

Methods for isolating, expanding, and characterizing umbilical cord mesenchymal stromal cells  
and their *in vitro* metabolism

by

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## Abstract

Mesenchymal stromal cells (MSCs) derived from the umbilical cord (UC-MSCs) have therapeutic applications and are studied to understand their potential uses and immunomodulatory properties. Research must identify good manufacturing process (GMP) compliant methods to isolate and expand UC-MSCs. In addition, MSCs metabolism characteristics in culture are unknown, warranting further investigation. Viability of MSCs decreases after cryopreservation, which is detrimental to clinical translation. Previously published methods used to isolate MSCs from the umbilical cord included open dissection steps and xenogeneic components. Here, I developed improved methods by eliminating dissection which reduces contamination risks. Instead, I used the whole umbilical cord and Miltenyi dissociator tubes to mechanically and enzymatically dissociate cells in a closed system. Xenogeneic components were decreased by using medium containing pooled human platelet lysate instead of fetal bovine serum. The cell numbers isolated from umbilical cord averaged  $2.68 \times 10^5$  per cm, which represents greater than 20 fold improvement over the previous method. Moreover, expansion cell numbers were increased using 10% pooled human platelet lysate supplemented media. The UC-MSCs generated here met the International Society of Cell Therapy (ISCT) definition of MSCs. Metabolism characteristics of MSCs indicated that glucose was the critical metabolite, maintaining cells longer in culture than glutamine. Cell death followed depletion of glucose, too. Finally, the average viability after thawing cryopreserved MSCs was more than 95%, higher than previous methods. The improvements I introduced to our methodology could speed clinical translation of MSCs as an allogeneic cellular therapy.

# Table of Contents

List of Figures .....	v
List of Tables .....	vi
Acknowledgements .....	vii
Dedication .....	viii
Chapter 1 - Review of Literature: Mesenchymal Stromal Cells .....	1
Introduction .....	1
Mesenchymal Stromal Cells .....	1
MSC Properties .....	3
Self-Renewal .....	3
Differentiation .....	4
Immune properties .....	5
Immune modulation .....	6
Preconditioning .....	7
Exosomes .....	8
Advantages of MSCs derived from umbilical cords .....	8
Isolating MSCs from umbilical cord .....	9
MSC clinical trials .....	10
Summary .....	11
Chapter 2 - Standardizing Umbilical Cord Mesenchymal Stromal Cells for Translation to Clinical Use: Selecting GMP-Compliant Medium and a Simplified Isolation Method .....	13
Abstract .....	13
Introduction .....	14
Materials and Methods .....	16
Umbilical Cords .....	16
Isolation Optimization Strategy .....	17
Final (Optimized) Isolation Method .....	19
Optimization of MSC Expansion .....	20
CFU-F Assay .....	21

Differentiation .....	22
Flow Cytometry .....	22
Statistics .....	23
Results.....	25
Umbilical Cords .....	25
Isolation Method Comparison.....	26
MSC Expansion Comparison.....	27
Evaluation of UC-MSC Characteristics .....	28
Colony Forming Unit-Fibroblast (CFU-F) .....	28
Differentiation.....	29
Flow Cytometry .....	29
Discussion.....	33
Chapter 3 - ' Analysis of umbilical cord mesenchymal stromal cells growth media and cryopreservation .....	43
Introduction.....	43
Growth Media .....	43
Cryopreservation.....	44
Methods .....	44
Growth Kinetics .....	44
Freezing and Thawing.....	45
Results.....	48
Growth Kinetic.....	48
Viability of Thawed Cells .....	50
Discussion.....	50
Chapter 4 - Discussion.....	53
References.....	56
Appendix A - Supplemental Figures and Tables .....	65

## List of Figures

Figure 2.1 Isolation Flow Chart .....	18
Figure 2.2 Isolation numbers and initial doubling time .....	25
Figure 2.3 UC-MSK expansion results .....	30
Figure 2.4 Differentiation and CFU-F results .....	31
Figure 2.5 Flow Cytometry Characterization .....	32
Figure 3.1 Metabolism characteristics over time .....	47
Figure 3.2 Images of media conditions .....	49
Figure 3.3 Cryopreservation per line and over time .....	52
Figure A.1 CFU-F plating condition and time .....	66
Figure A.2 Method for analyzing CFU-F .....	68

## List of Tables

Table 2.1 Individual Umbilical Cord Comparisons .....	24
Table A.1 surface marker expression data for six isolates.....	65
Table A.2 Comparison of frozen and never frozen isolates for surface marker expression. ....	65
Table A.3 Umbilical Cord Averages per variable Representation of the umbilical cord isolation averages for the protocol; total number of 37 umbilical cords used. ....	67

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## **Dedication**

I dedicate my thesis to my aunt, Susan Carns, and my late aunt, Donna Hynes. Both women struggled with diseases that affected their lives. Despite their hardships, they put others first. I hope that my research will lead to treatments and improve lives.

# **Chapter 1 - Review of Literature: Mesenchymal Stromal Cells**

## **Introduction**

Umbilical cord mesenchymal stromal cells (UC-MSCs) possess characteristics that make them useful as therapeutic cells. Although much is known about the cells themselves, good manufacturing practice production of UC-MSCs must be addressed. The considerable knowledge gained from studying UC-MSCs is moot if the cells cannot be used for treating patients. Standardized methods for isolating, expanding, and characterizing MSCs are necessary for clinical translation. To advance cells into human trials, a standardized manufacturing method with GMP compliance would provide convincing evidence that the cells are safe for use in trials. My goal here was to improve the original Weiss laboratory protocol developed for isolating, expanding, and characterizing UC-MSCs (Seshareddy *et al.*, 2008)) and develop methods that lend themselves to GMP.

## **Mesenchymal Stromal Cells**

Mesenchymal stromal cells (MSCs) were first described in 1966 by Friedenstein *et al.* who found fibroblastic cells in bone marrow aspirates and hypothesized that MSCs differed from hematopoietic cells in bone marrow. Later Friedenstein found that MSCs adhere to substrate and expand as a monolayer of fibroblastic colonies (Friedenstein *et al.*, 1970). Few MSCs were initially isolated. Over time they expanded (divided) to fill the culture plate, thus demonstrating a capacity for replication, or self-renewal. It was not until the 1980s that Owen and Friedenstein (1988) theorized that MSCs represent the stromal stem cells in bone marrow, thus suggesting that bone marrow contains a population of stem-like cells. The first stem cells discovered in bone marrow were hematopoietic stem cells (Till and McCulloch, 1961).

Following these discoveries, MSCs were derived from other tissues, among them umbilical cord blood (In't Anker *et al.*, 2003), umbilical cord tissue (Mitchell *et al.*, 2003), adipose tissue (Zuk *et al.*, 2002), and fetal tissues (Campagnoli *et al.*, 2001). Subsequently, Crisan *et al.* (2008) hypothesized that all connective tissues contain MSCs. Mesenchymal stromal cells are a heterogeneous population and include a subpopulation of stem cells. Caplan distinguished the stem cell subpopulation by their multilineage differentiation potential and apparent differentiation into various cell lineages (Caplan, 1991). Caplan identified the embryonic origin of mesenchymal stem cells in chick and found that they developed into mesodermal tissues. “Mesenchymal stem cell” was considered the appropriate term for both cell populations until 2005 when the International Society for Cellular Therapy (ISCT) MSC working committee clarified the terminology (Horwitz *et al.*, 2005). The ISCT MSC working committee defined bone marrow derived MSCs as multipotent mesenchymal stromal cells because they are a mix of stem cells, progenitors, and differentiated cells. In fact, only a subset of the population could be termed mesenchymal stem cells: those cells that form colonies as indicated by the efficiency of their colony forming unit fibroblast (CFU-F). Henceforth, the term MSC refers to the mixed population of stromal cells as defined by the ISCT (discussed below). Many researchers continue to refer to MSCs as stem cells despite the ISCT definition. This is a point of confusion in the scientific literature.

In 2006, the ISCT established a minimum criterion for identifying cells as MSCs. This criterion states that MSCs grow as an adherent cell layer to tissue culture-treated plastic; can differentiate into adipose, cartilage, and bony tissues; and stain positively for surface markers CD105, CD73, and CD90, but do not stain for surface markers CD45, CD34, CD14, or CD11b, CD79a, or CD 19, and human leukocyte antigen-antigen D related (HLA-DR) (Dominici *et al.*,

2006). This definition has been used for the last 10 years to identify MSCs. This basic definition, however, does not address the physiological interactions between MSCs and cells of the immune system. These physiological interactions provide rationale for using MSCs as a cellular therapy in immune related diseases.

## **MSC Properties**

Perhaps the most important properties of MSCs for use in cellular therapy are their interactions with the immune system. Those traits are described below.

### **Self-Renewal**

MSCs grow, that is, undergo mitosis, when placed into suitable *in vitro* conditions and may undergo several rounds of population doublings. As MSC populations double, they occupy more of the surface area of the tissue culture plate. When they cover approximately 70-80% of the surface, MSCs must be removed from the plate and given additional space to maintain their growth. Cell-to-cell contact limits continued growth, a condition called contact inhibition. Lifting cells from the substrate to provide more space for them to expand is called passaging of cells. The colony forming unit-fibroblast (CFU-F) assay is used to analyze proliferative capacity of MSCs. CFU-F efficiency is defined as the ratio of the number of MSCs plated to the number of observed colonies. For example, MSCs derived from the umbilical cord (UC-MSCs) have CFU-F efficiencies of 25.2 in 21% oxygen and 19.9 in 5% oxygen (López *et al.*, 2011), indicating that lower oxygen tension increases proliferative capacity.

MSCs have a finite life in culture. They are not immortal like some cancer cell lines. This suggests that, in culture, the stem cell subpopulation may be lost over time and that current cell culture conditions do not maintain the MSC stem cell population like *in vivo*. As the number of MSC population doublings increases, more cells reach cellular senescence, which means they no

longer expand. Cellular senescence depends upon culture conditions and how carefully MSC are handled. In general, the percentage of cells reaching senescence increases as more passages occur. Cheng *et al.* (2011) reported that bone marrow MSCs (BM-MSCs) began reaching senescence after 6 passages (16 population doublings). In contrast, UC-MSCs began reaching senescence after more than 10 passages (28 population doublings). Those findings were corroborated by Kern *et al.* (2006), who found senescence at passage 7 in BM-MSCs. Majore *et al.* (2009) identified a subpopulation of UC-MSCs that were smaller and had an increased proliferation rate and delayed cellular senescence. UC-MSCs produce the enzyme telomerase, which adds a telomere repeat sequence to the ends of telomeres. Since telomere shortening correlates with increased cellular senescence, telomerase activity may prevent senescence. Mitchell *et al.* (2003) speculated that telomerase activity in UC-MSCs prevents cellular senescence, which thus enhances replicative capacity. For clinical application, it may be preferable to use cells with reduced cellular senescence.

## **Differentiation**

As Arutyunyan *et al.* (2016) detailed, MSCs can form other cell types including osteogenic, chondrogenic, adipogenic, neurogenic, cardiac, dermal fibroblastic cells, and skeletal or smooth muscle cells. Some subpopulations of MSCs may have increased differentiation capacity. For example, Ishimine *et al.* (2013) found MSCs with surface expression of N-cadherin could differentiate more efficiently into cardiomyocytes. In contrast, Mitchell *et al.* (2003) described neural potential associated with UC-MSCs but could not determine a particular surface marker that allowed MSCs with neural differentiation capacity to be identified. Porcine UC-MSCs express transcription factors Oct-4, Sox-2, and Nanog, which are also found in undifferentiated embryonic stem cells (Lee *et al.*, 2006). These transcription factors, however, are

expressed at lower levels in MSCs than embryonic stem cells [OBJ] *et al.*, 2013)[OBJ] This expression of pluripotent transcription factors by MSCs may be a curiosity, not a marker of the pluripotent state. UC-MSCs expressed several markers associated with the trophectoderm and all three germ layers (Bex1/Rex3, Hand1, HEB, Nestin, CXCR4, vimentin, CD44, collagen X, Flk-1, PDX-1, and Islet-1) [OBJ] *et al.*, 2006)[OBJ] so UC-MSCs may have more open chromatin and thus more pleiotropic gene expression than adult tissues.expression than adult tissues.

### **Immune properties**

MSCs have low immunogenicity, their ability to induce an immune response when transplanted is lower than normal. When MSCs are transplanted to an allogeneic recipient, they have no histocompatibility generated immune response (Ankrum *et al.*, 2014). Clinical use of allogeneic MSCs relies on this lack of immune response on the part of the host (Di Nicola *et al.*, 2002). Without major histocompatibility class II (MHC) antigens on MSCs, the low expression of MHC class I antigens may allow allogeneic MSCs to avoid immune rejection (Le Blanc *et al.*, 2003). Therefore, allogeneic MSCs have been clinically tested for safety and efficacy in graft versus host disease (GvHD). Le Blanc and Ringden (2006) reported that allogeneic MSCs ameliorated GvHD clinical signs and were safe. Similarly, *in vitro* UC-MSCs suppress the proliferation of stimulated T-Cells and do not express the co-stimulatory markers CD40, CD80, and CD 86 (Weiss *et al.*, 2008). MSCs may thus be suitable as an allograft because of low immunogenicity and lack of cell-mediated lysis by natural killer cells (Sotiropoulou *et al.*, 2006; Weiss *et al.*, 2008). However, after allogeneic treatment in rat models, MSCs did cause an up-regulation of alloantigen (Schu *et al.*, 2012). MSCs injected into the pancreas of diabetes model rats were rejected (Gu *et al.*, 2015). Ankrum *et al.* (2014) reported that MSCs were cleared from

the body. Furthermore, MSCs occasionally triggered immune rejection, suggesting MSCs have greater immunogenicity than first thought (Ankrum *et al.*, 2014).

### **Immune modulation**

In 2013, the ISCT published an update to their initial report. This follow-up report identified potential immunological markers for assessing the potency of MSCs for immune modulation (Krampera *et al.*, 2013). Indoleamine-2, 3-dioxygenase (IDO) is important for MSC's immune modulation, as is Prostaglandin E<sub>2</sub> (PGE<sub>2</sub>), tumor necrosis factor-inducible gene 6 protein (TSG-6), transforming growth factor beta (TGF- $\beta$ ), Interleukin-6 (IL-6), and IL-10 (Krampera *et al.*, 2013; Kyurkchiev *et al.*, 2014).

As noted in Gao *et al.* (2016), MSCs suppress proliferation of T-cells and B-cells. This includes T-helper cells (Th1) 1 and 2 inflammatory and cytotoxic effects, T-regulatory cell functions, and production of B-cell antibody and co-stimulatory molecules. Mouse MSCs do this by direct cell-to-cell contact and through secretion of factors like IDO, PGE<sub>2</sub>, nitrous oxide, TGF- $\beta$ , TSG-6, and hepatocyte growth factor. The effects of MSCs extend to dendritic cells and natural killer cells by inhibiting their induction and activation (Gao *et al.*, 2016).

TSG-6 produced by MSCs can inhibit inflammation induced by lipopolysaccharide in microglial cells and can suppress Th1 cells, delaying diabetes onset in mouse models (Kota *et al.*, 2013; Liu *et al.*, 2014). The effects of TSG-6 can help heal even when MSCs are not directly located near the site of healing. In rat corneal injury models, MSCs injected *intravenous* or *intraoperative* improved healing of the cornea through production of TSG-6 (Roddy *et al.*, 2011).

Research has also shown that MSCs help wounds heal (Isakson *et al.*, 2015). MSCs express pentraxin 3 (PTX3), which affects MSCs' pericellular fibrinolysis and migration through

fibrinous tissue during wound healing (Cappuzzello *et al.*, 2016). When the gene for PTX-3 is silenced, MSCs had reduced ability to remodel wounds during healing, slowing wound healing. This may indicate a marker in MSCs that identifies wound healing capabilities. MSCs can express C3a and C5a anaphylatoxins, which help tissue regeneration and repair (Schraufstatter *et al.*, 2015). Schraufstatter hypothesized complement activation causes MSC trophic effects by enhancing MSC migration to wounds via chemo-attraction to complement component 3a (C3a) and C5a, to which MSCs are attracted *in vitro*. In rat cutaneous wound healing models, alginate gels seeded with MSCs or MSC supernatant improved the rate of wound healing when compared to fetal bovine serum or phosphate buffered saline controls (Wang *et al.*, 2016).

