of extracellular matrix will be added at the top and the cell-free area.

Gelatin degradation assay is a method that uses a tissue culture plate coated with gelatin stained with fluorescence. If there has been invadopodia, e.g., the ability of cells to invade and degrade extracellular matrix, the fluorescence will be diminished, and the trail or path of the cells revealed. For vertical gel invasion assay, cells are seeded on top of gel filled with liquid media. Cells will invade through the gel vertically.

For spheroid/monodispersed cells invasion assay, a spheroid of one cell type suspended in media will be co-cultured with another/the same cell type in the form of a single cell. A single cell will finally invade into the spheroid because of chemokines and chemotaxis. Spheroid confrontation assay uses the same principle as spheroid/monodispersed cells but with 2 different cell types are used to form the spheroids\textsuperscript{52}. To see whether cells can invade into spheroid formation, one must section the spheroid and stain with antibody to discriminate the invading cells or use flow cytometry after dissociating the cells with trypsin. Spheroid gel invasion assay is an assay where cells in spheroid shape are cultured in 3D gel covered with growth medium. One can quantify cells by observing whether cells can invade the structure or not. In summary, there are a number of methods to examine migration or invasion experimentally. In my work, I used a 3D invasion assay that was similar to platypus invasion assay. I schematic of my experimental set up is shown in figure 3-3.

\textbf{Comparison between 2D and 3D Culture}
Wang and colleagues compared the differentiation efficiency of 3D spheroid cultured MSCs to 2D conventional culture\textsuperscript{53}. Cells cultured in 3D spheroids can differentiate into osteocytes and adipocytes at higher efficacy than in the 2D method. Also, the gene profiles showed that cell cultured in 3D condition had higher expression of genes related to differentiation. However, there had decreased in expression level of genes associated with self-renewal. MSC chondrogenic differentiation between 3D and 2D was compared by Winter and colleagues\textsuperscript{54}. BM-MSCs and ASCs were used in their experiment. The result revealed that BM-MSCs cultured in spheroid had an improved chondrogenesis compared with 2D. In addition to the efficacy of differentiation, the stem cell markers, SOX2, OCT4 and Nanog had higher expression level in 3D spheroid condition in ASCs.

A mouse model of carbon tetrachloride induced (CCl\textsubscript{4}-induced) liver failure was used to study the therapeutic potential of 3D cultured MSCs. They showed that serum cytokines level of IFN-gamma, and IL-6 were increased, and TNF-alpha was decreased in response to cell infusion therapy of MSC after growth to 3D culture\textsuperscript{31,55}. Another study using 3D spheroid culture of MSCs in a rat stroke model showed that 3D culture reduced the infarction due to stroke embolism better than MSCs from 2D culture and there has better MSC engraftment at the injured area and improved neurological recovery \textsuperscript{31}. These findings indicate that MSC cultured in 3D conditions may have better clinical effect compared with those cultured in 2D condition.

**Clinical Translation and Clinical Application of MSCs**

MSCs act as anti-inflammatory agents\textsuperscript{56}, and many different clinical trials attempt to treat immune-related disorders using MSCs. The bioassay to prospectively identify MSCs with potent clinical effects is the *in vitro* inhibition by MSCs of activated lymphocyte proliferation\textsuperscript{57}. In rats with corneal injury, MSCs and MSC-conditioned medium reduced neovascularization,
corneal inflammation, IL-2, IFN-gamma production, and, CD4+ cell activation was reduced\textsuperscript{58}. In a mouse colitis model, which mimicked Crohn’s disease in humans, ASCs decreased inflammatory cytokine release, and clinical and histopathological severity after systemic infusion\textsuperscript{59}. In a phase II study of Crohn’s disease, allogenic MSCs infused in patients that were refractory to glucocorticoids reduced the severity of disease\textsuperscript{60}.

Physiological stimulation affects MSC properties \textit{in vitro}. For example, BM-MSC exposure to Toll-Like Receptor 4 (TLR 4) agonist, Lipopolysaccharide (LPS) for one hour induces the so-called “MSC1”, pro-inflammatory phenotype\textsuperscript{61}. In contrast, MSCs exposed to the TLR3 agonist, poly (I:C) for one hour display the MSC2, or anti-inflammatory phenotype. Therefore, it may be possible to enhance MSC culture conditions to improve their potency for clinical therapy\textsuperscript{62}.

As reviewed by Madrigal and colleagues, the therapeutic efficacy of MSCs after culture in 3D conditions may be higher than after culture in a 2D setting\textsuperscript{62}. Spheroid culture of BM-MSCs induces stanniocalcin-1 expression, which has anti-apoptotic and anti-inflammatory properties, greater than in 2D traditional culture\textsuperscript{63}. Also, when mice with peritonitis were infused with MSCs derived from either 2D or 3D culture, the 3D cultured MSCs decrease pro-inflammatory molecules, TNF-alpha, IL-1beta, and PGE-2 more than MSCs from 2D culture condition\textsuperscript{63}. When comparing media from MSCs cultured in 2D or 3D, medium from 3D cultured MSCs significantly decreased the viability of osteosarcoma cells compare to medium from MSCs cultured in 2D\textsuperscript{13}.

In summary, the therapeutic potency of MSCs might be improved by 3D culture. Moreover, using HPL gel as a 3D scaffold is easy, inexpensive and and does not expose the cells to
xenogeneic products. Moreover, HPL gel can be used for 3D invasion assay to test invasion response for stimuli.
Chapter 2 - Characterization of Human Umbilical Cord-Derived
Mesenchymal Stromal Cells Cultured in 3D Human Platelet Lysate

Gel

Abstract

Mesenchymal stromal cells (MSCs) are attractive candidates for regenerative medicine since they appear to be safe and effective for modulating diseases with an inflammatory component such as osteoarthritis and Crohn’s disease. The immune modulating and paracrine effects noted clinically cannot be explained solely by cellular engraftment since a small fraction of the injected MSCs can be recovered one month after transplantation. Traditional two-dimensional (2D) MSC culture evaluates cells expanding along a flat surface. In contrast, their natural environment, e.g., in vivo, is complex and three-dimensional (3D). We speculate that 3D cell culture may more accurately reflect in vivo cellular behavior. The objective of this study was to compare human umbilical cord-derived (HUC) MSCs cultured on a traditional 2D tissue culture plastic surface versus MSCs grown in a 3D human platelet gel model. We compared proliferation rate three ways (e.g., MTT Assay, population doubling time (PDT), CFU), expression of cell surface receptors by flow cytometry, and cellular differentiation to bone, cartilage and fat of HUC-MSCs. We found that cells grown in 3D had a lower proliferation rate than those from 2D but they still had ability to attach to plastic surfaces when seeded back in traditional 2D culture. The surface markers of cells after exposure to 3D HPL culture and collection by nattokinase shows that they express CD90, CD 73 more than 95% of the cells and thus meet ISCT’s MSC definition but CD 105 expression did not meet ISCT’s MSC criteria after 3D culture. However, when MSCs were cultured one additional passage in 2D, cells recovered of
expression of CD 105 and thus all positive MSC surface markers were expressed to ISCT MSC
definition. For trilineage differentiation, 3D cultured MSCs were similar to those cultured in 2D.

**Introduction**

Human umbilical cord-derived mesenchymal stromal cells (HUC-MSCs) are an attractive
cell source for the field of regenerative medicine. MSCs derived from bone marrow are the most
commonly used for cell-based therapy. Because of the limited amount of MSCs that are
obtained from bone marrow and the pain felt by donors during harvest, it is important to find an
alternative source of MSCs. HUC-MSCs are a good alternative source of MSCs because they are
isolated from a discarded tissue and the collection procedure is not invasive or painful. Therefore, the concern with the moral and ethical issues of tissue collection is reduced compared
to bone marrow derived-sources.

The traditional culture method of MSCs is two-dimensional in polystyrene tissue culture
plates. The three-dimensional tissue culture system is of interest because the environment of
MSCs in the human body is 3D. This leads to the speculation that the behavior of cells will
reflect the better biological response when cultured in 3D, and the physiology and morphology
mimics the *in vivo* environment better than 2D. The experimental results from cells cultured on
flat surface in a monolayer may lead to nonproductive results for an *in vivo* test.

The most commonly used MSC culture medium is Dulbecco’s modified eagle medium
(DMEM) supplemented with 10% fetal bovine serum (FBS) to support growth and
proliferation. For clinical application, FBS must be carefully tested to ensure it meets
requirements of good manufacturing practice (GMP) because FBS can cause cross-
contamination between species when using cells from humans, and can trigger a xenogenic
immune response. Also, FBS may suffer from lot-to-lot variation that can affect the
reproducibility of results\textsuperscript{71}. Therefore, human platelet lysate (HPL) has been suggested as a replacement for FBS in MSCs culture\textsuperscript{72-74}. BM-MSCs cultured in HPL have significantly better expansion than in FBS\textsuperscript{75-77} and cells expanded in HPL have already been used in clinical trials\textsuperscript{78,79}. Important growth factors can be found in HPL including platelet-derived growth factor (PDGF), epithelial growth factor (EGF), vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF), hepatocyte growth factor (HGF), and transforming growth factor β1 (TGF-β1)\textsuperscript{80}.

To prepare HPL, outdated platelet aliquots go through several freeze-thaw rounds to break the platelet membrane and thus release their growth factors\textsuperscript{81}. However, the medium requires the addition of heparin as an anticoagulant to keep the medium in the liquid form. Heparin can impair growth rate and adipogenic and osteogenic differentiation of BM-MSCs.\textsuperscript{82} To avoid the problems, and to imitate 3D environment \textit{in vivo}, HPL can form a hydrogel if heparin is omitted before exposure to calcium containing solutions, and thus HPL can be used for 3D cell culture.

After allowing MSCs to expand for a few days until they reach about 80-90\% confluence, they are dissociated or lifted from tissue culture plates by trypsinization. In HPL gel, nattokinase (NK) is added to dissolve the gel, since trypsin-EDTA is unable to disrupt the HPL gel. NK is a serine protease, fibrinolytic enzyme isolated from \textit{Bacillus subtilis} natto B-12 in traditional fermented Japanese soybean\textsuperscript{83}. NK has been used in clinical settings as thrombolytic supportive to treat and prevent cardiovascular diseases\textsuperscript{84}.

Here, we compare HUC-MSCs cultured in 3D HPL and exposed to NK with cells cultured traditionally in 2D. To set the MSC standard, we used ISCT’s minimal definition of MSCs\textsuperscript{3}: MSCs should (1) adhere to plastic and self-renew, (2) be positive for the surface markers
CD105, CD73, CD90, and negative for the surface markers CD45, CD34, CD14, CD11b, CD79 alpha, CD19, and HLA-DR, and (3) differentiate into adipocytes, chondrocytes and osteocytes.

**Materials and Methods**

**HUC-MSC Isolation**

After birth the umbilical cords were collected with a sterile technique and processed for isolation within 4 days. MSCs from 5 donors were isolated from discarded anonymous umbilical cords with a protocol previously described\(^8\). This experiment used human tissue reviewed by the Kansas State University Institutional Human Subjects Review Board (IRB review #5189). Universal precautions were used for working with potential human blood borne pathogens per containment recommendations from Occupational Safety and Health Administration (OSHA) 29 CFR. 1910.1030.

**Preparation of pooled HPL**

Expired human platelets were obtained from Community Blood Services (Kansas City, MO). Platelets from at least 25 qualified donors were pooled together and then frozen to -20°C. The pooled human platelets were thawed at room temperature until no ice crystals were observed. Then, the pooled platelets were re-frozen and thawed a second time. The pooled product, now called as human platelet lysate (HPL), was centrifuged at 4,000 Xg for 5 minutes at 4°C. Supernatant was collected and filtered through a 100 μm filter and a 0.22 μm filter and then aliquotted into 40 ml aliquots and kept at -20°C until use. Prior to use, randomly selected aliquots were sterility tested per USP 71 and tested for mycoplasma contamination using a nucleic acid based kit (Biological Industriues, EZ-PCR mycoplasma test kit). Once proved sterile and mycoplasma negative, the lot was released for cell culture use.
**Cell culture in 10% HPL medium and 30% HPL gel**

Five different HUC-MSCs lines between passages 4-8 were used. Cells were plated at 10,000 cells/cm² on CytoOne plates and grown at 37°C in a 5% CO₂, 90% humidity atmosphere (NuAire model 4950) until they reached 70-90 % confluence. For 2D culture, cell culture media consisted of Dulbecco’s Modified Eagle’s low glucose medium (DMEM, Gibco® by Thermo Fisher Scientific, Cat. No. 11885-084), 10% HPL, 1% GlutaMAX™ (Life Technologies, Cat. No. 35050), 1% antibiotic-antimycotic (Life Technologies, Cat. No. 15240-062), and 4 units/mL heparin (1000 USP U/mL). Once cells reached 70-90% confluence, medium was removed and, the cells were rinsed with Dulbecco’s Phosphate Buffered Saline (DPBS) without calcium and magnesium (Life Technologies, Cat. No. 14190-250) warmed to 37°C. After removing DPBS, 0.05% trypsin-EDTA (Lifetech, Cat. No. 25200-056) was added and cells were incubated for 3 minutes at 37°C. To neutralize the enzyme, 3x volume of 10% HPL medium was added. After centrifugation at 200 X g for 5 minutes at room temperature, the supernatant was discarded and the cell pellet was resuspended for a live cell count (Nexcelom Auto 2000 with ao/pi live/dead stain). For 3D culture, 30% HPL (v/v) was chosen in DMEM low glucose medium, 1% GlutaMAX™, 1% antibiotic-antimycotic. Sterile CaCl₂ was added to the media to a final concentration of 1mM and complete gelatinization occurred within 1 hour. After the hydrogel base layer was formed, cells suspended in 1 mL of media were added on top of the gel so that the medium containing the cells would be suspended in gel in contact with the previously formed gel and not the tissue culture plastic.

Once cells reached confluence, 800 uL of 1.75% NK was added for 30, 60, 90 or 120 minutes with shaking on an orbital shaker to disrupt the gel. Cells were then collected and centrifuged, and the supernatant was discarded and the cell pellet was resuspended as described...
above. After cells were cultured in HPL gel and exposed to NK for one passage, cells from 3D were ready for the experiment.