### **Preconditioning**

Preconditioning MSCs by stimulating them with immunogenic particles or markers can affect their cytokine expression. Depending on treatment, MSCs polarize to different phenotypes, such as MSC1 or MSC2, as demonstrated by Watermann *et al.* (2012). They found that MSCs activated by toll-like receptor 3 (TLR-3) or TLR-4 antagonists (poly I:C and lipopolysaccharide) produced two separate phenotypes. MSC1 phenotype from TLR-4 primed cells increased pro-inflammatory properties. MSC2 from TLR-3 primed cells increased immunosuppressive properties on activated T cells. The immunosuppression correlated with an increased expression of IDO and PGE2 in the MSC2 phenotype. Krampera *et al.* (2011) suggested that interferon gamma (IFN- $\gamma$ ) exposure would generate MSCs with the immune suppressive phenotype, which they termed “licensing” MSCs. For stronger anti-inflammatory action, MSCs could be exposed to IFN- $\gamma$  and tumor necrosis factor alpha, IL-1 $\alpha$ , or IL-1 $\beta$ , which increase inhibition of activated T-cells *in vitro* (Krampera, 2011). Exposure to IFN- $\gamma$  increases the surface expression of major histocompatibility complex (MHC) class II antigens on MSCs (Krampera *et al.*, 2013).

Expression of MHC class II like HLA-DR may trigger an alloreactive immune response in the patient, which is detrimental to clinical applications. MHC class II expression on the surface of MSCs may also cause increased immune surveillance and rejection by the host (Batsali *et al.*, 2013). The immunosuppressive phenotype would be preferable for treating cases of immune rejection, auto-immune conditions, or attempting to decrease inflammation.

### **Exosomes**

MSCs produce microvesicles and exosomes, which may have use as a treatment (Favaro *et al.*, 2014). *In vitro* exosomes derived from MSCs inhibit T-cell responses (Favaro *et al.*, 2014). UC- MSC-derived exosomes have been tested in animal models of myocardial infarction (Sun *et al.*, 2016).

### **Advantages of MSCs derived from umbilical cords**

MSCs were first identified in umbilical cord matrix (Wharton's jelly) in 1991 (Mcelreavey *et al.*, 1991), and MSCs derived from the umbilical cord were widely studied in the early 2000s (Mitchell *et al.*, 2003; Sarugaser *et al.*, 2005; Wang *et al.*, 2004). The umbilical cord may contain MSCs in five different regions: umbilical cord blood, perivascular tissues associated with the outer layer of the umbilical vessels, Wharton's jelly, subendothelial layer cells of the umbilical vein, and the cells from the subamniotic region (Troyer and Weiss, 2008).

Bone marrow is a common source of MSCs, but bone marrow aspiration is a painful, invasive procedure. Not only that, bone marrow produces fewer MSCs in culture than umbilical cord MSCs (Hua *et al.*, 2014). The umbilical cord is obtained painlessly from discarded fetal tissue (McGuirk *et al.*, 2015). Clearly, painless collection from a discarded tissue such as the umbilical cord is a better alternative to the painful aspiration of MSCs from bone marrow.

Furthermore, umbilical cord tissue could provide a virtually limitless supply of MSCs compared to the bone marrow.

Tissue source and age of tissue affects MSCs and their proliferative capacity (Hua *et al.*, 2014). MSCs derived from fetal tissues, particularly umbilical cord MSCs, can produce more MSCs and higher levels of CFU-Fs than placental, bone marrow, or adipose derived MSCs (Hass *et al.*, 2011; Li *et al.*, 2014). Hua *et al.* (2014) compared proliferation, differentiation and surface marker of MSCs from the umbilical cord (UC-MSCs), umbilical cord blood (UCB-MSCs) and bone marrow (BM-MSCs). UC-MSC's population doubling time was faster, with lower osteogenic differentiation capabilities, and they expressed CD105 and CD146 at higher levels than UCB- and BM-MSCs. Moreover, UC-MSCs have lower percentage of senescent cells than BM-MSCs (Cheng *et al.*, 2011). UC-MSCs suppress lymphocyte proliferation better than BM-MSCs and have lower HLA-DR expression (Barcia *et al.*, 2015). For those reasons, UC-MSCs are more suitable to clinical applications than BM-MSCs.

### **Isolating MSCs from umbilical cord**

Several methods are used to isolate MSCs from the umbilical cord (Han *et al.*, 2013; Hua *et al.*, 2014; Paladino *et al.*, 2016; Seshareddy *et al.*, 2008). The tissue explant isolation method is common. In the explant method, pieces of Wharton's jelly are plated in tissue culture plates after removing the umbilical vessels. Others methods combine blood vessel removal with enzymatic digestion of Wharton's jelly to liberate cells from the connective tissue (Han *et al.*, 2013; Hua *et al.*, 2014; Paladino *et al.*, 2016; Seshareddy *et al.*, 2008). Han *et al.* used a longer enzymatic digestion time of 16-20 hours to successfully isolate MSCs (Han *et al.*, 2013). Combining mechanical methods and enzymatic digestion of umbilical cord tissue, however, yielded more MSCs than the explant method (Seshareddy *et al.*, 2008). More recently, the whole

umbilical cord has been used with promising results for both mechanical methods and enzymatic digestion or a combination of the two (Marmotti *et al.*, 2012). Using the entire cord can save time since dissecting blood vessels is eliminated (Hendijani *et al.*, 2014). Although using the entire cord means a heterogeneous population of cells are initially isolated, MSCs were identified after expansion suggest the other cell types are removed from the population or did not grow in culture (Marmotti *et al.*, 2012). For this reason, isolating cells from the whole cord instead of targeting Wharton's jelly is more effective, reducing contamination risks and decreasing the time needed to isolate cells.

### **MSC clinical trials**

Bone marrow, umbilical cord blood, or adipose tissue are all sources for MSCs used in clinical trials (Sharma *et al.*, 2014). However, several trials using UC-MSCs are on-going (Arutyunyan *et al.*, 2016). The top three diseases targeted by MSC cell therapy are liver, cardiovascular, and autoimmune disorders (Arutyunyan *et al.*, 2016). Si *et al.* (2011) summarized several areas where MSCs are being assessed for clinical application and they identified several challenges. For example, they discussed that *in vitro* MSC results do not always translate *in vivo*, clinical long-term studies using MSCs are rare, the mechanism by which MSCs have their clinical effect is not well-known, and laboratories lack standardized methods, making results difficult to compare (Si *et al.*, 2011). Specific challenges must be targeted and addressed for GMP-compliant expansion for clinical trials. To speed clinical translation, closed MSC expansion systems should be considered for isolating and culturing MSCs, and cell culture medium that is GMP-compliant and contains no xenogenic components (Sensebe *et al.*, 2013). Other suggestions include using bioreactor systems to expand the cells. MSCs can be expanded on microcarriers in bioreactors and harvested (Cierpka *et al.*, 2013).

MSCs are commonly grown in media supplemented with fetal bovine serum (FBS) to provide necessary growth and attachment factors (Burnouf *et al.*, 2016)). FBS is a xenogeneic component and should, therefore, be eliminated from media used for human clinical trials. Recently pooled human platelet lysate (HPL) has been tested as a source of MSC growth factors. HPL supports the attachment and growth of MSCs and provides an alternative to xenogeneic components (Burnouf *et al.*, 2016). MSCs grown in HPL tested for quality and could be cryopreserved as multiple treatment doses for several patients. Cryopreserved MSCs may have impaired immunosuppressive effects and impaired licensing by IFN- $\gamma$  (Francois *et al.*, 2012). Although, MSCs do recover from freeze/thaw stress after a passage, the stress of freeze/thaw could effect their use *in vivo*. Concerns with cryopreservation must be addressed in order for MSCs to advance into the clinic.

UC-MSCs, like other MSCs, may be useful for treating acute graft versus host disease (GvHD). In GvHD allogeneic rat model, human UC-MSCs produced complete amelioration of GvHD (Yelica Lopez, PhD dissertation) Similar results have been found treating human GvHD patients with MSCs (Le Blanc and Ringden, 2006). In treating inflammatory bowel disease (IBD), autologous bone marrow MSCs improved outcomes by reducing symptoms (Duijvestein *et al.*, 2010). Recently, Crohn's patients with perianal fistulas showed improved remission when treated with allogeneic MSCs compared to a placebo (*et al.* Panes *et al.*, 2016).

## Summary

As outlined, UC-MSCs have characteristics that potentially make them a a better choice for use in cell therapy. UC-MSCs are fetal-derived, with outstanding proliferation capacity. Developing new MSC-based treatments relies on understanding MSC growth conditions and

generating a standardized GMP-compliant procedure. These two things can dramatically help in developing a therapeutic product using these cells.

## **Chapter 2 - <sup>1,2</sup>Standardizing Umbilical Cord Mesenchymal Stromal Cells for Translation to Clinical Use: Selecting GMP-Compliant Medium and a Simplified Isolation Method**

### **Abstract**

Umbilical cord derived mesenchymal stromal cells (UC-MSCs) are needed in clinical trials, but medical research lacks standardized methods for isolation and expansion. Previous isolation and expansion methods for UC-MSCs presented challenges to good manufacturing practices (GMP) for clinical translation. This paper describes a more standardized, simpler method for isolating and expanding UC-MSCs, eliminating dissection of blood vessels and using closed-vessel dissociation after enzymatic digestion to reduce both contamination and manipulation time. The new method produced more than 10 times the cells per cm of UC than previous methods. When biographical variables were compared, we found no significant differences between male and female donors or for type of birth. UC-MSCs were expanded in medium enriched with 2%, 5%, or 10% pooled human platelet lysate (HPL) eliminating the xenogeneic serum components. When the HPL concentrations were compared, media supplemented with 10% HPL had the highest growth rate, smallest cells, and the most viable cells at passage. UC-MSCs grown in 10% HPL had surface marker expressions typical of MSCs, high colony forming efficiency, and could undergo chondrogenic, adipogenic, and osteogenic

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<sup>1</sup> Reproduced with permission from Smith, J.R., K. Pfeifer, F. Petry, N. Powell, J. Delzeit, and M.L. Weiss. 2016. Standardizing Umbilical Cord Mesenchymal Stromal Cells for Translation to Clinical Use: Selection of GMP-Compliant Medium and a Simplified Isolation Method. *Stem Cells International* 2016:14.

<sup>2</sup> Abstract presented in part at Experimental Biology 2015. Smith, J.R., K. Pfeiffer, J. Delzeit, S. Akel, J. McGuirk, and M. Weiss. 2015. Umbilical Cord Mesenchymal Stromal Cell Isolation Expansion and Validation for GMP Compliance. *The FASEB Journal* 29:

differentiation. The new protocol standardizes manufacture of UC-MSCs and enables clinical translation.

## **Introduction**

The minimal criteria for defining mesenchymal stromal cells (MSCs) was provided by the International Society of Cellular Therapy (ISCT) MSC working group in 2006 and updated in 2013 with guidelines for characterizing MSC immune properties (Dominici *et al.*, 2006; Krampera *et al.*, 2013; Mendicino *et al.*, 2014; Sensebe *et al.*, 2013). The physiological properties of MSCs suggest a potential to treat diseases like graft versus host disease (GvHD) and Crohn's (Duijvestein *et al.*, 2010; McGuirk *et al.*, 2015; Ringden *et al.*, 2013). In addition, more than 500 clinical trials have tested the safety and efficacy of MSCs as listed on ClinicalTrial.GOV (ClinicalTrials.gov, 2015).

In 2014, about 53% of the MSC clinical trials world-wide used bone marrow-derived MSCs (BM-MSCs) (Bersenev, 2015). BM-MSCs may be used as an autologous cellular product, which is a distinct advantage over allogeneic MSC products. However, collecting bone marrow is a painful, invasive procedure, unlike collecting MSCs from umbilical cord stroma (UC-MSCs), which is collected painlessly from tissues that are discarded after birth. Furthermore, adult BM-MSCs have lower potential for expansion, lower capability for immunosuppression when co-cultured with activated T-cells, and perhaps less potential for differentiation than UC-MSCs (Baksh *et al.*, 2007; Barcia *et al.*, 2015; Wang *et al.*, 2009; Yoo *et al.*, 2009; Zhang *et al.*, 2005; Zhang *et al.*, 2009).

UC-MSCs also have advantages over BM-MSCs as an allogeneic MSC source. These advantages include a virtually limitless supply of starting material, available for producing tissue banks as an allogeneic matched product, similar to umbilical cord blood banks; collecting

umbilical cords is painless, and cord donors are a consistent, young age. *In vitro*, UC-MSCs have high proliferation potential, broad differentiation potential, and better immune modulation properties than other sources of MSCs (Barcia *et al.*, 2015; Deuse *et al.*, 2010; Weiss *et al.*, 2008; Zeddou *et al.*, 2010). For these reasons, the therapeutic potential of UC-MSCs bears testing, and 25 clinical trials worldwide were using UC-MSCs as of 2014 (Bersenev, 2015).

Producing MSCs that meet the requirements for clinical application is, however, a challenge (Mendicino *et al.*, 2014; Wang *et al.*, 2012). MSC manufacturing may be unable to keep pace with the number of MSC clinical studies (Sharma *et al.*, 2014; Wang *et al.*, 2012). One challenge is the lack of a standardized method for isolating, expanding, and validating MSCs. In fact, several methods to isolate UC-MSCs from umbilical cord stroma have been described (Bongso and Fong, 2013) that include the tissue explant method (Han *et al.*, 2013; Marmotti *et al.*, 2012), mechanical dissociation of the cord stroma followed by enzymatic digestion (Barcia *et al.*, 2015; Han *et al.*, 2013; Seshareddy *et al.*, 2008), isolation of MSCs from the entire umbilical cord including the blood vessels (Bongso and Fong, 2013; Marmotti *et al.*, 2012), enzymatic digestion of the tissue immediately surrounding the umbilical blood vessels (Romanov *et al.*, 2003), or mincing and enzymatic digestion of the stroma (Wharton's jelly) without the blood vessels (Seshareddy *et al.*, 2008). Several of these methods require dissection of the umbilical vessels. This dissection step increases processing time and the risk of contamination.

Thus, the goal of this research was to develop an isolation method that would decrease the risk of contamination as well as isolation time and increase the yield of MSCs. While developing this protocol, we identified an additional goal: to provide a more reliable measure than length for comparing biological units. Length has been used to compare umbilical cords, but the great variability from cord to cord makes this potentially less reliable than weight. Moreover,

in reviewing previous MSC expansion protocols, we determined that formulating the media required many ingredients, complicating the process of clinical manufacturing (Seshareddy *et al.*, 2008). Therefore, the second goal was to identify a simplified medium that would provide robust expansion of MSCs, decrease xenogeneic components, and remain suitable for clinical manufacturing.

## **Materials and Methods**

### **Umbilical Cords**

At first, the institutional review board of Kansas State University deemed this research nonhuman subjects research, a designation since discarded, anonymous human tissue with all identifying linkages broken was used (IRB #5189). Tissue processing was performed inside a biological safety cabinet in a BSL2 laboratory using universal precautions recommended by Occupational Safety and Health Administration (OHS) for containing blood borne pathogens in 29 CFR. 1910.1030.