**Differentiation**

Tri-lineage differentiation of the cells was performed according to the manufacturer’s protocol for adipogenic, chondrogenic, and osteogenic differentiation medium (StemPro, Life Technologies, Cat. Nos. A10070-01, A10071-01, and A10072-01, respectively). Cells grown in 2D culture conditions and cells grown in 3D cultures (both greater than passage 5) were placed in a 12-well plate (CytoOne, USA Scientific, Cat No. CC7682-7512). After 21-28 days of differentiation, the differentiation media was removed and cells were washed with DPBS without calcium and magnesium (Corning Cat. No. 21-030-CV). Then the cells were fixed with freshly prepared 4% paraformaldehyde in phosphate buffer (pH 7.4) for 30 minutes at room temperature. For adipocytes, fixed cells were stained with Oil Red (Sigma Aldrich Cat. No. HT904-8F0Z). For osteocytes, cells were stained with Alizarin Red S (Sigma Aldrich Cat. No. A5533-25G). For chondrocytes, cells were stained with Safranin O (Sigma Aldrich Cat. No. O0625-100G). The area of positive staining was measured using ImageJ (Dr. Wayne Rasband, NIH).

**Flow Cytometry Analysis**

Flow cytometry was performed using the BD Biosciences’ MSCs characterization kit (Cat. No. 562245). Cells from 2D, 3D, and 3D then cultured another passage in 2D were prepared and stained with antibodies: CD40 FITC, CD105 PerCP-Cy5.5, CD73 APC as compensation controls. mIgG1, k FITC, mIgG1, k PerCP-Cy5.5, and mIgG1, k APC were served as hMSC Positive Isotype Control Cocktail. mIgG1, k PE and mIgG2a, k PE were served as PE hMSC Negative Isotype Control Cocktail. hMSC Positive cocktail contained CD90 FITC, CD105 PerCP-Cy5.5 and CD73 APC. PE hMSC Negative Cocktail had CD34, CD11b, CD19,
CD45, and HLA-DR. Isotype controls were prepared (e.g., PE Mouse IgG2b, k) to identify and gate cells in the context of compensation settings for multiple fluorochromes. Cells were labeled and analyzed following manufacturer’s protocol (BD Biosciences, Cat. No. 562245). Then samples were analyzed using a BD FACSCalibur flow cytometer with negative gates (e.g., isotype controls) set at 1% positive.

**Scanning Electron Microscope**

3D HPL gel was fixed using Trump’s fixative (e.g., VWR 100503-726) at 4 °C overnight. After removing fixative, samples were triple rinsed with 0.1 M phosphate buffer for 5 minutes, and triple rinsed with double distilled water for 5 minutes. Then they were put in desiccators under vacuum for 48 hours. Samples were imaged with Hitachi S-3500N Scanning Electron Microscope.

**Metabolic Activity**

Methyl thiazolyl tetrazolium (MTT) assay (Vybrant™ Cat. No. V-13154) was performed to measure metabolic activity. Cells either cultured in either 2D condition or 3D condition (previous passage) were plated at a density of 3,200 cells per well in 96-well plate (CytoOne Cat. No. CC7682-7596) with DMEM supplemented with 10% HPL, 1% GlutaMax, 1% antibiotic/antimycotic but without phenol red. After allowing cells to attach and expand for 48 hours, 10 uL of 12 mM MTT was added in each well and incubated for 4 hours at 37 °C. Next, 100 uL of 0.01 mM HCl containing 10 mg SDS was added in each well and incubated at 37 °C for 16 hours. Absorbance was read at 570 nm using a SpectraMax i3x plate reader (Molecular Devices) and a standard curve was generated after averaging technical triplicates.
**Colony-Forming Unit Assay**

Cells either from 2D and 3D group were plated in 2D culture dish at low concentration, 10 cells/cm² in DMEM low glucose supplemented with 10% HPL, 1% GlutaMax, 1% antibiotic/antimycotic. Plates were placed into the incubator and left untouched for seven days to allow the cells to attach and form colonies. Medium was then removed and cells were washed with DPBS. Cells were fixed with freshly prepared 4% paraformaldehyde in phosphate buffer for 30 minutes at room temperature. After rinsing with DPBS again, 1% methylene blue was used for staining the cell colonies. The colonies, defined by groups of > 12 cells, were counted and averaged from technical triplicates. Colony forming efficiency was calculated by dividing the plating density by the average number of colonies.

**Statistical Analysis**

Statistical analysis was performed using Sigma Plot v 12.5 (Systat Software, Inc). When analysis of variance (ANOVA) assumptions were met, it was used to evaluate main effects and interactions. When ANOVA assumptions were not met, simple mathematical transformations of the data were applied to attempt to render the data normal or unimodal, so that ANOVA could be performed. When transformed data did not meet ANOVA requirements, non parametric statistics were used to evaluate the null hypothesis. When a significant main effect or interaction were found by ANOVA, post hoc analysis was performed to compare pre-planned comparisons using the Bonferroni correction. Then graphs were made in Sigma Plot showing averages (means) and Least Squares standard error of the mean. The graphs were saved in EMF format. EMF files were opened and edited using Canvas v 15 (ACD systems of America, Inc) for figure preparation. Two tailed analysis was performed and p < 0.05 was called “statistically significant”.
Results

HPL gel functions as a scaffold for HUC-MSCs

HUC-MSCs grown in 3D reached 70-90% confluence in 3-5 days (see figure 2-1). To prevent attachment of cells to tissue culture plate, HPL gel was placed on bottom and gelled before seeding HUC-MSC suspended in HPL gel on top. Scanning electron micrographs of 20%, 30%, and 40% HPL showed a surface with fibrin matrix forming pores of different sizes (see figure 2-2). Freeze/ fracture was not performed.

![Figure 2-1: HUC-MSCs culture in 2D and 3D pictures](image-url) HUC-MSCs plated in a standard tissue culture plate in 2D at day 1 (a) and day 3 (b) and HUC-MSCs plated in 3D HPL gel at day 1 (c) and day 3 (d).
Nattokinase (NK) was able to retrieve HUC-MSCs from HPL gel

After pilot studies with the different concentrations of NK to retrieve HUC-MSCs out of 30% HPL gel, 1.75% NK was used in follow-up experiments. Different incubation times of NK from 30, 60, 90, or 120 minutes were used to retrieve HUC-MSCs from the HPL gel. Although there was no statistical difference in the number of MSCs retrieved from different NK incubation times, 1.75% NK at 60 minutes was used for following up because 30 minutes incubation in NK did not dissolve the entire gel, whereas other incubation times dissolved the gel (see figure 2-3(a)). NK exposure for 60 minutes was useful to remove HUC-MSCs from HPL and there was no advantage to exposing HPL gel to NK for longer than 60 minutes. When cell numbers were compared between HUC-MSCs cultured in 10% HPL (liquid) medium in 2D with those cultured in 30% HPL gel (e.g., in 3D), there were significantly more cells in 2D than in 3D (see figure 2-3(a)). There was no difference in the viability of the cells from 2D which were lifted by 0.05% trypsin-EDTA for 3 minutes or the cells that were extracted from the 3D gel using 1.75% NK for 60 minutes (see figure 2-3(b)). In any case, when HUC-MSCs from 3D culture were harvested and returned to 2D culture, they attached to plastic and grew normally (see figure 2-4).
Figure 2-3: Cell number and viability after extracted from 2D (10% HPL) and 3D (30% HPL). Cells were seeded at 10,000 cells/cm² in 2 wells of 12-well plate and dissociated after 72 hours of growing. Cells from 2D culture are released by 0.05% trypsin-EDTA, 3 minutes. Cells from 3D culture are incubated with 1.75% NK at 30, 60, 90, and 120 minutes. Effect of incubation time of 3D HPL gel by NK and 2D by trypsin-EDTA on cell numbers (a) and viability (b).

Figure 2-4: HUC-MSCs at day 3 (40x): After culturing in 3D in the 30% HPL gel and dissociated by NK, HUC-MSCs returned to 2D culture conditions attached to the tissue culture plate and expanded normally.

HUC-MSCs from 2D culture grew faster, e.g., had a higher proliferation rate, than cells from 3D culture.

Proliferation rate of HUC-MSCs was analyzed using the MTT proliferation assay. Figure 2-5 summarizes the results from five independent HUC-MSCs lines that were grown in 2D and 3D conditions for at least one passage before their proliferation was assessed using MTT. There
was significantly more cells, approximately 3 times more cells, in the 2D culture condition than in the 3D culture condition. This suggests that HUC-MSCs grow more slowly in 3D conditions.

Next, population doubling time analysis (PDT) was performed in five independent cell lines grown for at least three passages in both 2D and 3D conditions. On average from the five lines, HUC-MSCs cultured in 10% HPL in 2D culture in a traditional culture plate had the lowest PDT (21.94 hr ± 0.66) and those were cultured in 3D in HPL gel had the highest PDT (101.63 hr ± 13.71), a statistically significant change (p < 0.05) in proliferation. This indicates that cells grow approximately 4.5 times slower in 3D than in 2D conditions. This result is similar to what we observed with MTT (see figure 2-5). To determine whether the slower growth in 3D culture was due to an inhibitory effect of 30% HPL vs 10% HPL, the PDT for the same lines was compared in 2D culture when HUC-MSCs were grown in 30% HPL supplemented medium with heparin in 2D. While the HUC-MSCs did grow significantly slower in 30% HPL in liquid medium in 2D compared to 10% HPL in 2D, they still grew significantly faster than 3D (see figure 2-6). To determine whether the cells were capable of faster growth in 2D after 3D culture, their PDT was evaluated after 3D culture when returned to 2D. As shown in figure 2-6, returning HUC-MSCs to 2D culture after growing them in 3D resulted in a return to significantly faster proliferation.

Colony Forming Unit-fibroblast assay results were determined seven days after seeding in 2D tissue culture plate. The results indicated that HUC-MSCs grown in 2D culture had ability to form more colonies, approximately 3 times more colonies, than cells from 3D culture (see figure 2-7).
Figure 2-5: Proliferation Analysis HUC-MSCs 5 lines were analysed by MTT Assay

Figure 2-6: Average Population Doubling Time from HUC-MSCs 5 lines Cells were cultured in 2D 10% HPL (yellow), 2D 30% HPL (red), 3D 30% HPL (beige), and 2D 10% HPL after exposed to 3D 30% HPL gel and NK for 1 passage (purple).
Figure 2-7: Colony Forming Unit-fibroblast representative pictures of CFU-F plate either from 2D (a) and 3D (b) seeded at 10 cells/cm². Average colony number calculated from technical triplicates from 5 HUC-MSC lines between 2D and 3D culture (c)

HUC-MSCs cultured in 30% HPL gel in 3D and exposed to NK expressed MSC surface markers and maintained tri-lineage differentiation

Cell surface markers expression was analyzed by flow cytometry in three lines. Those same lines of HUC-MSCs cultured in traditional 2D culture served as a control. HUC-MSCs either cultured in 3D HPL gel and those culture in 3D in 30% HPL, harvested and then cultured 1 additional passage in 2D culture were compared. CD 73, CD 90, and CD 105 were found on more than 95% of the cells (see figure 2-8) and CD34, CD 11b, CD 19, CD 45, and HLA-DR were detected on less than 2% of the cells in control group (figure 2-8 (a)). After cells were grown in 3D HPL gel were immediately analyzed by flow cytometry, HUC-MSCs expressed all the MSC positive markers on more than 95% of the cells except for CD 105. The CD 105 surface
marker showed lower level of expression and failed to meet the ISCT criteria of > 95% positive. Moreover, 3D HPL culture HUC-MSCs were positive (>2% positive) for PE negative cocktail CD34, CD 11b, CD 19, CD 45, and HLA-DR (figure 2-8 (b)). To determine whether the effect of 3D culture on surface marker expression was reversible, the HUC-MSCs were cultured one additional passage in 2D culture, e.g., the traditional setting. In all three HUC-MSC lines analyze, the positive and negative cell surface markers expression was not different from the control group grown in 2D (figure 2-8 (c)). This indicates that 3D culture conditions alter surface marker expression by HUC-MSCs.

*In vitro* differentiation for adipogenesis, chondrogenesis, and osteogenesis were assessed in 3 independent HUC-MSC lines after cultured in 2D and 3D conditions. Oil Red O was positive for lipid droplets in both 2D and 3D groups exposed to adipogenic differentiation cocktail for > 2 weeks. Cartilage-like structures were found after exposure to chondrogenic differentiation medium with the positive of glycosaminoglycan from Safranin O staining in both 2D and 3D groups. Calcium deposition, stained by Alizarin Red, was positive in both 2D and 3D groups after HUC-MSCs were exposed to osteogenic differentiation media for > 2 weeks. Quantitative data analysis using ImageJ to assess the positive pixel area in each condition showed no significant difference in tri-lineage differentiation between HUC-MSCs from 2D and 3D culture condition (see figure 2-9).
Figure 2-8: Flow Cytometry data HUC-MSCs with the cultured in 2D traditional culture (a), 3D HPL gel and dissociated by NK (b), 3D HPL gel and then dissociated by NK and cultured in 2D plate for an additional passage before performing flow cytometry (c). hMSC Positive Cocktail contained CD 90 (FITC), CD 105 (PerCP Cy5.5), CD 73 (APC). PE hMSC Negative Cocktail (a, b, c) was composed of CD 34 (PE), CD 11b (PE), CD 19 (PE), CD 45 (PE), HLA-DR (PE). Flow cytometry results were summarized in bar graph (d).
Figure 2-9: Tri-lineage differentiation HUC-MSCs from 2D culture (a) and 3D culture (b) are positive for Oil Red O staining after induction by adipogenic differentiation media. HUC-MSCs from 2D culture (c) and 3D culture (d) are positive for Alizarin Red staining after induction by osteogenic differentiation media. HUC-MSCs from 2D culture (e) and 3D culture (f) are positive for Safranin O after induction by chondrogenic differentiation media. Quantitative analysis of positive area staining of fat, bone, cartilage (g).
Discussion

The International Society for Cellular Therapy (ISCT) defines minimal criteria of being MSCs\(^3\). The criteria include plastic adherence and self-renewal, MSC surface marker expression, and differentiation capability. Here, we assessed whether HUC-MSCs that were cultured in 30% HPL gel could maintained the ISCT minimal criteria for MSCs.

We showed that HUC-MSCs can be cultured and expanded in 30% HPL gel as a 3D scaffold that can invest and support MSC growth. We observed MSC migration through the hydrogel associated with expansion. HPL hydrogel, a fibrin-based structure which polymerizes during blood clotting, forms a non-fluorescent (data not shown), transparent soft hydrogel that supports MSC expansion (figure 2-1). The polymerized fibrin forms a network which is visible in SEM, and shows a decreased pore size as the concentration of the HPL in the gel increases from 10 to 30% (see figure 2-2). Based upon the minimal time to liquify completely the gel, we selected 1.75% NK at 60 minutes incubation to retrieve HUC-MSCs. After culture in 3D and dissociation by NK, HUC-MSCs retain the ability to adhere to plastic tissue culture plates and grow in 2D at a similar rate to their growth in 2D prior to culture in 3D hydrogel. In contrast to 2D culture, when cells are cultured in 3D hydrogel, they had slower proliferation than 2D, as demonstrated by MTT assay, which show that the metabolic activity than cells from 3D condition is less and PDT and CFU results which show the cell cycle time is longer in 3D than 2D. 3D hydrogel expansion of HUC-MSCs affected their cell surface marker expression by lowering CD 105 level expression and increasing negative phenotypes, CD 34, CD 11b, CD19, CD45, and HLA-DR. Interestingly, the expression of these surface markers can be rescued by growing the MSCs in an additional passage in 2D using standard culture conditions. HUC-MSCs
grown in either 2D or 3D culture conditions demonstrated similar tri-lineage differentiation capability.