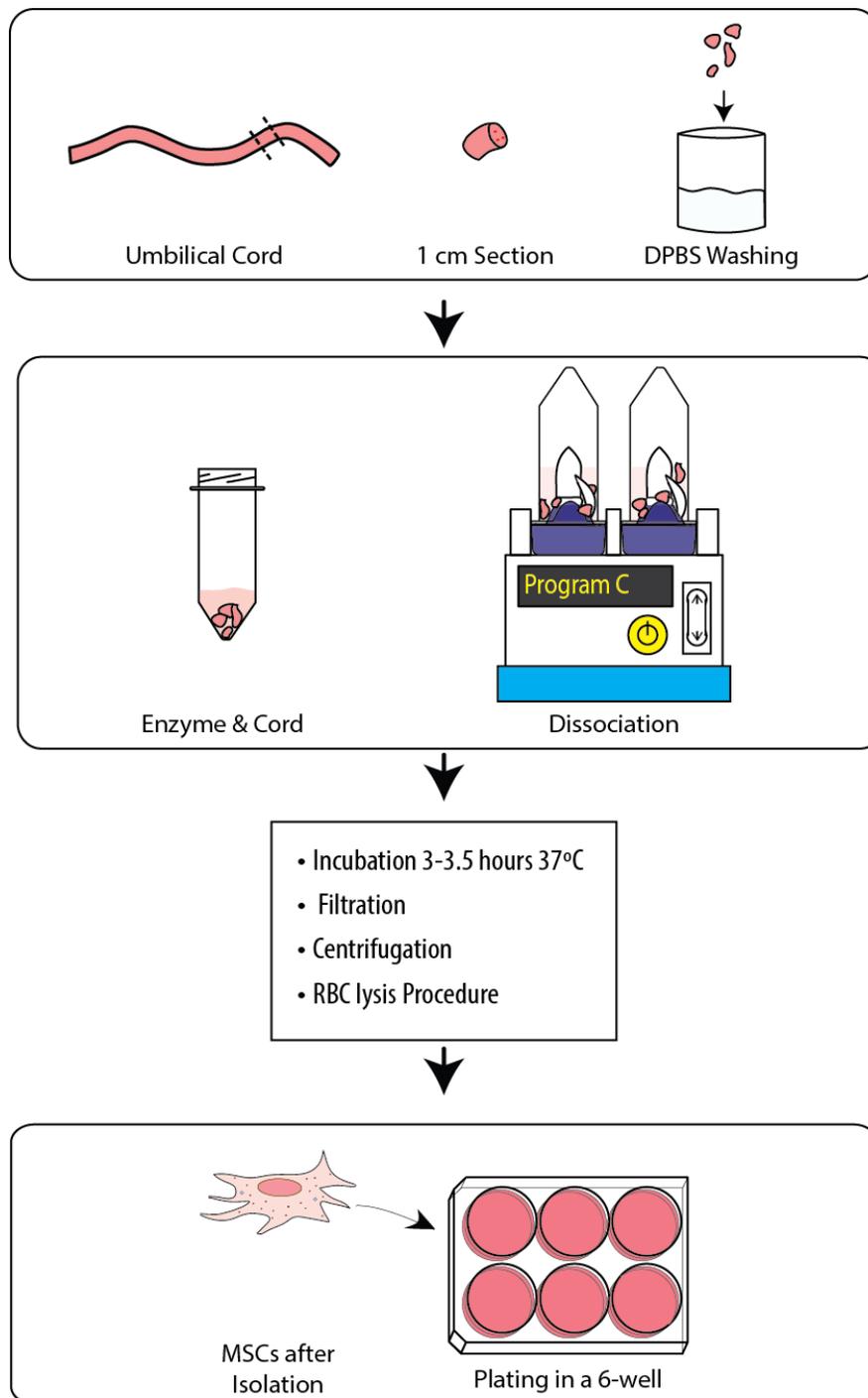
In a pilot study (data not presented), 8 umbilical cords were used to identify optimization variables. In the pilot study, umbilical cords were stored up to 5 days before extracting MSCs; however, no testing was performed to determine whether storage alters the quality of the product.

Once variables were determined for this study, 24 umbilical cords (11 females and 13 males) from vaginal births or Caesarean-section births were obtained and stored in a sterile tissue sample container in saline solution at 4°C until use. Isolation procedures were performed within 4 days after birth. To randomize treatment effects, no prescreening was done, and cord samples (biological replicates) were randomly assigned to each experimental variable to control for cord-to-cord variability and potential differences associated with birth or sex.

## **Isolation Optimization Strategy**

A previously described protocol (Seshareddy *et al.*, 2008) was optimized to decrease contamination risk, increase yield, and improve GMP compatibility. For each umbilical cord (the biological unit), eight samples were isolated. Each sample was 1cm randomly selected from the cord. These samples were used to test the effect of the experimental variables identified in the pilot work. Two to four variables were evaluated per cord using technical duplicates, and the results were averaged for each experimental variable per umbilical cord before comparing the results.

First, a mechanical disruption of the tissue was tested using a Miltenyi GentleMACS Dissociator (#130-093-235) with preprogrammed settings A, B, C, D, and E (corresponding to weakest to strongest dissociation). Next, tissue dissociation was tested both before and after enzymatic digestion. Then, the effect of mincing the tissue samples was compared to tissue dissociation. Next, the effect of filtering using 100  $\mu\text{m}$  cell strainers (Fisherbrand #22-363-549) and 60  $\mu\text{m}$  Steriflip tubes (Millipore #SCNY00060) was tested. Finally, the concentration of enzyme was varied to determine the effect on yield. The technical duplicates or triplicates were averaged for each variable per cord sample. Each procedural optimization variable was evaluated using at least three different cord replicates. Choices for processing were based on process yield (more live cells) or increased process efficiency (reducing number of processing steps, reducing time, or reducing contamination risk).



**Figure 2.1 Isolation Flow Chart**<sup>3</sup> Schematic of the optimized isolation method. The was umbilical cord selected, a 1 cm section taken and cut into 4 equal pieces, cord pieces were rinsed in DPBS. The cord pieces inside a C-tube were immersed in enzyme solution and dissociated with C-tubes and Miltenyi Dissociator. Steps following dissociation prior to plating the isolated cells, and the isolated cells initial plating at P0 in a 6 well plate.

<sup>3</sup> Updated from the original figure for *Current Protocols in Stem Cell Biology: manuscript accepted* Smith, J.R., A. Cromer, and M.L. Weiss. 2017 Human Umbilical Cord Mesenchymal Stromal Cell Isolation, Expansion, Cryopreservation, and Characterization *Current Protocols in Stem Cell Biology* 2017

## **Final (Optimized) Isolation Method**

A schematic of the revised method is shown in Figure 2.1. Umbilical cords were rinsed to remove surface blood using 37°C DPBS with 1% Antibiotic-Antimycotic (Dulbecco's Phosphate Buffered Saline, Life Technologies #14190-250; Antibiotic-Antimycotic, Life Technologies #15240-062). The cords were then treated with 0.5% Betadine (Dynarex, Providone Iodine Solution, #1416) in DPBS for 5 minutes at room temperature. Inside the biological safety cabinet (BSC), the cord was cut into 1 cm lengths and rinsed repeatedly with 3 volumes of DPBS until no further surface blood could be seen. Each 1 cm length of tissue was cut into four equal pieces and placed into a Miltenyi Biotec Dissociator C-Tube with enzyme solution (Miltenyi #130-096-334). The tissue weight was calculated by subtracting the tare weight of the C-tube with 9 mL of enzyme solution from the weight after adding tissue. The C-tubes were placed in a Miltenyi Dissociator, processed using program C, and incubated for 3-3.5 hours at 37°C with constant 12 rpm rotation. Following the 3-3.5-hour incubation, the tissues were dissociated using program B and filtered through 60  $\mu$ m Steriflip filter (Millipore #SCNY00060) to remove tissue debris. The cells were pelleted by centrifugation at  $200 \times g$  for 5 minutes at room temperature, and the supernatant was discarded. The cells were suspended in 0.5 mL of growth media. To remove red blood cell contamination, 0.5 mL RBC lysing solution (Sigma's RBC lysis solution, #R7757-100ML) was added. The cells were mixed gently for one minute, and then 8 mL of DPBS were added. Cells were centrifuged again at  $200 \times g$  for 5 minutes at room temperature, and the supernatant was discarded. The cells were suspended in 1 mL of medium, and the number of live cells was determined using a Nexcelom Auto 2000 Cellometer (immune cells program, low RBC) using the procedure ViaStain AOPI (acridine orange and propidium iodide)

viability staining (Nexcelom cat. #CS2-D106-5ML). Cells were plated at 10,000 to 15,000 live cells per cm<sup>2</sup> on tissue culture treated plastic (CytoOne 6-well plates, #CC7682-7506).

### **Optimization of MSC Expansion**

We used our previously described method MSC expansion medium as the standard for comparison. The medium used in that method contains more than 10 components (Seshareddy *et al.*, 2008), so our goal was to reduce the number of medium components while maintaining MSC attachment at isolation/startup and while maintaining MSC expansion, CFU-F efficiency, multipotent differentiation potential, MSC surface marker expression, and cellular morphology.

For testing, we used a solution of low glucose Dulbecco's Modified Eagle's Medium (DMEM Life Technologies cat. #14190) supplemented with 1% GlutaMAX (Life Technologies cat. #35050), 1% Antibiotic-Antimycotic, and, by volume, 2, 5, or 10% pooled human platelet lysate (HPL; from more than 25 outdated platelet donors; supplied by Kansas University Medical Center diagnostic laboratory, Dr. Lowell Tilzer, director), and 4 units/mL heparin. The cells were plated at 10 to 15,000 cells per cm<sup>2</sup> in CytoOne flat bottom 6-well plates treated with tissue cultures; the cells were expanded for 5 passages. Cells were incubated and grown as a monolayer at 37°C at 5% CO<sub>2</sub> and 90% humidity (Nuair AutoFlow 4950 or Heracell 150i). Once the cells reached approximately 80–90% confluence, they were lifted and plated in fresh medium. To lift the cells, the medium was removed, and cells were washed with 37°C DPBS. The DPBS was removed and replaced with 37°C 0.05% trypsin-EDTA (Lifetech #25200-056). Following a 3–5-minute incubation at 37°C, the plates were tapped to release cells, and the enzymatic digestion was terminated with 3 volumes of medium. Cells were pelleted by centrifuging at 200 × g for 5 minutes at room temperature. Supernatant was discarded, and 1 mL of media was used to suspend the cells. Cells were counted using the Nexcelom Auto 2000 Cellometer and ViaStain

AOPI staining reagent following the manufacturer's protocol and pre-programmed settings. At passage, the number of cells, percentage of live cells, cell size, and number of hours in culture were recorded. Cells were initially plated at a density of approximately 10,000 cells/cm<sup>2</sup>; using this as the initial cell number and the number of cells at harvest as the final cell number and culture time, population doubling time was calculated using the standard formula:

$$\text{Population Doubling Time} = \text{Culture Time} \times \frac{\log(2)}{\log\left(\frac{\text{Final Cell Number}}{\text{Cell Number Plated}}\right)}$$

Some extra cells were frozen for later use. To freeze, cells were cryopreserved using a 1:1 ratio of HPL media and cryopreservative (Globalstem #GSM-4200) and held on ice until they were transferred to a controlled rate freezing device (Mr. Frosty); they were then placed in a -80°C freezer overnight. The next day, the vials were moved to the vapor phase of liquid nitrogen for long term storage.

### **CFU-F Assay**

MSCs were plated at 10, 50, or 100 cells per cm<sup>2</sup> in duplicate in 6-well CytoOne tissue culture plates in 2, 5, and 10% HPL-enriched DMEM, as described above. Four cell lines were expanded 4 days in culture before fixation and methylene blue staining. Subsequent tests used 4 to 7 days of culture at densities of 5, 10, or 50 cells per cm<sup>2</sup>. After the required culture period, the medium was removed, and the cells were washed with DPBS. They were then fixed for 5 minutes in 4°C 100% methanol. The cells were washed again with DPBS, stained with 0.5% methylene blue for 15 minutes, rinsed several times with distilled water, and air dried. The stained colonies were counted manually at 40x final magnification. Colonies were defined as isolated groups (clonal groups) of at least 10 cells. Colony number was determined by averaging the number of colonies in the technical replicates at each plating density for a given expansion

period. Colony forming efficiency was calculated by dividing the number of plated cells by the number of colonies. (For a diagram of the CFU-F 6-well plate assay, see Figure A.2 in the appendix).

## **Differentiation**

Differentiation of MSCs was induced by replacing the expansion medium with MSC differentiation medium (StemPro, Life Technologies #s A10070-01, A10071-01, and A10072-01 for adipogenic, chondrogenic, and osteogenic differentiation) and following the manufacturer's protocol. After approximately 21 days of differentiation, the medium was removed; MSCs were washed with DPBS, fixed with 4% paraformaldehyde for 10 minutes, and stained with Oil Red for analysis of adipose cells, Safranin O for chondrogenic cells, or Alizarin Red S for osteogenic cells. Micrographs were taken using an Evos FL Auto microscope (Life Technologies).

## **Flow Cytometry**

The BD human MSCs flow cytometry characterization kit was used for positive and negative surface marker staining (#562245). Using the manufacturer's recommended protocol, MSC samples were stained with four fluorochromes together for both positive and negative staining cocktails. The positive marker cocktail stained for CD90, CD105, and CD73 (defined as >95% positive staining). The negative cocktail (all antibodies were stained using a single fluorochrome, PE) stained for CD34, CD45, CD11b, CD19, and HLA-DR (defined as <2% positive staining). A CD44 labeled PE antibody was used as positive control for the negative cocktail to set the compensation and gating of the negative cocktail. For each flow cytometry run, fluorescence minus one controls for each fluorochrome and isotype controls for each antibody were used for compensation and nonspecific fluorescence analysis. Samples were washed with 1% BSA solution before and after staining. An FACSCalibur (BD Biosciences) was

used for flow cytometry, and analysis was conducted using FCS software. Negative staining gate of the isotype control was set at 1% positive staining.

## **Statistics**

After confirmation that ANOVA assumptions of normality and homogeneity of variance were met, ANOVA was used to evaluate significant differences between optimization variables. If assumptions were violated, the dataset was transformed mathematically and again tested to see if it met ANOVA assumptions. Hypothesis testing was two-tailed (*e.g.*, mean 1  $\neq$  mean 2). After running ANOVA and finding significant main effect(s) or interaction terms, post hoc means testing of planned comparisons was conducted using either the Bonferroni correction or Holm-Sidak method. Significance was set at  $p < 0.05$ . Data is presented as average (mean) plus/minus one standard error. SigmaPlot v.12.5 (Systat software) was used for statistics and to create graphs. The graphs created in SigmaPlot were saved as EPS files and moved into a vector-based graphics package (Adobe InDesign or Adobe Illustrator CS6) for editing and rendering.

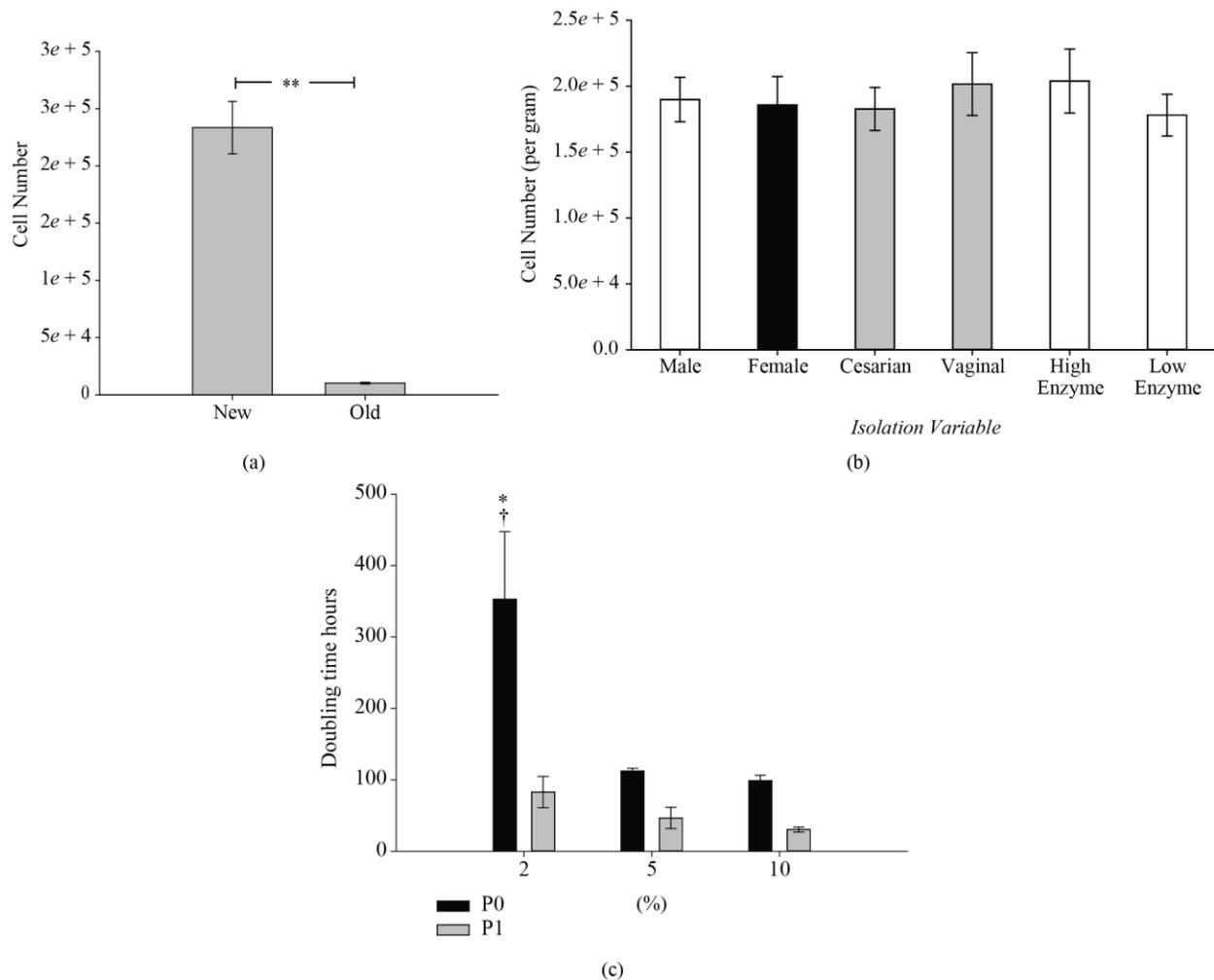
**Table 2.1 Individual Umbilical Cord Comparisons**

UC-MS	Sex	Birth	Length (cm)	Enzyme	Weight (g)	Gram per cm	Viability	± SE	Live cells per cm	± SE	live cells per gram	± SE	Theoretical Cell Yield
241	F	V	46	High	67.9	1.5	76.7%	0.6%	3.7E+05	2.3E+05	3.2E+05	5.6E+04	2.17E+07
242	M	V	43	High	60.7	1.4	58.0%	1.9%	2.4E+05	2.9E+04	1.7E+05	2.2E+04	1.06E+07
243	F	V	57	High	81.1	1.4	62.8%	2.0%	3.9E+05	7.4E+04	2.7E+05	6.4E+04	2.17E+07
244	M	C-S	35	High	66.0	1.9	58.6%	2.6%	2.0E+05	2.4E+04	1.2E+05	1.8E+04	7.85E+06
245	M	V	61	High	76.0	1.2	50.6%	6.4%	3.3E+05	9.9E+04	2.7E+05	9.2E+04	2.06E+07
246	M	C-S	41	High	60.9	1.5	66.0%	0.9%	1.5E+05	4.3E+03	8.5E+04	8.2E+03	5.19E+06
248	F	C-S	47	High	72.6	1.5	68.3%	3.2%	2.9E+05	4.2E+04	1.2E+05	4.3E+04	8.66E+06
249	F	C-S	32	High	37.7	1.2	64.2%	10.7%	1.3E+05	4.9E+04	1.2E+05	4.3E+04	4.50E+06
250	M	V	26	High	43.3	1.7	84.2%	0.5%	5.3E+05	4.1E+04	3.1E+05	8.8E+03	1.34E+07
251	F	V	28	High	30.4	1.1	74.4%	3.8%	9.9E+04	5.7E+02	8.8E+04	1.7E+04	2.69E+06
252	M	C-S	54	High	83.6	1.5	79.8%	3.9%	1.9E+05	3.9E+04	1.3E+05	5.0E+04	1.11E+07
253	M	V	61	low	48.8	0.8	58.0%	3.7%	3.4E+05	9.8E+04	2.3E+05	6.5E+04	1.11E+07
254	F	C-S	38	low	59.3	1.6	60.9%	3.9%	1.1E+05	5.3E+04	7.9E+04	4.0E+04	4.68E+06
255	F	C-S	47	low	82.3	1.8	75.1%	8.6%	7.7E+04	5.2E+03	4.7E+04	4.0E+03	3.85E+06
256	M	C-S	45	low	49.0	1.1	70.2%	1.1%	2.4E+05	5.8E+04	2.1E+05	3.3E+04	1.02E+07
257	M	C-S	43	low	105.2	2.4	66.6%	3.0%	3.5E+05	2.4E+04	1.5E+05	1.4E+04	1.56E+07
258	M	C-S	37	low	45.1	1.2	62.5%	4.6%	1.9E+05	3.8E+04	1.6E+05	2.7E+04	7.01E+06
259	M	C-S	31	low	57.8	1.9	62.2%	3.5%	2.7E+05	2.6E+04	1.5E+05	2.2E+04	8.56E+06
260	F	C-S	67	low	100.6	1.5	64.4%	1.8%	1.7E+05	3.9E+04	1.1E+05	2.8E+04	1.11E+07
261	M	C-S	51	low	92.8	1.8	72.3%	2.1%	2.2E+05	1.9E+04	1.2E+05	8.8E+03	1.10E+07
262	F	C-S	28.5	low	25.1	0.9	50.5%	4.1%	1.0E+05	2.7E+04	1.5E+05	5.7E+04	3.81E+06
263	F	C-S	28	low	23.8	0.9	65.3%	3.8%	2.0E+05	5.4E+04	2.6E+05	8.9E+04	6.27E+06
264	F	C-S	32	low	45.7	1.4	59.8%	3.4%	4.5E+05	4.7E+04	3.2E+05	4.3E+04	1.46E+07
265	M	C-S	38	low	38.7	1.0	54.8%	3.2%	1.5E+05	3.0E+04	1.5E+05	3.5E+04	5.81E+06

24 cords were compared below

	Length	Weight	g per cm	Viability	Live cells per cm	Live cells per gram	Theo. Yield
Mean	42.4	60.6	1.4	65.25%	2.42E + 05	1.72E + 05	1.01E + 07
Standard dev.	11.6	22.9	0.4	8.33%	1.16E + 05	7.48E + 04	5.00E + 06
Coefficiency of var.	27.5%	37.7%	26.9	12.8%	47.9%	43.5%	49.7%

F = female, M = male, C-S = Caesarean-section, and V = vaginal. The enzyme concentration: low was 300 U/mL and high was 532 U/mL of collagenase. SE = standard error, calculated after averaging the technical replicates for each umbilical cord. Live cells per gram were calculated from the live cell number for each tube divided by the weight of the tube. Theoretical yield calculation represents cell numbers achieved assuming the entire umbilical cord was processed and expanded.



**Figure 2.2 Isolation numbers and initial doubling time** Effect of various experimental variables on UC-MSCs isolation. (a) The new methods average cell number per cm of umbilical cord isolated, compared to the old cell number isolated per cm ( \*\* means  $p < 0.001$ ). (b) Comparing different experimental variables, there were no significant differences (see Table A.3 for the data) updated from original publication. (c) Population doubling time for passage 0 (initial isolation) or passage 1 (first passage of expansion phase). \* represents  $p < 0.05$  for 2% HPL media compared to 5% and 10% media. † represents  $p < 0.05$  for the passages (P0 compared to P1).

## Results

### Umbilical Cords

Umbilical cord from Caesarean-section delivery ( $n = 17$ ) or vaginal delivery ( $n = 7$ ) were used. Table 2.1 shows the biographic data.

## Isolation Method Comparison

Note that the MSC expansion was evaluated for passages 1-5, and passage 0 was considered part of the MSC isolation. Results obtained from our previous method (historical data from 27 umbilical cords (Seshareddy *et al.*, 2008)) and our optimized method were compared. As Figure 2.2 shows, the optimized method yielded on average more than 10 times the MSCs per cm length than the original method (New method  $233,404 \pm 22,796$  cells per cm; Old method  $10,223 \pm 292$  cells per cm) and yielded MSCs in 100% of the UC samples (n=24). In the pilot study, while identifying variables to optimize, in two cases MSC isolation failed, but even then, MSCs were isolated from the same UC in different samples (*e.g.*, in no case did the UC not contain viable MSCs). While testing was not done for bacterial, viral, or fungal contamination, no break in sterility was apparent (no obvious contamination was observed, and no cultures were discarded due to contamination). Table 2.1 shows live cells per cm of length or per gram; the coefficient of variation was less for live cells per gram. The optimized method used a closed processing system for tissue disruption and requires 4 hours with an additional 3-hour enzyme extraction step to isolate the umbilical cord MSCs.

MSC attachment was observed within 24 hours of isolation, and proliferation was observed in all three HPL media enrichment conditions. As Figure 2.2(c) shows, during the isolation phase (P0) UC-MSCs grew more quickly when plated in 5% or 10% HPL enriched DMEM than UC-MSCs plated in 2% HPL enriched DMEM. It is possible that UC-MSCs grown in 5 or 10% HPL enriched DMEM attached more quickly than those grown in 2% HPL enriched DMEM in P0. The growth rate difference for 2% HPL enriched medium was statistically different (slower) at P0 than later passages (see figures 2.2(c) and 2.3(a)) and differed significantly (slower) from 5 and 10% HPL enriched media at isolation and during expansion.

## MSC Expansion Comparison

MSC expansion was compared for passages 1-5, but passage 0 was part of MSC isolation. UC-MSCs were expanded for passages 1-5 and evaluated in 3 different growth conditions: DMEM supplemented with either 2% HPL, 5% HPL, or 10% HPL. A two-way ANOVA (main effects: HPL level and expansion over time) found HPL concentration was a significant main effect on attachment and expansion. In post hoc testing, significantly more cells were found; about 30% more were obtained when cells were expanded in 10% HPL enriched DMEM medium than 5% HPL enriched medium ( $9.4 \times 10^5 \pm 6.2 \times 10^4$  cells per  $\text{cm}^2$  versus  $6.6 \times 10^5 \pm 3.8 \times 10^4$ )(Figure 2.3(b)). Similarly, post hoc testing showed significantly shorter population doubling times when MSCs were expanded in 10% HPL,  $32.4 \pm 2.5$  hours, compared to  $40.7 \pm 4.1$  hours for 5% HPL and  $100.9 \pm 14.8$  hours for 2% HPL enriched medium (see Figure 2.3(c)). As Figure 2.3(d) shows, MSCs grown in 10% HPL enriched DMEM were on average 17% smaller than those grown in 2% HPL ( $14.7 \pm 0.2 \mu\text{m}$  versus  $17.6 \pm 0.4 \mu\text{m}$ ) and 10% smaller than cells grown in 5% HPL medium ( $16.1 \pm 0.3 \mu\text{m}$ ). The trends in MSC size across HPL medium conditions became noticeable after the second passage (Figure 2.3(e)). HPL medium enrichment affected the viability of the cells noted at passage (see Figure 2.3(c)). Subtle but significant differences were found in viability at passage among the three medium conditions: MSCs in expanded in DMEM supplemented with 10% HPL had higher viability than those grown in DMEM supplemented with 2% HPL ( $92.2 \pm 0.9\%$  versus  $84.9 \pm 1.7\%$ ) and 5% HPL medium had significantly greater viability than 2% HPL medium ( $90.4 \pm 0.9\%$ ; see Figure 2.3(c)).

The theoretical cell yield was calculated assuming the entire umbilical cord was isolated and expanded in each medium condition to passage 5. As Figure 2.3(f) shows, the total yield was

estimated as exceeding  $10^{12}$  MSCs (a trillion cells) at passage 5 for UC-MSCs expanded in 10% HPL supplemented medium and exceed  $10^{11}$  MSCs for UC-MSCs expanded in medium supplemented with 5% HPL (Figure 2.3(f)).

### **Evaluation of UC-MSC Characteristics**

Sex of the donor had no effect on number of MSCs isolated (Figure 2.2(b)) or the estimated number of MSCs obtained after expansion (data not shown). On average, more cells were isolated for UC-MSCs isolated from normal vaginal delivery than Caesarean-section delivery (see Figure 2.2(b)).

### **Colony Forming Unit-Fibroblast (CFU-F)**

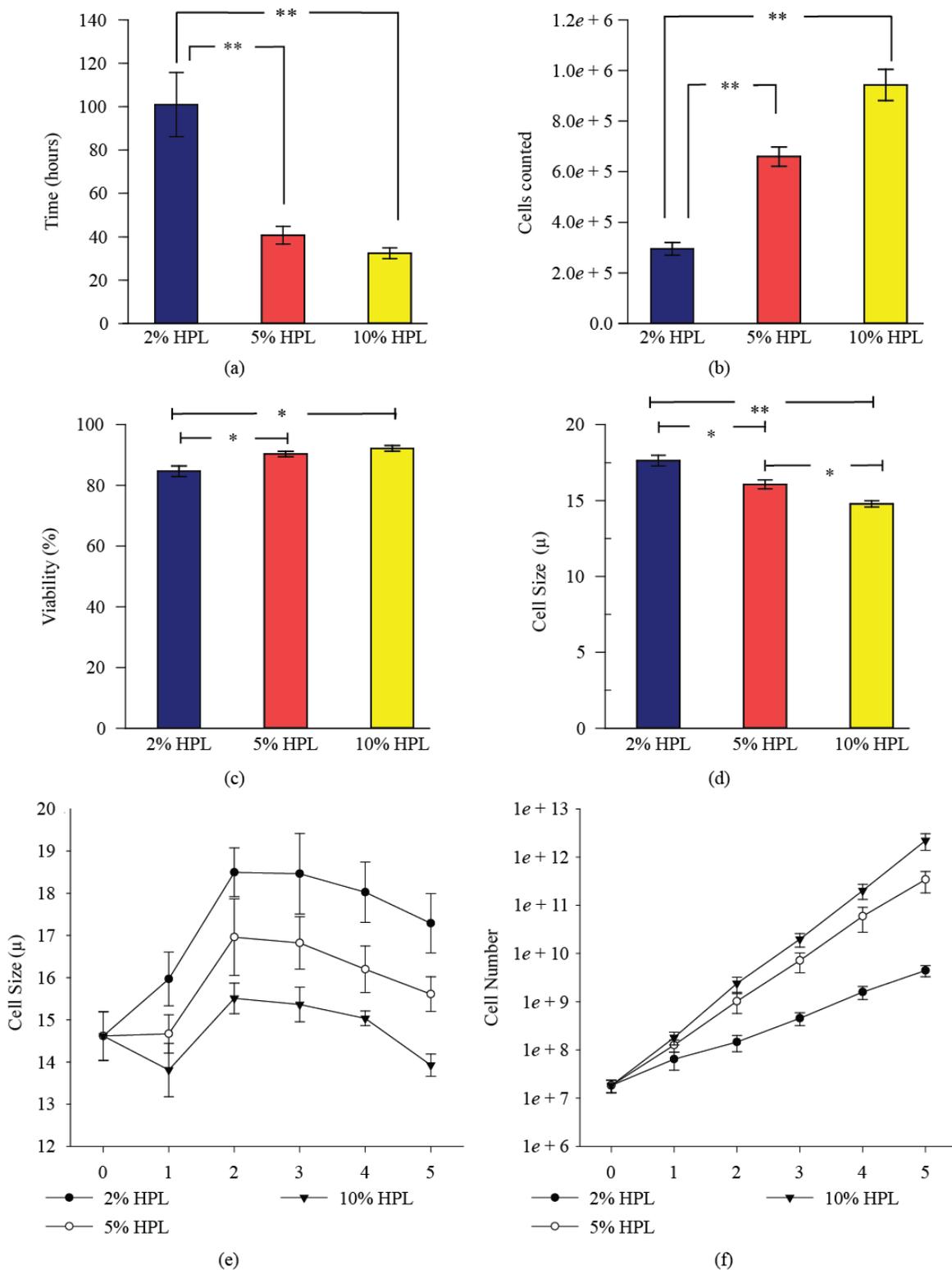
Data is presented as a normalized unit: colony forming efficiency (CFE;  $CFE = \text{Number of plated cells} / \text{number of colonies}$ ). As Figure 2.4(e) shows, the concentration of HPL supplementation had no effect on CFE at 10 cells/cm<sup>2</sup> (100 cells per well of a 6-well plate) after 4 days of expansion in culture. In contrast, when plated at a density of 50 cells per cm<sup>2</sup> and 4 days of expansion in culture, 10% HPL supplementation showed higher CFE than either 2 or 5% HPL: 2 to 4 MSCs were needed to form a colony when plated in medium supplemented with 10% HPL (Figure 2.4(e)). Figure 2.4(e), and as previously reported (López *et al.*, 2011; Sarugaser *et al.*, 2005), shows plating density affects CFE with higher efficiency at lower plating density. Therefore, we determined whether higher efficiency occurred at plating density below 10 cells/cm<sup>2</sup> after plating in HPL. The highest CFE occurred when MSCs were plated at 5 cells/cm<sup>2</sup> for 6 days (50 cells per well of a 6-well plate); in medium supplemented with 10% HPL, on average, one out of two MSCs formed a colony (see Appendix, Figure A.2)