Pilot studies (data not shown) demonstrated that the HPL hydrogel was not disrupted with 0.05% trypsin-EDTA. For the HUC-MSCs expanded in 3D HPL hydrogel to be characterized, we needed to retrieve the cells. We used nattokinase, a fibrinolytic enzyme at 1.75% concentration for 60 minutes. Although there is no significant difference in cell numbers after 30-120 minutes incubation, at 30 minutes we observed that the gel was not fully dissolved. We found that 60 minutes was the minimum time needed to disrupt 30% HPL hydrogel when using 1.75% NK, and incubation times longer than 60 minutes did not retrieve more cells. Our data suggest that NK is not harmful to HUC-MSCs based on cell number and cell viability over the 30-120 minutes incubation. As described by Carrion and colleagues, NK was able to extract MSCs out of a fibrin gel better than trypsin or TrypLE at the same incubation period\textsuperscript{44}. They also reported that the numbers of MSCs in both 2D and 3D was comparable, whereas our experiments found significantly more MSCs when grown in 2D than 3D hydrogel when both are seeded identically on day 0. One possible explanation of our observation is the cells cultured in 3D may need to adapt themselves after 2D culture for >5 passages and may need more time to enter the exponential growth phase. Our MTT assay revealed that 2D culture had higher proliferation rate than 3D HPL gel. However, MTT is one of many methods to determine cell proliferation and it is an indirect method to measure proliferation detected by color change. So, to confirm that observation, we evaluated PDT and CFU-F. Those results show the same trend as the MTT assay: that MSC cultured in 3D for 1 passage have significantly higher PDT and significantly lower CFU-F from 2D. Our results of MTT assay, PDT, and CFU is in contrast to the results of Walenda and colleagues\textsuperscript{39}. They found that proliferation analyzed by MTT assay was higher on
HPL gel culture than traditional 2D tissue culture plate and the result of PDT in 3D HPL culture was similar to 2D culture. For CFU analysis, they revealed that cells from HPL gel has higher CFU than cells expanded in 2D cell culture. Wallenda et al., reported that HUC-MSCs were embedded in HPL gel, but their methods suggested that they plated MSCs on top of the HPL gel, and within a liquid HPL medium and not embedded within the matrix of the hydrogel. Moreover, for MTT assay, they did not separate cells from 3D HPL gel to measure proliferation rate. In contrast, we retrieved cells from both 2D and 3D culture and seeded them in 2D tissue culture plates to perform the MTT assay. For gel extraction to get cells for processing to the next passage, they used pipetting to collect cells but we used NK. Moreover, they used bone marrow derived MSCs. All of these differences may account for the contrasting observations between our two reports.

Next, we assessed the ability of HUC-MSCs to express surface markers after culture in 3D HPL gel and extraction by nattokinase. This study showed cells from 3D culture had high expression (>95%) of surface markers CD90, CD44, and CD73 but not for CD105 (it was found to be 76.07 ± 10.46 %). For, HSC markers that should be negative for MSCs, (e.g., <2%) CD34, CD 11b, CD19, CD45, and HLA-DR, cells from 3D and exposed to NK express markers that should be negative (as defined here as < 2% positive). In contrast we observed 61.33 ± 17.39 % positive for the negative markers immediately after 3D culture (see figure 2-8). We observed that after culture for an additional passage in 2D, MSC positive and negative surface markers were “rescued”. This suggests that culture in the soft hydrogel, which altered the population doubling times and metabolism, also may temporarily affect MSC surface marker expression.

We then investigated the ability of three HUC-MSC lines to undergo tri-lineage differentiation. Previous research showed MSCs after culture in platelet lysate had the ability to
undergo tri-lineage differentiation\textsuperscript{39}. Our qualitative analysis indicates that MSCs either from 2D or 3D culture and then plated in 2D on a plastic tissue culture plate were able to differentiate into fat, bone, and cartilage after 21-28 days of differentiation. This is a limitation of the present work. In the future, a quantitative analysis, perhaps made by measuring calcium deposition for osteogenic differentiation, or GAG contents for cartilage differentiation, would provide a better means of comparison of differentiation potential between the different culture conditions. Here, we used the positive staining areas as determined by ImageJ to compare. Oil red O is positive for lipid droplets accumulated in the cytoplasm after adipogenic differentiation. Safranin O stains for cartilage associated with glycosaminoglycan after chondrogenic differentiation. Alizarin red stains calcium deposition for osteogenic differentiation. We found no significant differences of positive area between HUC-MSCs from 2D and 3D culture conditions in tri-lineage differentiation. We observed a trend of positive area in cells from 2D than 3D in chondrogenic and osteogenic differentiation. As mentioned above, our method to quantify differentiation potential should be improved upon before solid conclusions can be made.
Chapter 3 - A Customizable Assay for Testing Cell Invasion

Abstract

Expanding cells in a three dimensional (3D) microenvironment for tissue engineering and in vitro testing has certain challenges. A variety of substrates have been tested but few substrates meet all the design requirements. For example, the substrate should permit cell anchorage, be self supporting, be biocompatible so the cells survive within it, be porous to permit cell migration, be suitable for 3D printing, and the polymerization conditions must be compatible with cell viability. Here, we tested a hydrogel based upon human platelet lysate (HPL) that includes these specifications and determined that it supported the expansion of undifferentiated human mesenchymal stromal cells (MSCs) in 3D culture. MSCs grown in 3D were tested for their ability invade the hydrogel for use as a cell invasion model. The HPL gel was liquified by 1.75% nattokinase treatment for 60 min, liberating the cells. The MSCs could be suspended in the hydrogel formed in 3D within the matrix (e.g., before they fell to the bottom of the plate). We tested a range of gel concentrations from 20-40% and MSC grown in 30% HPL was used. 30% HPL gelled in 1-1.5 hours after mixing with Dulbecco’s modified eagle medium. The gel is transparent and non-fluorescent. MSC grew as individual cells or as colonies that showed a similar morphology of fibroblastic cells. Cells grown in 3D demonstrated positive trophism to certain test solutions, e.g., chemotaxis. This suggests that 3D culture is useful for drug development or for encapsulation of cells for clinical use.
Introduction

By industry standards, mesenchymal stromal cells (MSCs) are expanded as adherent cells attached to tissue culture treated plastic dishes in 2 dimensions (2D). Recently, Prockop’s group reported that MSCs might be grown as colonies or spheroids either by placing them in low-attachment dishes or by using the hanging drop culture method. Their work suggested that the 3D microenvironment impacts MSC physiology.