## **Differentiation**

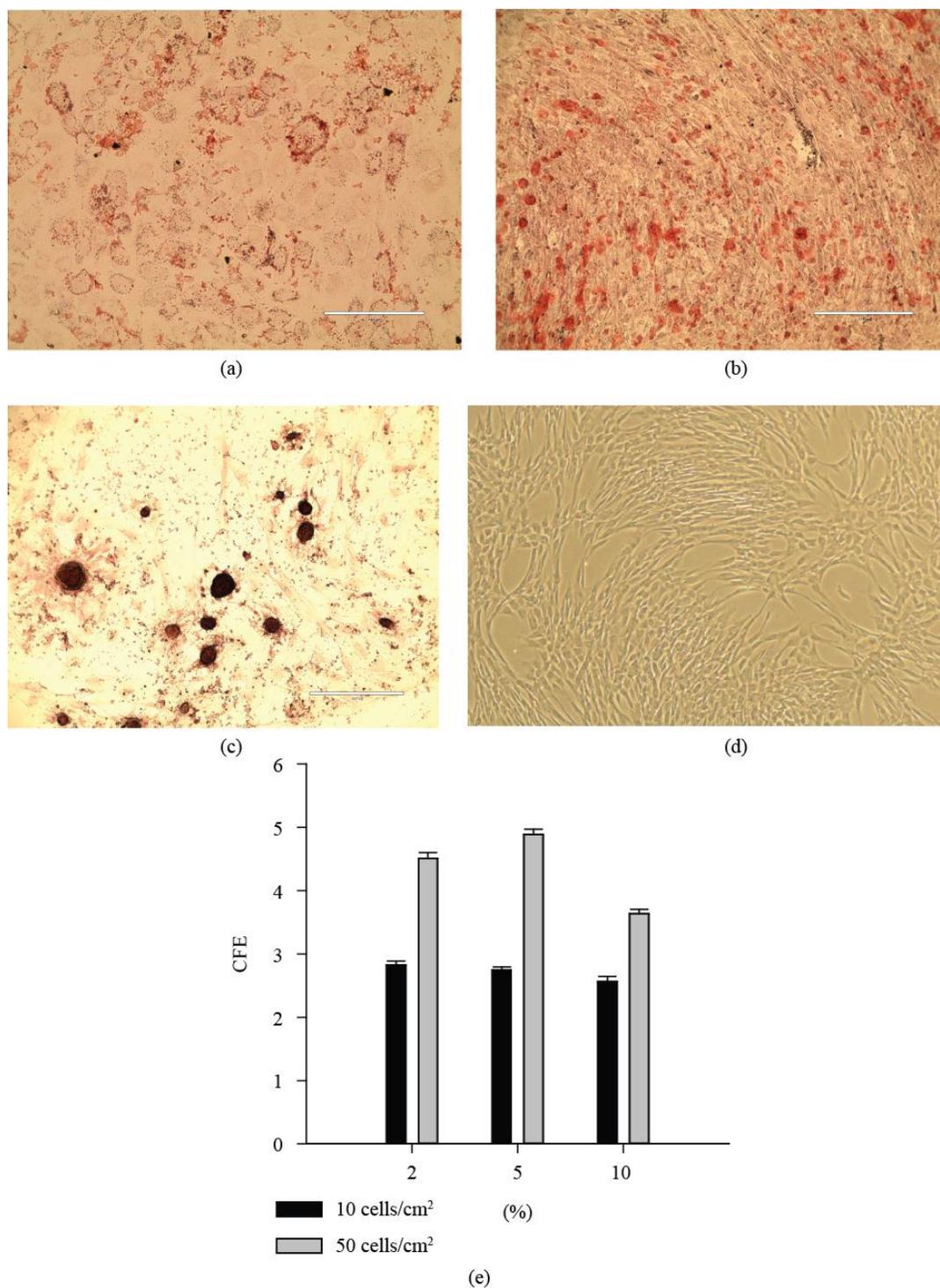
MSCs isolated and expanded using the optimized method differentiate into the three mesenchymal lineages, bone, cartilage, and fat, after exposure to differentiation medium conditions for 3 weeks. Figure 2.4 shows MSCs differentiated to fat and chondrogenic and osteogenic lineages using the closed isolation method and expansion to passage 5 in 10% HPL supplemented DMEM. Exposure to adipogenic differentiation medium caused lipid droplets to form in MSCs that stained with Oil Red (Figure 2.4(a)). Exposure to osteogenic differentiation conditions caused calcium deposits to form in MSCs that stained with Alizarin Red S (Figure 2.4(b)). Cartilage like tissue formation was observed in clusters of cells after they were exposed to differentiation medium; this was indicated by glycosaminoglycan staining by Safranin O for chondrogenic cells (Figure 2.4(c))

## **Flow Cytometry**

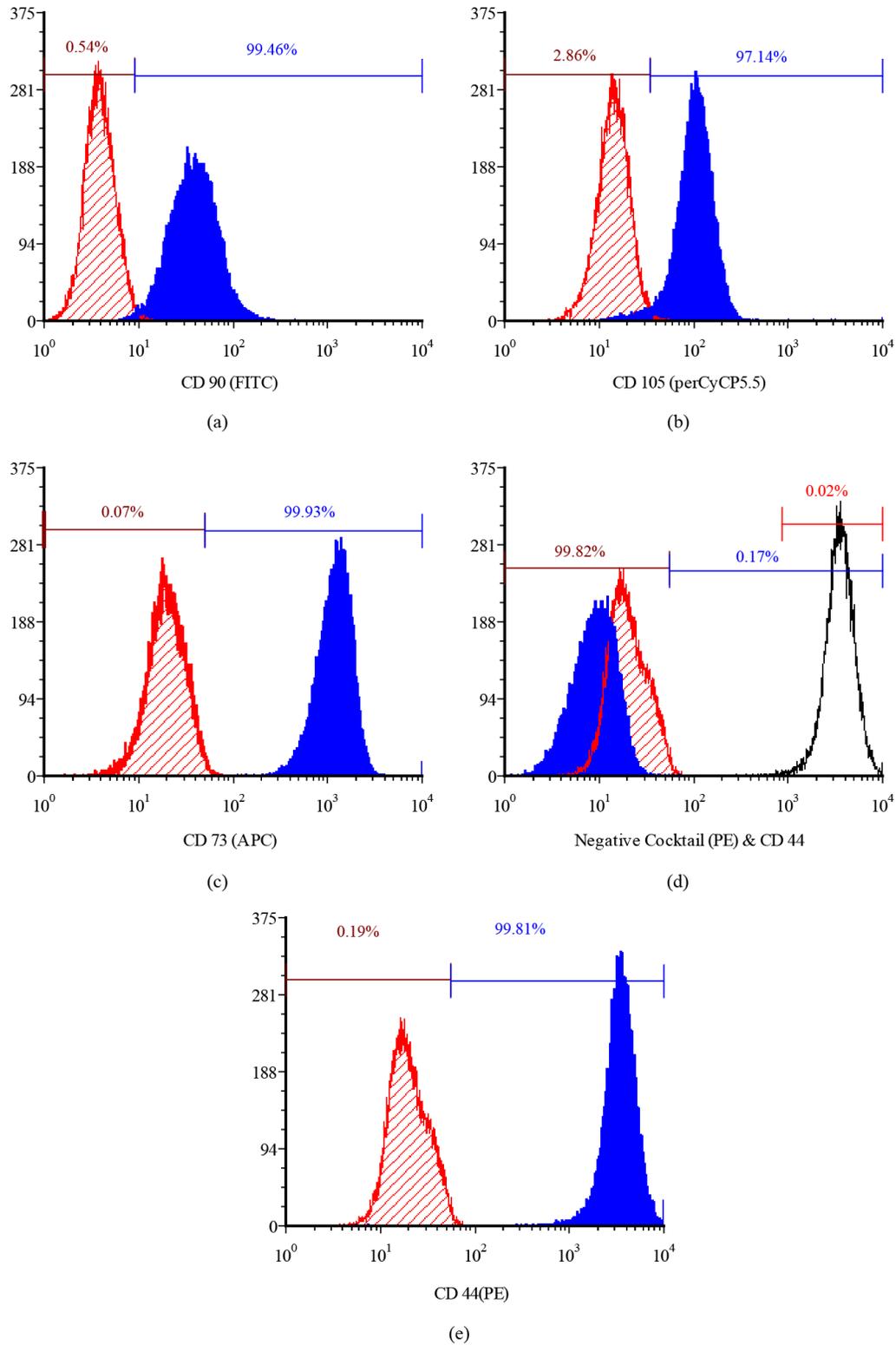
Following expansion using the 10% HPL supplemented DMEM for 5 passages, flow cytometry was used to analyze the surface marker expression in six MSC lines. High surface marker expression (>95% of the cells are positive) for CD73, CD90, CD105, and CD44 was observed (see Figure 2.5 for representative results; see Appendix Table A.1 for all flow cytometry data). Low surface marker expression (<0.5% positive) was observed for CD34, CD45, CD11b, CD19, and HLA-DRT (Supplemental Table 1). To evaluate how freezing and thawing affected MSCs for surface marker expression, four MSCs lines were evaluated before and after a freeze/thaw cycle. Frozen/thawed MSCs did not differ significantly in surface marker expression from never frozen MSCs (See Appendix, Table A.2).



**Figure 2.3 UC-MSC expansion results** Effect of HPL concentration on expansion. (a–d) UC-MSC (e,f) expansion results combined for passages 1–5. (a) Population doubling times for the 3 media conditions. (b) Number of cells counted at passage for each medium condition. (c) Cell viability at passage for each medium condition. (d) The average size of the cell for each medium condition at passage, measured by the Nexcelom during cell counts. (e) Cell size over 5 passages for each medium condition. (f) The theoretical yield if an entire umbilical cord was isolated and grown to confluence at each passage. \* means  $p < 0.05$  and \*\* means  $p < 0.001$ .



**Figure 2.4 Differentiation and CFU-F results** Differentiation and colony forming unit fibroblast (CFU-F) results for the characterization of UC-MSCs. (a) After adipogenic differentiation, MSCs were stained with Oil Red, which binds to lipid droplets (20x objective magnification; scale bar = 200 micrometers). (b) After osteogenic differentiation, MSCs were stained with Alizarin Red S, which binds to calcium deposits. (c) After chondrogenic differentiation, MSCs were stained with Safranin O, which binds to glycosaminoglycans in cartilage ((b) and (c) at 10x objective magnification; scale bar = 400 micrometers). (d) UC-MSCs in normal growth conditions (control) phase contrast micrograph at 4x objective magnification. (e) CFU-F efficiency was calculated by dividing the number of plated cells by the number of CFU-F colonies observed. Panel (e) shows CFE versus human pooled platelet lysate (HPL) concentration in medium (2, 5, or 10% HPL) after plating at 5 (black bars) or 10 (gray bars) cells per cm<sup>2</sup> and 4 days in culture.



**Figure 2.5 Flow Cytometry Characterization** Histograms of flow cytometry; blue solid filled overlay represents the test sample; red diagonal line filled overlay represents the isotype control. For each histogram, the negative gate (red bar) was set to include 99% of the isotype. Percentages shown in histograms are for the test samples. (a–c) All are markers in the positive cocktail CD90, CD105, and CD73. The positive gate percentages are shown in blue for each sample. (d) The negative cocktail with CD44 was included as a positive control (unfilled overlay); positive percentage is black. (e) CD44 marker was separate from the positive cocktail.

## Discussion

The advances of MSCs in clinical trials in regenerative medicine have renewed the effort to standardize production and characterization of MSCs in GMP-compliant standard operating procedures (SOPs). UC-MSCs have a number of advantages as a potential source for allogeneic MSCs for cellular therapy, as indicated by trends in UC-MSC clinical trials (Arutyunyan *et al.*, 2016).

Our goal was to reduce processing time and contamination risks. We reasoned the method for isolating UC-MSCs could be standardized. To develop an SOP for GMP production of UC-MSCs, several limitations of a previously described method for UC-MSC isolation and expansion had created barriers to GMP production. First, the previous isolation method required a lengthy dissection step, cutting open the umbilical cord to manually remove the vessels before mincing the Wharton's jelly. This is time consuming and increases the risk of contamination. Second, the previously used UC-MSC medium, originally described by Catherine Verfaillie's laboratory for expanding multipotent adult progenitor cells (MAPCs), was complicated, with more than 10 components, and it furthermore contained 2% FBS, a xenogeneic product (Reyes *et al.*, 2002). Identifying a simpler medium with fewer components, one free of xenogeneic materials, was desirable because of compliance concerns.

We chose to test a medium enriched with human platelet lysate (HPL) to eliminate concerns about xenogeneic materials. Previous research had indicated that HPL could be produced in a GMP-compliant format and produce good expansion of MSCs (Burnouf *et al.*, 2016; Castiglia *et al.*, 2014; Hemeda *et al.*, 2014). Our results indicated that 5 or 10% HPL enrichment improved MSC attachment time in the initial isolation (P0). Furthermore, we observed robust cell growth over passages 1–5. Therefore, using HPL-enriched medium

eliminated two barriers to GMP compliant manufacturing of UC-MSCs. However, using a pooled human blood product is not without risks. Burnouf *et al.* (2016), reviewed HPL, identifying several risks, including donor derived pathogens, allogeneic proteins causing MSCs to generate immune reactions, potential influence of blood group on the platelet lysate, and donor-to-donor protein content variability.

Sample-to-sample variability means pooling platelet lysate is essential to produce a uniform product (Schallmoser *et al.*, 2009). The platelet lysate could be pooled by blood type, expanding cell lines that match patient blood type, although in our study, we did not separate by blood type. Moreover, human pathogens that escape screening by providers may contaminate HPL samples. One way to ameliorate this risk would be to inactivate pathogens in HPL (Fazzina *et al.*, 2015). The HPL used here was not inactivated, but the repeated freeze-thaw process followed by the filtration through a 0.2 µm filter should kill and remove bacteria. While gamma irradiation is something that may be considered to reduce viral risk, the platelet units used here were outdated units obtained from a blood bank for clinical use. Therefore, the HPL met blood screening safety measures.

The heparin used here is technically a xenogeneic component, derived from porcine. However, the form used is pharmaceutical grade and a commonly used drug; therefore, it should not cause a significant compliance burden (Burnouf *et al.*, 2016). Alternatively, synthetic heparin could be used to supplement the media; this was not tested. Heparin is a critical component, preventing the gelling of the media that happens because of fibrins in the HPL. Fibrin depleted HPL has been investigated as an alternative, and results indicated an improvement in immunosuppressive properties of MSCs (Copland *et al.*, 2013).

In our study, 1 cm lengths of cord were used to optimize the isolation protocol. The sections of umbilical cord provided enough cells for isolation and expansion, allowing several experimental variables to be examined for each cord (the biological variable). The assumption was a 1 cm length of cord represented the whole umbilical cord (that cellular distribution and umbilical cord extracellular matrix are homogenous). We did not attempt to validate this assumption here, and to my knowledge, no investigation has either supported or denied this assumption. Umbilical cords display tremendous biological variation in density (weight per unit length), diameter, and physical mechanical properties perhaps due to the amount of extracellular matrix surrounding the vessels (see Table 2.1). The gram per cm measurements vary in each technical replicate from a single cord and between different umbilical cords as well. This indicates that multiple biological and technical replicates are important when optimizing methodologies using umbilical cords. The number of cells varied considerably for each umbilical cord as did the density and amount of extracellular matrix. Thus, the biological variation may limit the ability to manufacture a standardized cellular therapy product, one that could guarantee consistent results for cell numbers and viability. Here, our hypothesis suggested isolated cells have similar physiology for each cord, but this is based on an assumption that requires testing. To date, every UC-MSC cell line tested for characterization has met the criteria for MSCs as stated by the ISCT (Dominici, 2006).

Several protocols for isolating MSCs from umbilical cord have been published (Mitchell *et al.*, 2003; Sarugaser *et al.*, 2005; Seshareddy *et al.*, 2008; Troyer and Weiss, 2008). These protocols require dissecting different parts of the umbilical cord and a variety of methods to enrich MSCs from the primary isolation population. This explains much of the variation in the number of cells in the primary isolation and their ability to undergo expansion in culture. We

saw no visual differences between MSCs isolated from the Wharton's jelly matrix following blood vessel dissection in the old protocol and those using the entire cord for isolation with the new protocol, which indicates the procedure can be simplified. Isolation using 1 cm lengths of cord with the methods outlined here gave a >10-fold increase in the number of input cells for the primary culture. This may be due to the reduced manipulation of the tissues (elaborate dissection negatively affects attachment and expansion) and the more efficient removal of red blood cell contamination (blood negatively affects the viability, attachment, and expansion of MSCs).

In earlier research, measuring the cord length was used to compare yield of cords (Seshareddy *et al.*, 2008). Here, both cord length and weight were collected to determine which proved the better predictor of cell yield in initial isolation. The variation between umbilical cords for both length and weight is provided in Table 2.1. Table 2.1 shows live cell numbers per weight in grams varied less than live cell numbers per cm, suggesting weight is more reliable for measuring cells isolated for the whole cord. Additional research could determine differences between the predicted total cell yield of an umbilical cord and the estimated value. Cells have not been isolated from an entire umbilical cord; therefore, the accuracy of these estimates cannot be confirmed.

The optimized protocol produced more cells, which may be due to faster processing and reduced dissection. By not removing the blood vessels, the optimized method is a significant departure from previously described methods. This may mean the isolated cell population may be different, and the MSCs obtained using the optimized methods may or may not differ from those obtained using previous methods. Several different methods for obtaining MSCs from the umbilical cord have been described, but it is unclear whether each isolates the same cells. Our data does not directly address this question, but we demonstrate here that following evaluation of

6 umbilical cord MSC isolates, the cells from the optimized methods conform to ISCT criteria for MSCs (Dominici *et al.*, 2006).

Earlier research indicated there may be a significant difference between UC-MSC isolation efficiency from cords obtained from vaginal births and Caesarean-section births. The results indicated vaginal birth umbilical cords had more approximately 41% cells per after isolation (Figure 2.2(b)). After the initial study, further isolations showed fewer differences between vaginal and Caesarean-section birth isolation numbers in the statistical models (Figure 2.4 (b)). Thus, delivery method does not significantly affect isolation numbers although on average, vaginal birth umbilical cords produce more cells numbers at isolation. Before these observations, researchers preferred to use Caesarean-section umbilical cords for MSC isolation, assuming surgical collection would reduce the contamination risk compared to cords collected following passage through the birth canal. Our study showed no differences in contamination between umbilical cords from vaginal births or Caesarean sections. Moreover, cords obtained from vaginal births provided a lower volume of umbilical cord blood than Caesarean-section births (Noh *et al.*, 2014; Solves *et al.*, 2003). Gender had no effect on the number of cells isolated or MSC expansion rate or number. Enzymatic digestion using a high concentration of digestive enzymes tended to give more yield at isolation (Figure 2.2(b)). Visually, more samples treated with higher concentrations of enzymes appeared to have less debris at initial plating than samples treated with lower enzyme concentrations.

After the initial plating of cells during the isolation protocol, cell attachment is delayed. Attachment to the substrate is a defining characteristic of MSCs and appears to be necessary for MSCs expansion. We found that after MSCs were isolated, in passages 1–5, MSCs attach and begin to expand within 24 hours of plating. In contrast, other methods see delays in attachment

and thus the time to reach confluence for the isolation and initial passage is significantly affected. Here, data for P0 was included with the isolation of MSCs, and passages 1-5 were considered the expansion phase of the MSCs. When viability was higher at initial isolation, the cells attached more rapidly, leading to faster expansion. In previous research, cell viability was not recorded at the initial isolation (Seshareddy *et al.*, 2008). In this study, we used the Nexcelom and ViaStain AOPI viability assay as a quantitative method for counting cells. This method uses two fluorescent dyes: acridium orange to identify all cells and propidium iodide to identify cells with disrupted membranes (indicating cell death). This gives more consistent results than trypan blue manual counting using a hemocytometer, which is how cells were counted in earlier research. Automated cell counting lends itself to optimizing SOPs.

To advance cells to clinical trials, cryopreserving UC-MSCs is necessary when expanding the cells in culture. Studies have reported cryopreservation affects cells surface marker expression or viability (Francois *et al.*, 2012). For that reason, surface marker expression was evaluated in never frozen cells and in cells subjected to a freeze-thaw cycle. The analysis showed no difference in surface marker expression between fresh cells (those never frozen) and frozen, then thawed, cells for six umbilical cord MSCs lines. Therefore, we suggest further testing to confirm these results in a larger sample size. Clinical trials will require freezing cells before use; using fresh cells in clinical trials is not feasible considering the rigorous quality control and release testing that must be done to determine if cells meet the standards for clinical use.

Here, human platelet lysate enriched media at three different concentrations (2%, 5%, and 10%) were used to analyze the effect on the initial isolation (P0) and growth of the MSCs for passages 1–5. MSCs through passage 5 were evaluated to characterize expansion potential. The

highest variation in growth rate was observed for P0 to P1 (see figures 2.2(c) and 2.3(a)). Results from five umbilical cords showed no difference in passages 1 through 5 for population doubling time, number of cells at passage, and viability at passage (data not shown). However, the three media conditions did affect these variables. Media with 2% HPL enrichment differed significantly from 5% and 10% HPL media for population doubling times, cell size, cell numbers, and viability. Enrichment with 2% HPL doubled the population more slowly, with fewer cells at passage, larger cells, and a lower percentage of viable cells at passage. Although 5% and 10% showed no truly significant differences, 10% HPL was observed to have more favorable values, quicker population doubling, more cells at passage, smaller cells, and higher viability at passage. For this reason, 10% HPL was selected as the new standard medium condition for growing UC-MSCs. We did not expect cell size in the UC-MSCs to vary significantly among media conditions, but we did see variation and a significant difference in cell size at higher HPL concentrations. Cell size initially increased after the first passage, and then decreased over subsequent passages in all media conditions (Figures 2.3(d)-2.3(e)). Further research is needed to assess whether cell size is affected by passage. Senescent cells may become more prevalent over multiple passages; previous observations suggest senescent cells are larger, so decreasing cell size over passage, as we saw in this research, goes against convention (Cheng *et al.*, 2011). If, however, more rapidly dividing cells are smaller, then the cell size data could support our conclusion that 10% HPL is optimal for cell growth of UC-MSCs.

Previous research had also indicated that smaller MSCs with a rapidly dividing phenotype could be identified by plating at a density of 3 cells per cm<sup>2</sup> (Prockop *et al.*, 2001). The effect of plating density on proliferation in HPL enriched medium was not evaluated. Future

work might evaluate the interaction between plating density and 10% HPL media to optimize manufacturing efficiency.

UC-MSCs were characterized by assessing cell surface markers with flow cytometry, CFU-F, and differentiation capacity. Six UC-MSC lines were analyzed with flow cytometry, and all had high levels of the surface markers known to be associated with MSCs. The percentage of positive cells is comparable to previously published isolation methods (Seshareddy *et al.*, 2008). These results suggest a homogenous cell population even though the blood vessels were not removed during the isolation step. Differentiation ability was assessed in five cell lines, and all displayed multipotent differentiation capacity. The capacity for adipogenic differentiation was analyzed by Oil Red O staining for lipid droplet accumulation within the differentiated cells cytoplasm. Analysis showed multiple lipid droplets forming within many of the cells (Figure 2.4(a)). Cell death during differentiation led to the space between the adipogenic cells. Osteogenic differentiated MSCs were stained with Alizarin Red S to analyze calcium deposit formation. Staining was observed in calcium deposits on the cells and within the cells as seen in Figure 2.4(b). Chondrogenic differentiated cells were stained with Safranin O to assess if cartilaginous tissue associated with glycosaminoglycan. This differentiation yielded circular colonies, often remaining adhered to the plate, and they robustly stained for Safranin O. Typically, histological sections of microcolonies are used to assess chondrogenic lineage differentiation. Our results indicate this may not be necessary when small colonies of cells adhere to the plate (Figure 2.4(c)). Although the results are not quantified, the quality of the staining and duplication between multiple lines provides evidence that MSCs isolated and expanded by the new method have robust multipotent differentiation capability *in vitro*.

We used CFE to analyze for the self-renewal potential of UC-MSCs. Compared to previous research where UC-MSCs expanded in 21% oxygen, our research showed fewer cells were needed to form a colony when using the method described here, suggesting a higher CFE (López *et al.*, 2011). Because of the difficulty of counting cells at 50 cells per cm<sup>2</sup>, plating 10 cells per cm<sup>2</sup> was considered a more reliable measure for the CFU-F effect of HPL concentration.

The fast growth rate in the 10% HPL enriched medium made our previous CFU-F protocols unreliable because the cells grew too fast. Growth conditions were tested for measuring CFE by analyzing days from plating versus colony counts. The number of cells needed to form colonies decreased with each day of growth; the exception was 50 cells per cm<sup>2</sup> which increased the number of cells needed. The highest CFU-F efficiency was for 5 cells per cm<sup>2</sup> grown for 6 days. The use of both 10 and 50 cells per cm<sup>2</sup> yielded consistent data for CFU-F. The self-renewal data (CFE) is important when estimating the expansion potential of an MSC line. Higher CFU-F efficiencies are associated with MSC lines with a more robust growth potential. Determining this method for analyzing CFU-Fs in these fast-growing cells allows analysis of growth potential for future research using UC-MSCs.

In this research, a new optimized method to isolate and expand UC-MSCs was developed. Compared to previous methods, we saw an increase in total MSC yield at the initial isolation of more than 10 times, and less time was needed to isolate MSCs from the umbilical cord. Additionally, this method reduced the overall expansion by reducing the amount of population doubling needed to meet our production target of 2–10 billion cells per batch. The method used a closed system for initial isolation with minimal dissection of the cord and, thus, reduced contamination risk, while simultaneously reducing processing time. The method used a

simplified (5 component) medium that can be upgraded to GMP-compliant components. Characterization of MSCs produced using this optimized processing protocol and simplified medium included *in vitro* expansion; colony forming efficiency; multipotent differentiation to osteogenic, chondrogenic, and adipogenic lineages; and surface marker expression by flow cytometry, all indicating that MSCs were produced by this method. Further research is needed to confirm that MSCs isolated and expanded using this method will perform *in vivo* as a cellular therapy. This procedure should speed clinical translation of UC-MSCs by providing a foundation for the chemistry and manufacturing controls (CMC) portion of an investigation of new drug application (IND).

# Chapter 3 - <sup>4,5</sup> Analysis of umbilical cord mesenchymal stromal cells growth media and cryopreservation

## Introduction

Therapeutic uses for UC-MSCs have been identified for several diseases, both *in vitro* and *in vivo* (Arutyunyan *et al.*, 2016). UC-MSCs are a non-controversial source, easily obtainable from discarded tissue. Those isolated using the recently standardized protocol meet the ISCT designations for MSCs (Dominici *et al.*, 2006; Smith *et al.*, 2016). These cells were expanded efficiently in medium using pooled human platelet lysate (HPL) as the primary supplement, eliminating xenogeneic components (Seshareddy *et al.*, 2008). Two areas require further study to optimize UC-MSCs and advance them to clinical use: the characteristics of UC-MSCs metabolism in culture and their post thaw viability.

## Growth Media

HPL supplemented media have been used in efficiently expanding different types of MSCs (Hemeda *et al.*, 2014). The evidence suggests that, as the primary supplement, HPL provides more favorable conditions for growing UC-MSCs than fetal bovine serum (FBS) . As more researchers use HPL enriched media to grow MSCs, we must understand the characteristics of MSC metabolism grown under this medium. In conjunction with the initial isolation, expansion, and characterization of UC-MSCs (Smith *et al.*, 2016), Petry *et al.* (2016) explored their growth using microcarriers in a 3D fluid growth model, which could be scaled up to

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<sup>4</sup> Abstract presented in part at ISSCR annual meeting 2016.

<sup>5</sup> Research described in part in *manuscript accepted* Smith, J.R., A. Cromer, and M.L. Weiss. 2017 Human Umbilical Cord Mesenchymal Stromal Cell Isolation, Expansion, Cryopreservation, and Characterization *Current Protocols in Stem Cell Biology* 2017

bioreactors *et al.*, 2016). Theoretically, bioreactors could generate a batch of MSCs large enough to cryopreserve multiple doses for patients, thus decreasing the required quality assurance tests (Gibson *et al.*, 2013; Jung *et al.*, 2012; Sensebe *et al.*, 2013). In this study, we analyzed four conditions for the impact of different components (antibiotics, GlutaMAX, and glucose) on the metabolism of UC-MSCs.

## **Cryopreservation**

One concern with MSCs involves cryopreservation and whether UC-MSCs maintain the same characteristics after freezing. If they do not, this could limit the ability to use UC-MSCs in the clinic. One report shows MSC growth rate decreases during the first passage after thawing as well as changes in MSC immune suppressive abilities; IFN- $\gamma$  preconditioning effects decreased in MSCs post thaw (Francois *et al.*, 2012). Furthermore, Ginis *et al.* (2012) reported a marked decrease in MSC viability after 1 month in cryostorage; even cryostorage for 1 week could decrease MSC viability from 4 to 8%. In our study, we further analyzed the data from freezing and thawing UC-MSCs to assess the effects cryostorage might have on viability.

## **Methods**

### **Growth Kinetics**

UC-MSCs were isolated using the methods described in Chapter 2 (Smith JR, SCI, 2016). Cells were grown 1 passage after thawing before they were used in the growth kinetics. All cell lines used were from passage 3. Cells were plated at 10,000 cells/cm<sup>2</sup> after passage under 4 different media conditions in 6-well plates (CytoOne 6-well plates, #CC7682-7506).

Media Conditions used:

Control: 1 g/L (low glucose) DMEM (Fisher, cat no. 11-995) with 10% HPL, 4 U/mL heparin, 2 mM GlutaMAX (Fisher, cat no. 35050061) for approximately 6mM glutamine total at start, with an added 1% antibiotic-antimycotic (Fisher, cat no. 15240062).

High glucose: 4 g/L (high glucose) DMEM (Fisher, cat no. 11-885); other components were the same as control.

No GlutaMAX: GlutaMAX was omitted; 4 mM glutamine were added instead of 6 mM; other components were the same as control.

No Antibiotics: Antibiotic-antimycotic was omitted; other components were the same as control.