In contrast to 2D culture, in vivo MSCs grow in three dimensions (3D) and the 3D microenvironment may influence migration, self-renewal, patterning, and cell fate decisions through both integrin-based cell-ECM signaling, cadherin-based cell-cell interactions, mechanical traction via interaction with the substrate, and perhaps other factors. MSCs stromal cell/ stem cell fate decisions may involve signals via ligands such as cadherins, integrins, cytokines, growth factors and nutrients, and gradients thereof, and mechanical properties such as stiffness or elasticity or porosity of the substrate. Therefore, the 2D environment fails to mimic the MSC’s in vivo microenvironment since the cells in a 2D colony have planar-type interactions and chemical gradients compared to the 3D arrangement within the body. Our goal here was to develop novel 3D culture methods for MSCs and test a new substrate for its potential for biocompatibility, tissue engineering and 3D molding or bioprinting applications.

Other laboratories have successfully encapsulated MSCs in 3D, for example, by using alginate microcapsules. Since alginate lacks an attachment moiety for MSCs, the cells grow as spheroids within the capsule. In contrast, here we used a fibrinogen-based hydrogel derived from polymerization of fibrin in human platelet lysate (HPL). Previous work suggested that pooled platelet lysate is a good medium supplement for MSCs. Gelation of HPL occurs when
platelet lysate is exposed to the calcium found in culture medium, and gelation of HPL can be inhibited by the addition of heparin.

In previous work, our group reported that MCF-7 breast cancer cells could be encapsulated in a synthetic peptide hydrogel. Here, we investigate the feasibility of 3D encapsulation of human umbilical cord derived MSCs (HUC-MSCs) in the pooled platelet lysate gel. Our data shows that HPL can be cast into molds prior to gelling, and the the HPL gels are biocompatible. We found small physical differences in the HPL when varying concentration from 20-40% (v/v), and that HPL gel is suitable for MSC expansion in 3D culture. We demonstrate found that by adding HPL mixed with MSCs into a calcium solution, one may 3D “print” a MSC gel construct for tissue engineering applications. The HPL formulations used here were not self-supporting and the 3D constructs slumped after printing. We developed a 3D invasion assay that demonstrated MSC migration towards TLR3 and TLR4 agonists. Further work is needed to determine additives that can strengthen HPL gel so that it is self supporting and has greater stiffness.

**Methods**

**Human MSCs.**

The KSU human subjects review committee reviewed our protocol (IRB #3996 and #5189). Here, previously isolated and characterized HUC-MSCs from five lines were used. Low passage (p3-4) MSCs were thawed, expanded one passage in 2D prior to use here.

**2D cell culture**

Human HUC-MSCs were cultured in Dulbecco’s modified eagle medium (low glucose, DMEM, (Gibco® by Thermo Fisher Scientific, Cat. No. 11885-084). DMEM was supplemented with 2mM GlutaMax (Life Technologies, Cat. No. 35050), 1% antibiotic/antimycotic Life
Technologies, Cat. No. 15240-062) with 10% (v/v) pooled human platelet lysate (manufactured in-house) as previously described in chapter 2. Cells were plated at 10-15,000 cells per cm² on tissue culture plastic plates (CytoOne 6 or 12 well plates) and incubated at 37°C in a 5% CO₂, 90% humidity atmosphere (NuAire model 4950). Cells were passaged every 3-5 days by removing media and rinsing with Dulbecco’s phosphate buffered saline without calcium or magnesium (DPBS), exposed to 0.05% trypsin-EDTA at 37°C until they lifted off the plate, followed by inactivation of the trypsin by adding 1.5 volumes of medium.

3D cell culture

We adapted the methods previously described to encapsulate breast cancer cells in 3D, here, to encapsulate MSCs. The human platelet lysate medium was prepared and the cells were added at the desired density. Gel formation begins within 1.5 h at 37°C. To passage the cells, the hydrogel and MSCs were treated with 1.75% of nattokinase for one hour. Once the gel liquefied, the solution was centrifuged and the cells pelleted by low speed centrifugation (100-200 Xg for 5 minutes at room temperature). The supernatant was discarded and the cell pellet was resuspended in DPBS. The cells were counted using AO/PI using a Nexelom Auto 2000.

**Figure 3-1.** Mold for making 3D hydrogel for the 3D invasion model. The lid of the 12 well plate created by 3D printing of ABS using the FlashForge Creator Pro printer (left). Schematic of the three voids created by the positive mold (right). The central void was loaded with mesenchymal stromal cells, the left void was filled with medium only (control), the right void was filled with medium with either Toll-like receptor (TLR) 4 or 3 agonists. Cell migration was observed for up to five days.
3D invasion model

As shown in figure 3-1, a customize lid was printed using 1.5 mm ABS in a FlashForge Creator Pro 3D printer (an example 3d files is found on the NIH 3D print exchange website https://3dprint.nih.gov/discover/3dpx-003524) to fit 12-well plate to create voids in the hydrogel of the casted gel. To the central void 75,000 MSCs were added. To the left void, DMEM with 10% HPL was added (control). To the right void, DMEM with 10% HPL with either the Toll-like receptor 4 (TLR4) agonist lipopolysaccharide (LPS, 10 ng/mL) or the TLR3 agonist Poly (I:C), 1 ug/mL was added as described in Waterman and colleagues’ (2010). Five days after plating, MSCs were collected and counted.

Static compression testing of platelet lysate hydrogel in DMEM medium

The spring constant and the elasticity of hydrogels were determined on an Instrom 5940. All mechanical tests were performed at room temperature (22-24°C). A compressive stress strain curve was generated using a speed of 1.20 mm/min using gels cast either immediately (on day 0, at least 2 hrs after casting), or 3 or 7 days later. To determine the hydrogel compressive strength, spring constant and elasticity, 20-40% gel samples were prepared in triplicate. The gel was tested at day 0, 3, and 7 by transferring to the Instron and allowed to sit for stabilization for 1 min and then subjected to compressive strain for 1 min. The linear part of the Load/ compressive extension curve was used to determine the spring constant (delta load/delta extension) or the elasticity using the formula Young’s modulus (MPa) = (Force (at max linear load) * max linear extension) / (delta extension * Area of probe). The values for three samples were measured and averaged.
**Statistical Analysis**

Statistical analysis was performed using Sigma Plot v 12.5 (Systat Software, Inc). When appropriate analysis of variance (ANOVA) was used to evaluate main effects and interactions. When a significant main effect or interaction was found by ANOVA, post hoc analysis was used to compare pre-planned comparisons using the Bonferroni correction. Graphs were made in Sigma Plot showing averages (means) and Least Squares standard error of the mean. These graphs were saved in EMF format, and then edited using Canvas v. 15 (ACD systems of America, Inc) for figure preparation. Unless indicated otherwise, two tailed analysis was performed and p < 0.05 was deemed “significant”.

**Results**

**Platelet lysate gel physical properties**

Compression test were conducted on day 0, 3, and 7. There were no significant differences in elasticity between 20-40% (v/v) gels or between day 0-7 (figure 3-2). The spring constant K was found to be about 124 N/mm for 20% HPL, 122 N/mm for 30% HPL, and 118 N/mm for 40% HPL on day 0. On day 3, 20% HPL spring constant was 118 N/mm, 30% HPL was 119 N/mm and 40% HPL was 115 N/mm. This indicates a decrease in the spring constant. Furthermore, by day 7, 20% HPL’s spring constant had decreased to 86 N/mm, 30% HPL’s spring constant was 120, and 40% HPL was 114 N/mm. So, both 20 and 40% showed a clear trend to reduction of spring constant with gel aging. In figure 3-2 gel elasticity measures are shown. All three gels the elasticity was in the 1000 kPa range, a strength that is similar to gelatin hydrogels (Xing Q, Yates K, Vogt C, Qian Z, Frost MC, Zhao F. Sci Rep 2014; 4: 4706). We used 30% HPL for 3D cell culture experiements below.
Figure 3-2: Mechanical test of platelet hydrogel. Young modulus (left) and Spring Constant (right) of 20, 30, and 40% HPL at day 0, 3, and 7.

Effect of Gel concentration on MSC growth

The gel is transparent in visible wavelengths and non-fluorescent at standard wavelengths for fluorescent staining. This permitted observation of cell growth and morphology of the live encapsulated MSCs in 3D culture using phase contrast or epifluorescence microscopy. We tested gels ranging in strength from 20-40%. MSCs grow more slowly in the 30% hydrogel in 3D than they did in medium enriched with 10% or 30% HPL in 2D, based upon the number of days until passage (3 days in 2D and 4 days in 3D, data not shown).

MSC morphology in 3D culture

Cells encapsulated within the 30% HPL gel proliferated. The human MSCs became visible as small colonies usually two days after plating (see figure 3-3). When plating dissociated single cells, the colonies in a particular passage were of consistent size. The colony’s morphology was irregular, and more highly refractile colonies with occasional dark spots (see figure 3-3). The colonies increase in size (grow) over time in culture and usually became ready
for passage by day 4-5. Individual cells formed “bridges” between colonies and the cells appear to invade the gel and migrate from the edges over time in 3D conditions.

**Figure 3-3**: Picture of HUC-MSCs cultured in 3D HPL gel at day 3 (40x).

**Invasion of MSCs in 3D culture**

To investigate whether MSCs demonstrate chemotaxis or trophism, e.g., migrate through the 3D gel in response to chemical signals, MSCs were plated such that they were positioned between two test wells (see figure 3-4 (a) and (b)). The day after plating MSCs, substances were added to the test ports. For example, in the left well, medium only was added (as a control sample). In the right well, either the Toll like receptor-4 (TLR) agonist lipopolysaccharide (LPS) or the TLR3 agonist poly (I:C) was added. The two compounds were added daily thereafter to maintain the chemical gradient. Each day after adding the test compounds, MSC invasion was evaluated by taking a photomontage using the EVOs FL Auto imaging system to determine whether MSCs had migrated into the HPL gel and which direction they were moving. We observed that MSCs invaded through the 3D matrix to reach the test well containing either LPS or poly (I:C) compared with control side (figure 3-4). After five to seven days, we counted the number of recovered cells in the control and test wells. As shown in figure 3-4 c the LPS stimulant tended to increase the number of MSCs in the test well, but this did not reach a
significant difference in migration. In contrast, as seen in figure 3-4 d, poly (I:C) stimulation produced a significant increase in the number of MSCs found in the test well. These observations show that MSCs move through the 3D HPL matrix and respond chemotactic cues.

Figure 3-4: Cell invasion Model HUC-MSCs in invasion model compared between control (left well) and LPS (right well) (a,c). HUC-MSCs in invasion model compared between control (left well) and poly (I:C) (right well) (b,d).
Discussion

It is well-known that MSCs or stem cells receive three clearly defined types of signaling that impact cell fate and self-renewal: 1) cell-cell signaling that are mediated by cadherins via Wnt, catenin / p120 pathway and 2) cell-ECM signaling that are mediated by integrins, and 3) not fully understood traction forces or mechanical interactions that are mediated by actin and or tubulin in the cytoskeleton. Our goal was to develop a 3D hydrogel matrix for embedding or encapsulating MSCs or stem cells that would provide ECM-stem cell interactions and be compatible with 3D printing methods. Ideally, this material would gel at room temperature without requiring toxic crosslinking agents and the gelation would be reversible to allow the cells to be harvested and passaged. Ideally, the material should be transparent, non-fluorescent, porous, stable and biocompatible for long-term expansion of human MSCs. Here, we showed that human MSCs grow in a 3D culture system encapsulated within a HPL gel and that we can release the cells by liquefying the gel using nattokinase for passage.

Currently, the standard 2D protocol for expansion of MSCs involves the interaction of MSCs with tissue culture treated plates. While recent publications have indicated that MSCs grown in low adherence plates, and thus free from ECM interactions, may have enhance immune physiological properties, expansion of MSCs for clinical application til date has involved laminin, collagen, heparin sulfate proteoglycans and other factors.

In human embryonic stem cells, cell-cell interactions are critical and in the early years human ESCs culture, dissociation of ESCs to single cells during a passaged resulted in massive cell death (> 90% of the ESCs died due to passage). Recently, the use of the actin-myosin inhibitor ROCK has reduced cell death in human ESCs at passage. Rat and mouse ESCs grown on inactivated mouse embryonic fibroblasts (MEFs) in medium containing MEK 1/2 and
GSK3β inhibitors have different colony morphology compared to human ESCs grown on MEFs with bFGF supplemented medium: rat and mouse ESCs form circular, domed, phase-bright colonies compared to the flat, irregular human ESC colonies\(^94\). We reported that rat ESCs have two types of colonies, colonies that attach firmly to the MEFs and colonies that are loosely attached to MEFs and appear to be floating\(^95\). A similar observation was provided by QL Ying’s laboratory\(^96\).

The HPL hydrogel used here is easy to handle, transparent, rapidly polymerized, and could be dissolved by nattokinase for harvesting cells. Surface treatments, UV-exposure or potentially toxic cross-linking agents were not required. We observed thermal stability, transparency and biocompatibility of the HPL gel without apparent batch to batch variability. The fabricated HPL gels with a Young’s modulus of approximately 1000 KPa within the range reported for untreated collagen gels\(^97\) and gelatin (see reference above).

In addition to the HPL gel, we tested a synthetic peptide hydrogel (PGMatrix, data not shown). PGMatrix is another soft gel of approximately 1000 KPa that is transparent during the first 24 hrs of culture. PGMatrix also has the property of shear thinning (reversibility) and re-gelation without the need of dissolution agents. The shear-thinning and rapid recovery property of the PGmatrix hydrogel system has been reported before\(^98,99\). This property is benefited from the unique peptide primary structure\(^98-100\) and lead to a critical injectable character for various biomedical applications\(^98,101\) including the easy-to-use protocol described in Huang et al for 3D breast cancer culture\(^89\). The PGMatrix was also suitable for MSC culture, but the gel was susceptible to disruption after 3 or 4 days of culture, perhaps due to some factor released by MSCs.
In our 3D invasion model, we tested the chemotaxis of MSC to LPS and poly (I:C) gradients. LPS and poly (I:C) represent chemical signals used *in vivo*, for example, for gram negative bacteria infection and viral infection, respectively. We found that HUC-MSCs appear to respond to chemical gradients of LPS and poly (I:C), however, significantly different positive chemotrophism was found for poly (I:C) and not for LPS. One possibility to explain this situation is MSCs may act via paracrine effect without the need of invasion to the site of infection\textsuperscript{102}. We can clearly see MSCs invade toward poly (I:C) \textsuperscript{91}. On the other hand, there was a trend for MSCs to invade toward LPS, but it did not reach statistical significances.
Chapter 4 - Conclusion