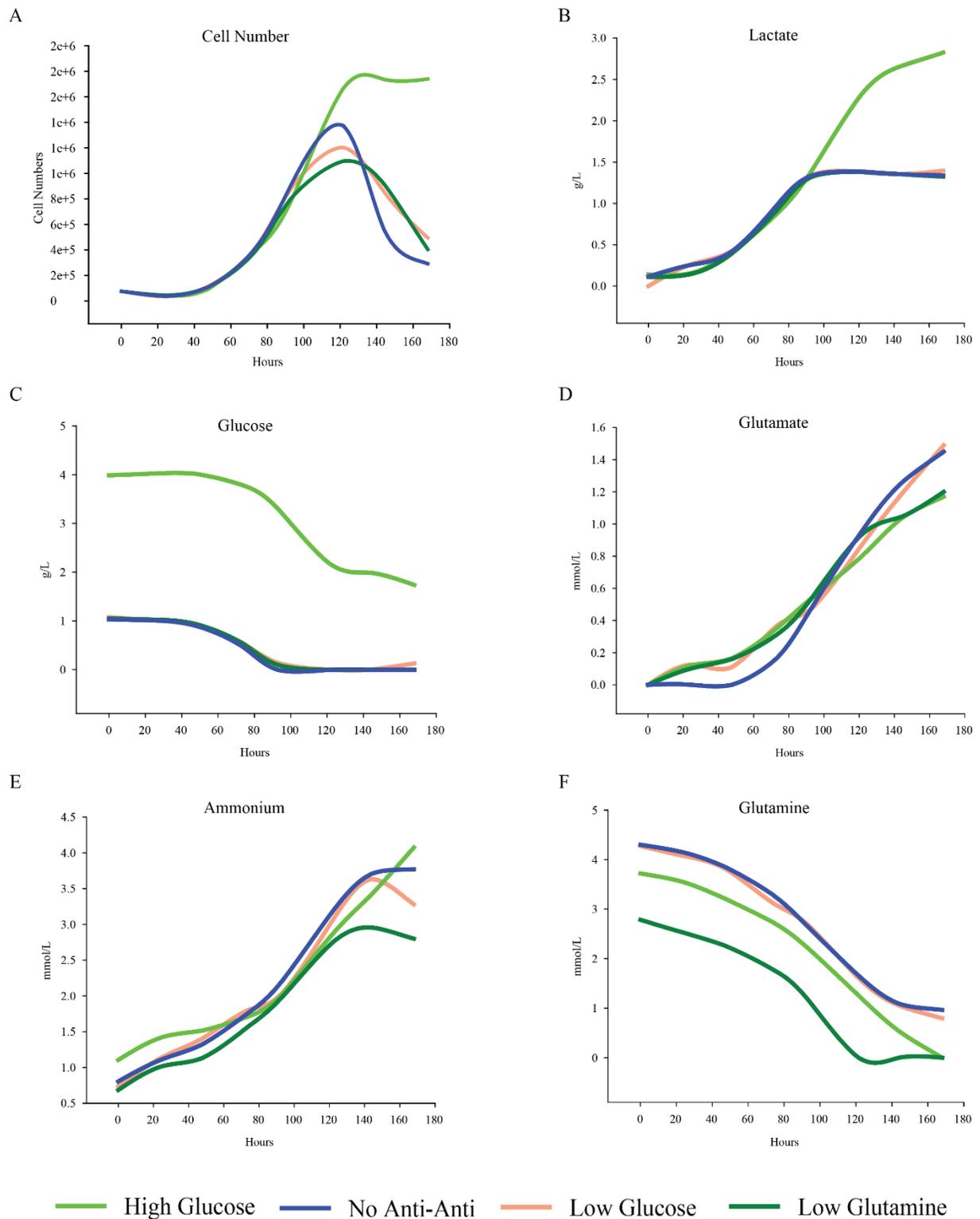
The UC-MSCs were expanded over seven days; samples were taken every day and frozen for later metabolite analysis with a Nova Bioprofile 400. Each day, wells were passaged for each condition, and photographs were taken before passaging. Cell numbers were counted using Nexcelom Auto 2000 and recorded every day, using the protocol described in Chapter 2. Duplicates were run for each line, and data from 2 lines were used here. Growth kinetic experiments were run using cells from passage 3. All UC-MSCs were validated as MSCs as described by Smith *et al.* 2016.

### **Freezing and Thawing**

Passaging and cryopreserving cells required the methods described in Chapter 2. After passage, cells were cryopreserved in a 1:1 dilution of ESfreeze media (GlobalStem GSM-4200) and 10% HPL growth media composed of 10% HPL enriched low glucose DMEM with 4 U/mL heparin, 1% GlutaMAX, and 1% antibiotic-antimycotic. Cells were frozen at passages 3-5. Cryotubes were placed in a Mr. Frosty freezing device and transferred to a -80°C freezer immediately after adding freeze medium. Within 24 hours, the tubes were transferred to vapor phase of a liquid nitrogen tank for long term storage.

Cells were collected after tubes were thawed; tubes were placed up to the middle of the tube into a water bath at 37°C for approximately 1 minute. Cells in their solution were then removed from the cryotube and placed into 3 mL of 10% HPL growth medium prewarmed to 37°C. Care was taken to remove cells from the cryotubes immediately after the last ice crystals had melted. Cells were centrifuged for 5 minutes 200 x g at room temperature. After centrifugation, supernatant was discarded, and cells were resuspended in 1 mL of 10% HPL growth medium. The cells were counted using the protocol described in Chapter 2 using the Nexcelom Auto 2000 and AOPI Viastain. All cell lines were expanded post thaw.

For each individual UC-MSK line tested, several tubes were frozen from the same passage to thaw for later analysis. At least 2 vials were thawed per line analyzed. Up to 5 million cells per mL were frozen in cryotubes (Fisher, 03-337-7D) and cryotubes were never filled over 1.4 mL because of safety concerns.



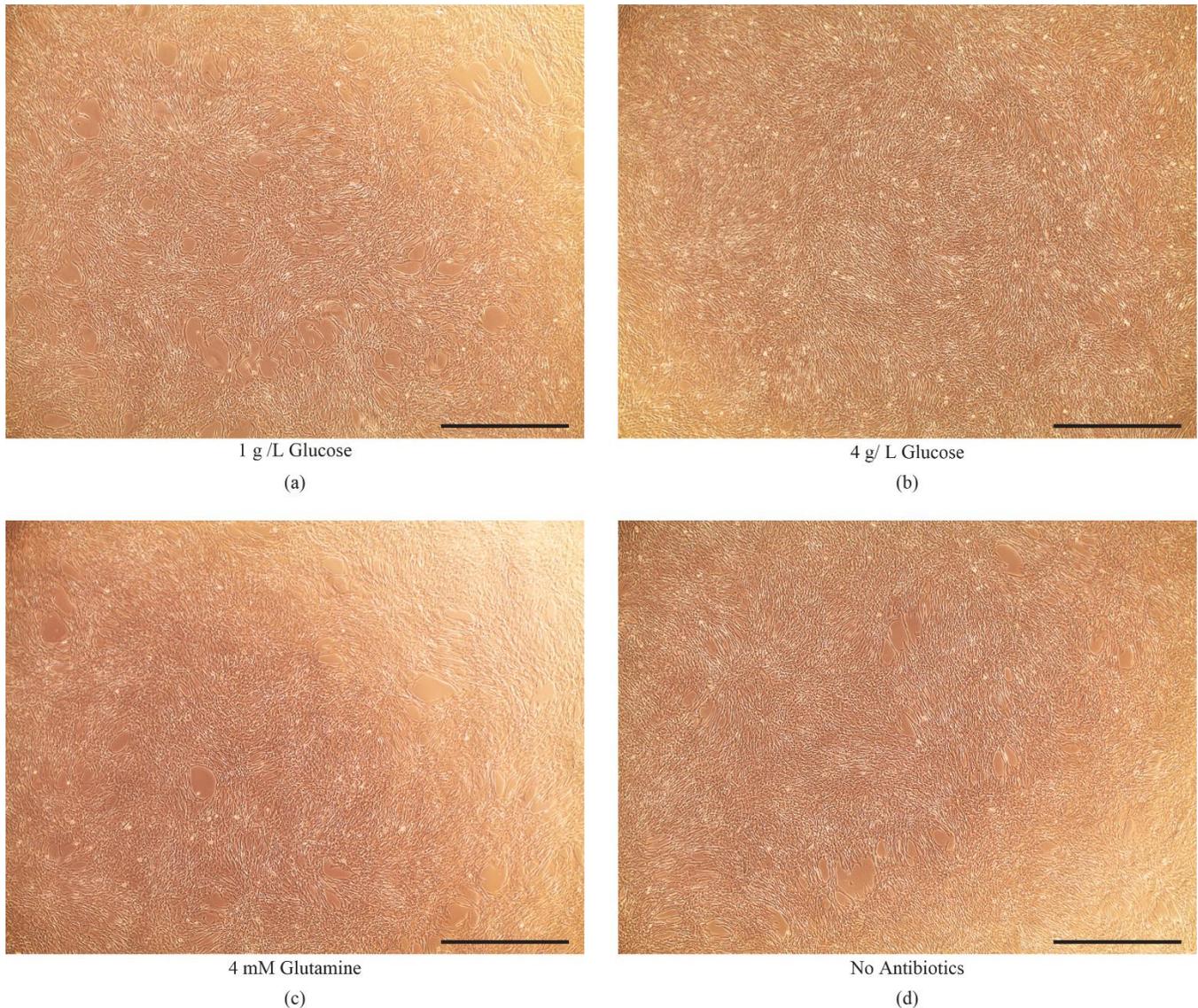
**Figure 3.1 Metabolism characteristics over time** Effect of Different Media Conditions. (A) Cell numbers, (B) Lactate concentration g/L, (C) Glucose concentration g/L, (D) Glutamate concentration mmol/L, (E) Ammonium concentration mmol/L, (F) Glutamine concentration mmol/L. For all conditions, n = 2 (biologic units).

## Results

### Growth Kinetic

The 4 g/L (high) glucose DMEM medium showed more cell growth after freezing and thawing than under other conditions. High glucose had more cells at 120 hours after thawing and could maintain growth in culture for longer; the other three media conditions showed fewer cells after 120 hours (Figure 3.1(a)). This correlated with consumption of glucose in the media and the production of lactate, which is associated with metabolizing glucose (Figure 3.1(c) and 3.1(b)). Lactate production (see Figure 3.1(b)) increased in the medium with 1 g/L (low) glucose until approximately 100 hours; high glucose was the only medium condition where lactate continued to be produced after 100 hours. In Figure 3.2(a, c, d) UC-MSCs started to detach from the plate in the three media conditions with 1 g/L glucose on day 4 (96 hours). The high glucose condition, Figure 3.2(b), cells were confluent at day 4 but did not detach from the plate. Metabolite analysis of the medium shows glucose levels correlated to the ability of UC-MSCs to grow in culture longer. The medium condition with no antibiotic had cell numbers for UC-MSCs comparable to the other media conditions with low glucose DMEM. Without antibiotics, the glutamate production was delayed, and once confluent, the cells without antibiotics detached more quickly from the plates. Glutamine concentration decreased under all media conditions. Only the high glucose and no GlutaMAX conditions used all glutamine by the end of 7 days (168 hours) although the high glucose medium condition had a lower starting glutamine concentration than the other two media conditions with GlutaMAX. Waste products, ammonium, lactate, and glutamate, did not appear to inhibit cell growth during culture over the observation period (Figure 3.1(b, e, f)). Ammonium concentration continued to increase under all media conditions, with the highest concentration in high glucose and lowest in the no GlutaMAX medium

condition (Figure 3.1 (e)). Glutamate, see Figure 3.1 (d), increased in all media conditions over the 7 days (168 hours); low glutamine and high glucose had the lowest final concentrations.



**Figure 3.2 Images of media conditions** Cells at day 4 (96 hours) during the growth kinetic. All images are at 40x magnification, scale bar = 1000  $\mu\text{m}$ . (a) 1 g/L Glucose, standard medium conditions. (b) 4 g/L Glucose, high glucose version of standard medium. (c) 4 mM Glutamine, medium without GlutaMAX. (d) No antibiotics, standard medium without antibiotic-anti-mycotic.

## Viability of Thawed Cells

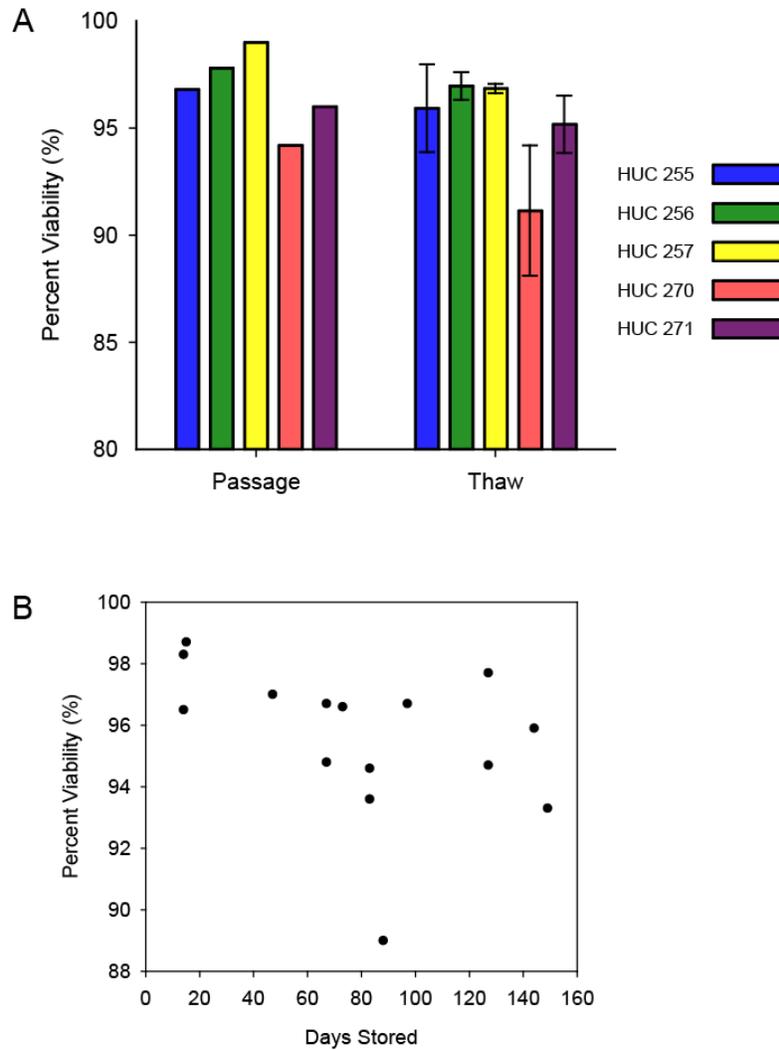
Before freezing cells, Figure 3.3(A) shows their viability averaged 96.9%, and post thaw, viability averaged 95.6%. No cell lines dropped below an average viability of 90% after thawing. The lowest post-thaw viability was found for cell line HUC 270, which correlated with the lowest viability at passage. In Figure 3.3(A), the results for each cell line suggest pre-freezing viability influences post thaw viability. Figure 3.3(B) shows little change for the viabilities of the cells over 150 days; the lone sample below 90% was also from the HUC 270 cells.

## Discussion

Findings here suggest 4 g/L glucose DMEM medium supplemented with 10% HPL provides a foundation for the most growth of UC-MSCs. Cells grown in the other three media conditions, 1 g/L glucose, 4mM glutamine (no GlutaMAX), and no antibiotics exhibited a markedly decreased life span in culture and overall lower cell numbers. GlutaMAX-containing medium provided similar results to the 4 mM glutamine-containing medium, indicating that adding glutamine in the form of GlutaMAX is not necessary to maintain MSC growth in culture. The medium without antibiotic-antimycotic was comparable to the control media with antibiotic-antimycotic, indicating that antibiotics do not affect the growth of UC-MSCs. For growing UC-MSCs, 4 g/L glucose DMEM, 10% HPL, antibiotic-antimycotic with no additional glutamine could be a more effective medium condition although this was not confirmed here and requires further research to validate.

Viability findings suggest the method used for freezing cells in this study was more effective than previously used methods. Seshraeddy *et al.* (2008) analyzed several storage media for freezing UC-MSCs and obtained a viability of 70-80%. Here, viability was > 95% post thaw, higher than expected considering Ginis *et al.*'s (2012) study of the effects of cryostorage on

MSCs. Furthermore, UC-MSCs viability post thaw was maintained even after 150 days in cryostorage. Long-term cryostorage is necessary for advancing cell therapies to the clinic. Ideally, treatment doses could be frozen for later administration to patients with little change in cell number. The long term cryostorage stability of MSCs has not been well-described; therefore, further work is needed to analyze shelf life and stability in the cryostorage. In Chapter 2, MSCs showed that they maintained characteristic surface marker expression after cryopreservation. Together, these results suggest the methods described here can support clinical translation of MSCs.