*In vitro* cell culture is a tool to expand cells and to study their biology and physiology. Ideally an *in vitro* culture system mimics the *in vivo* environment so our scientific observations are not colored by artifact due to the unnatural environment and reflect the cells’ nature *in vivo*. MSCs are adult cells that can be isolated, expanded *in vitro*, and used clinically for treating diseases, such as Crohn’s disease or acute graft versus host disease\(^6\). To reach clinical testing, it is important to understand MSC physiology so as to produce cells of optimal clinical effect and safety. Here, we expanded HUC-MSCs in 3D culture to mimic the *in vivo* environment and compared the properties of 3D expanded cells with those expanded conventionally, e.g., on tissue culture plastic in 2D. We made four important observations. First, not all the MSC surface markers found in 2D were expressed when cells are cultured in 3D. Specifically, after 3D culture the expression of CD 105 was lower than MSC criterion of \(>95\%\) positive. Second, we observed that the growth rate of MSCs was slower when they grew in 3D in comparison to 2D. This observation was confirmed by metabolic assay, MTT, by cell counting and calculation of PDT, and finally by CFU-F assay. Third, MSC differentiation potential to bone, cartilage and fats was not grossly different between 2D and 3D culture. Fourth, we observed that MSCs could respond to chemotactic signals and migrate through the HPL matrix along a poly (I:C) gradient, and tended to orient and invade along an LPS gradient, too. These observations suggest that HPL hydrogel can support MSC expansion and may modify their physiology, as has been suggested by other reports indicating that MSCs grown in 3D may have altered characteristics.

There are many sources of MSCs. We used HUC-MSCs since this source has advantages over bone-marrow-derived MSCs. For example, the umbilical cord donors are of the same young age and the MSCs are isolated from a discarded tissue, painlessly. It is possible that using MSCs
from another tissue source would produce different observations. Since we did not test adipose
derived MSCs or bone marrow derived MSCs, our conclusion might be different using MSCs
from those sources.

Liquid cell culture medium is used conventionally to expand MSCs. Commonly, fetal
bovine serum (FBS) is used as a medium supplement since it provides both attachment factors
and growth factors. However, using FBS causes cross contamination between species when
human MSCs are expanded, and xenogenic immune responses triggered by FBS and zoonotic
disease are safety considerations in clinical trials. Therefore, we tested human platelet lysate
(HPL) as alternative medium supplement for MSC culture. HPL is prepared using outdated
human apheresis platelets that are repeatedly frozen and thawed to break the platelets to release
their attachment and growth factors. Addition of calcium solutions to HPL supplemented
medium causes polymerization of fibrin similar to the coagulation cascade, and results in a soft
hydrogel being formed. To keep HPL supplemented media as a liquid, > 1 USP U/ ml heparin
should be added before adding HPL. Without adding heparin, HPL supplemented DMEM media
will form a hydrogel. Here, we tested MSC encapsulation in 20-40% HPL (v/v) supplementation
of DMEM. We found that 20% HPL may take longer than 4 hours to gel. In contrast 30% HPL
gelled in 1 - 1.5 hr. While 40% HPL gelled in 1 hr, the gel formed was no stiffer than 20 or 30%
HPL gel. Therefore, we choose to use 30% HPL for our experiment.

To test whether 30% HPL gel could be used to culture MSCs and also could be used for
studying cell invasion, we created a customized lid for 12 well tissue culture plates that was
used as a mold to created voids (or wells) of specific size in the hydrogel. The resulting casted
hydrogel was evaluated for a 3D invasion model and to test MSC responses to two different TLR
agonists. We used TLR4 and TLR3 agonists, two different stimuli that might be found in a
wound in vivo. LPS, the principle component of gram-negative bacteria cell wall, and interacts with Toll-like receptor 4, and poly (I:C), a synthetic analog of double-stranded RNA interacts with TLR3 and thus provides signals seen during a viral infection. The results show that MSCs migrate or invade through the gel to the depot where poly (I:C) was placed more than to the control depot site. While we did not find a statistical difference between the depot site where LPS was placed and the control depot site, there was a trend to indicate that MSCs migrated towards that chemotactic cue, too. These observations suggest strongly that our 3D invasion model is useful for testing the cellular migration response to various test substances. Therefore, this 3D invasion model system can be used to test other drugs and activators of MSCs in the future.

After expanding the MSC to confluence, it is necessary to lift the cells from the plate or liberate the cells encapsulated in 3D matrix to count them, or for assaying them or for passage. In 2D culture, this usually involves adding trypsin-EDTA solution to lift MSCs from the tissue culture plate. In 3D culture within the HPL hydrogel, the fibrin-structure was not dissociated or liquified by trypsin-EDTA but was by nattokinase. Previous work confirmed our observation that trypsin-EDTA was ineffective, but the enzyme derived from natto, a fermented soybean product, with Bacillus subtilis was effective44. Nattokinase (NK) has been tested in patients with thromboembolism as a potential treatment. Here, I showed that NK was safe and effective to retrieve MSCs encapsulated or encased in HPL hydrogel. We identified 1.75% NK at 60 minutes of incubation as optimal for extracting MSCs from 30% HPL gel because longer time incubation did not increase cell yield and shorter incubation times did not dissolve HPL completely. We compared the cell viability of HUC-MSCs extracted from 3D HPL gel with 1.75% NK for 60 minutes and those same cells lifted from 2D tissue culture by 0.05% trypsin-EDTA for 3 minutes
and found no significant difference (both > 95%). This indicates that NK can safely extract cells from 3D HPL gel.

Future work is needed to determine whether NK extraction affects the surface marker expression of MSC surface markers since we observed a shift in the MSCs marker expression after NK treatment. This shift in marker expression was lost after the MSCs were grown in 2D culture for one additional passage. Therefore, extraction using NK vs trypsin-EDTA might explain the differences observed by flow cytometry. Our observations that CD105 expression was lower after 3D hydrogel culture is similar to observations by another group who grew MSCs in suspension culture\(^\text{RW:ERROR - Unable to find reference:doc:5ad970d4e4b0a553e073cb5c}\). Therefore, we are unable to determine whether changes in surface marker expression as due to NK or trypsin treatment or are due to 2D vs 3D culture conditions.

MSCs must meet the ISCT’s minimal MSC definition criteria, e.g., they should (1) self-renew after attaching to tissue culture treated plastic plates, (2) have positive surface staining for CD105, CD73, CD90, and negative surface staining for CD45, CD34, CD14 or CD11b, CD79 alpha or CD19, HLA-DR, and (3) differentiate into adipocytes, chondrocytes and osteocytes. This definition derives from early work and was designed to discriminate between hematopoietic cells, including hematopoietic stem cells found in bone marrow from mesenchymal stromal cells also found there. It is difficult to apply this minimal definition if you grow cells in 3D because MSCs are not “attached to plastic”. After we culture HUC-MSCs in 3D for one passange and then we seed back to 2D on tissue culture plates, they retain their ability to attach and expand, although when compare the proliferation assay between cells from 2D and 3D, MSC grown attached in 2D always had a higher proliferation rate than 3D, whether assayed by MTT, PDT, or
CFU-F. Upon 2D culture, MSCs expanded in 3D HPL for 1 passage were able to differentiate to be fat, cartilage, and bone and meet the ISCT positive and negative cell surface markers.

In summary, my work has demonstrated the utility of growing MSCs in 3D using an HPL-based hydrogel. While the 3D matrix made by HPL’s polyermization has many positive properties, such as crosslinking at room temperature with the need for harsh chemical cross-linking agents, it lacks other properties such as rapid polyermization and rapidy liquification for passage and gels of sufficient strength to be self supporting. Therefore future work is needed to further optimize the HPL gel for 3D culture of MSCs.
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