**Figure 3.3 Cryopreservation per line and over time** (A) Comparison of cells prior to freezing at passage, and after thawing. At least 2 samples were averaged for each cell line. (B) Viability overtime for 15 individual samples.

<sup>6</sup>Figure from *manuscript accepted* Smith, J.R., A. Cromer, and M.L. Weiss. 2017 Human Umbilical Cord Mesenchymal Stromal Cell Isolation, Expansion, Cryopreservation, and Characterization *Current Protocols in Stem Cell Biology* 2017

## Chapter 4 - Discussion

Umbilical cord mesenchymal stromal cells are a promising cellular therapeutic product. Although considerable knowledge has been found by studying MSCs, concerns with GMP-manufacturing exist. Methods that address these concerns are necessary for translating UC-MSCs to the clinic. My goal was to produce an improved method for isolating, expanding, characterizing and cryopreserving UC-MSCs which may be applied for GMP-manufacturing.

Improving the original protocol meant developing methods that could be easily scaled-up for clinical trials, produce more cells, and maintain physiologically beneficial effects. While other groups have focused their research on understanding MSC immunological properties, the variety in the procedures between different laboratories may impair comparison of results. Advancing UC-MSCs towards a cellular therapeutic product requires considerable modification to the method. Here, I developed a reliable method with GMP considerations in mind. In addition, my method produced more UC-MSCs upon initial isolation and an improved expansion rate.

The new methods for isolating and expanding UC-MSCs address some challenges to clinical translation, but questions still remain. For example, it is unclear whether vaginal births resulted in a higher MSC yield than Caesarean-section births upon initial isolation from the umbilical cord. Originally, my work suggested a difference. However, after including more samples, the variability decreased and no differences between groups were found (see supplemental Table A.3). The improved isolation method provides the necessary cell yield for banking and uses a closed system. Using the Miltenyi C-tubes allows the method to be scaled-up easily. Moreover, the expansion medium supplemented with 10% HPL improved the growth rate of MSCs. These improvements may support GMP manufacturing of UC-MSCs. To

completely validate this method, functional testing for immune modulation or regenerative potential testing is needed. Emmett *et al.*'s research supports the use of Miltenyi C-tubes to isolate MSCs to comply with GMP demands, but their protocol does not use enzymatic digestion (Emmett *et al.*, 2016). My method uses enzymatic digestion and mechanical dissociation. The method also produced a higher percentage of viable MSCs after freeze /thaw and increased the number of MSCs that can be frozen per vial. The increased percentage of viable cells after thawing as well as after storing cells more than 150 days lends credibility to a scaled up version of this method for clinical production.

Traditional MSC expansion uses medium supplemented with fetal bovine serum (FBS). Recently, several studies have reported that MSCs grow more efficiently in HPL supplemented media. Some studies have even suggested that HPL could replace FBS as the primary supplement for human cell expansion, eliminating xenogeneic material concerns (Burnouf *et al.*, 2016). One potential issue is that HPL enriched media it that it may negatively affect the immunosuppressive properties in MSCs (Abdelrazik *et al.*, 2011). Further research of UC-MSCs to understand growth characteristics and *in vitro* properties is warranted.

UC-MSCs, as isolated and expanded in this study, have also been studied in 3D spinner flask models (Petry *et al.*, 2016). Our preliminary work used spinner flasks from 100 mL up to a 3L disposable bioreactor system (Petry *et al.*, 2016). This shows that UC-MSCs maintain their characterization after 3D growth and that optimization for scale-up in a bioreactor model is feasible. Furthermore, potential treatment doses as suggested by Jung *et al.* could be generated using the improved method developed in this study in combination with the bioreactor model (Jung *et al.*, 2012).

UC-MSCs from the protocol described in Chapter 2 have been used as a treatment *in vitro* and *in vivo* for abdominal aortic aneurism (AAA)(Sharma *et al.*, 2016). In this medical condition, the aortic wall degrades because of inflammation, causing high mortality in males. Sharma *et al.* showed UC-MSCs could suppress proteins associated with AAA, including high mobility group box 1 (HMGB1), matrix metalloproteinases (MMP) 2, and MMP9 as studied in human aortic tissue *in vitro* and *in vivo* mouse models. Furthermore, UC-MSCs decreased inflammatory effects in AAA and suppressed activation of CD4<sup>+</sup> T-cells and macrophages. These results show the utility of UC-MSCs isolated, expanded, and characterized using the methods described in Chapter 2.

Several other preclinical models have also evaluated the use of UC-MSCs including graft-versus-host disease as an allogeneic therapy (Guo *et al.*, 2011), inflammatory bowel disease (Lin *et al.*, 2015), myocardial infarction (Lopez *et al.*, 2013), and Parkinson's disease (Weiss *et al.*, 2006). These studies demonstrate UC-MSCs can be used to treat several conditions. Furthermore, bioreactor and spinner flask expansion using micro-carriers with cells shows that the method can be scaled up, suggesting these cells can be made available for clinical applications. The research described here improves production of UC-MSCs while addressing several compliance concerns, thus improving the potential use of the cells in clinical applications.

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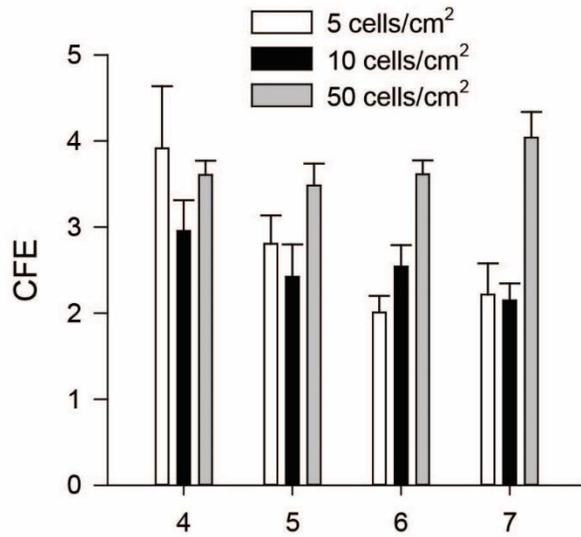
## Appendix A - Supplemental Figures and Tables

**Table A.1 surface marker expression data for six isolates**

	<b>248</b>	<b>255</b>	<b>256</b>	<b>257</b>	<b>260</b>	<b>262</b>	<b>Average</b>
CD44	99.47	99.5	98.76	98.03	99.91	99.84	<b>99.26% ± 0.30</b>
CD73	99.19	98.1	98.06	99.29	99.01	99.20	<b>98.80% ± 0.24</b>
CD90	99.32	97.9	99.48	99.70	99.68	99.86	<b>99.32% ± 0.30</b>
CD105	99.30	97.9	97.54	99.36	99.19	99.36	<b>98.77% ± 0.34</b>
Negative Cocktail	0.05	0.17	0.51	0.22	0.03	0.04	<b>0.17% ± 0.08</b>

**Table A.2 Comparison of frozen and never frozen isolates for surface marker expression.**

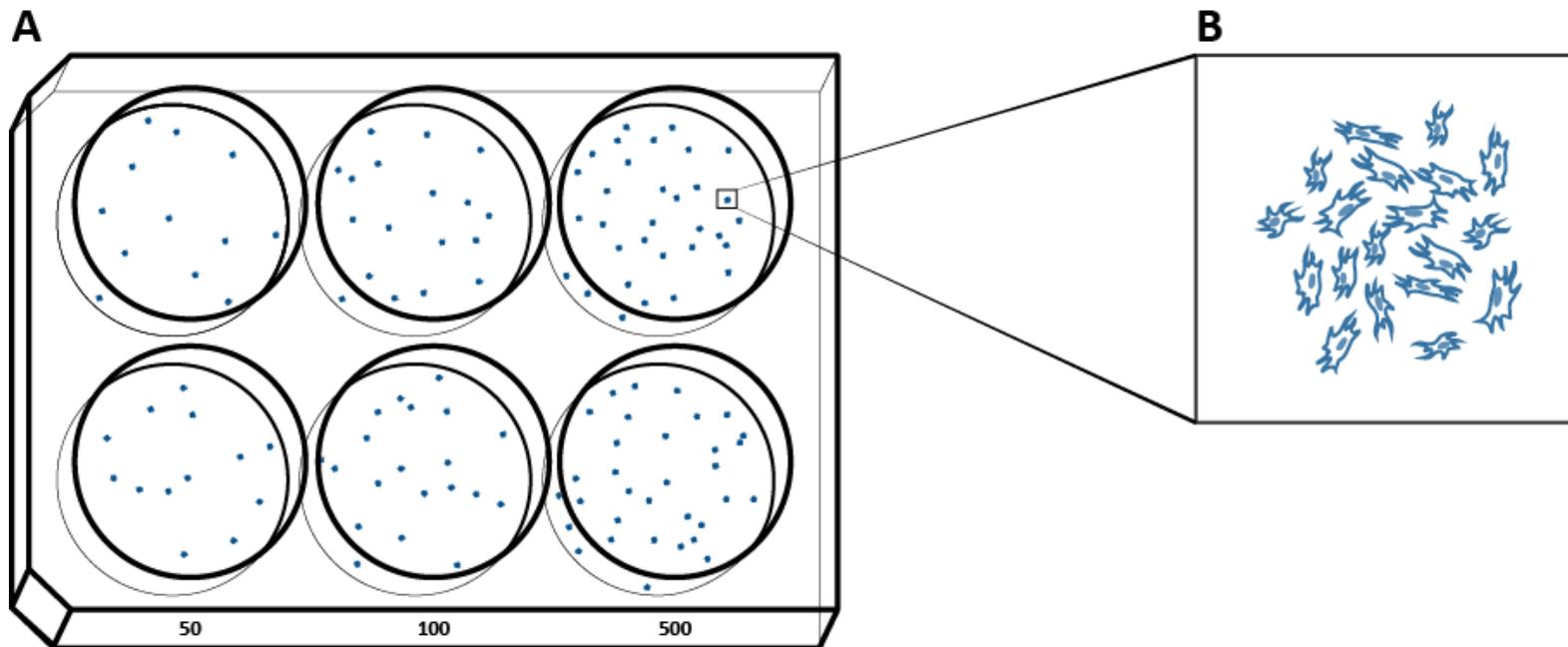
	<b>255</b>	<b>256</b>
CD44	99.73%	N/A
CD73	99.89%	99.82%
CD90	98.54%	99.82%
CD105	99.47%	98.99%
Negative Cocktail	0.05%	0.04%



**Figure A.0.1 CFU-F plating condition and time** CFU-F expression of UC-MSCs evaluated at different times after plating. Based upon this experiment, day 6 at 5 cell per cm<sup>2</sup> yields the highest colony forming efficiency.

**Table A.3 Umbilical Cord Averages per variable** Representation of the umbilical cord isolation averages for the protocol; total number of 37 umbilical cords used.

	<b>Number Cords</b>	<b>Ave. Cord Weight(g)</b>	<b>Ave. Viability</b>	<b>Ave. Total Cells</b>	<b>Ave. Live cells</b>	<b>Ave. Tube Weight(g)</b>	<b>Ave. Live size <math>\mu\text{m}</math></b>	<b>Ave. Dead size <math>\mu\text{m}</math></b>	<b>Cells per Gram</b>	<b>Live Cells per cord</b>
<b>Cesarean</b>	<b>27</b>	<b>62.2</b>	<b>64.6%</b>	<b>4.00 x10<sup>5</sup></b>	<b>2.56 x10<sup>5</sup></b>	<b>1.4</b>	<b>13.9</b>	<b>6.6</b>	<b>1.83 x10<sup>5</sup></b>	<b>1.11 x10<sup>7</sup></b>
<i>Standard Error</i>		$\pm 4.4$	$\pm 1.9\%$	$\pm 3.63 \times 10^4$	$\pm 2.61 \times 10^4$	$\pm 0.1$	$\pm 0.4$	$\pm 0.1$	$\pm 1.63 \times 10^4$	$\pm 1.33 \times 10^6$
<b>Vaginal</b>	<b>10</b>	<b>58.4</b>	<b>62.3%</b>	<b>5.13 x10<sup>5</sup></b>	<b>3.00 x10<sup>5</sup></b>	<b>1.4</b>	<b>13.8</b>	<b>6.5</b>	<b>2.02 x10<sup>5</sup></b>	<b>1.23 x10<sup>7</sup></b>
<i>Standard Error</i>		$\pm 4.9$	$\pm 4.3\%$	$\pm 7.85 \times 10^4$	$\pm 4.32 \times 10^4$	$\pm 0.1$	$\pm 0.3$	$\pm 0.2$	$\pm 2.38 \times 10^4$	$\pm 1.89 \times 10^6$
<b>High Enzyme</b>	<b>14</b>	<b>64.3</b>	<b>67.5%</b>	<b>4.71 x10<sup>5</sup></b>	<b>3.15 x10<sup>5</sup></b>	<b>1.5</b>	<b>13.9</b>	<b>6.6</b>	<b>2.04 x10<sup>5</sup></b>	<b>1.38 x10<sup>7</sup></b>
<i>Standard Error</i>		$\pm 5.0$	$\pm 2.6\%$	$\pm 6.21 \times 10^4$	$\pm 4.38 \times 10^4$	$\pm 0.1$	$\pm 0.3$	$\pm 0.1$	$\pm 2.43 \times 10^4$	$\pm 2.34 \times 10^6$
<b>Low Enzyme</b>	<b>23</b>	<b>59.2</b>	<b>61.9%</b>	<b>4.06 x10<sup>5</sup></b>	<b>2.40 x10<sup>5</sup></b>	<b>1.4</b>	<b>13.8</b>	<b>6.6</b>	<b>1.78 x10<sup>5</sup></b>	<b>9.94 x10<sup>6</sup></b>
<i>Standard Error</i>		$\pm 4.6$	$\pm 2.3\%$	$\pm 4.06 \times 10^4$	$\pm 2.28 \times 10^4$	$\pm 0.1$	$\pm 0.4$	$\pm 0.2$	$\pm 1.58 \times 10^4$	$\pm 9.33 \times 10^5$
<b>Female</b>	<b>18</b>	<b>56.7</b>	<b>65.1%</b>	<b>3.81 x10<sup>5</sup></b>	<b>2.46 x10<sup>5</sup></b>	<b>1.3</b>	<b>13.6</b>	<b>6.5</b>	<b>1.86 x10<sup>5</sup></b>	<b>1.08 x10<sup>7</sup></b>
<i>Standard Error</i>		$\pm 5.6$	$\pm 2.1\%$	$\pm 5.30 \times 10^4$	$\pm 3.66 \times 10^4$	$\pm 0.1$	$\pm 0.5$	$\pm 0.2$	$\pm 2.16 \times 10^4$	$\pm 1.97 \times 10^6$
<b>Male</b>	<b>19</b>	<b>65.3</b>	<b>62.9%</b>	<b>4.77 x10<sup>5</sup></b>	<b>2.89 x10<sup>5</sup></b>	<b>1.6</b>	<b>14.1</b>	<b>6.7</b>	<b>1.90 x10<sup>5</sup></b>	<b>1.20 x10<sup>7</sup></b>
<i>Standard Error</i>		$\pm 4.0$	$\pm 2.9\%$	$\pm 4.28 \times 10^4$	$\pm 2.62 \times 10^4$	$\pm 0.1$	$\pm 0.3$	$\pm 0.1$	$\pm 1.68 \times 10^4$	$\pm 1.03 \times 10^6$
<b>All Cords</b>	<b>37</b>	<b>61.2</b>	<b>64.0%</b>	<b>4.31 x10<sup>5</sup></b>	<b>2.68 x10<sup>5</sup></b>	<b>1.4</b>	<b>13.8</b>	<b>6.6</b>	<b>1.88 x10<sup>5</sup></b>	<b>1.14 x10<sup>7</sup></b>
<i>Standard Error</i>		$\pm 3.3$	$\pm 1.7\%$	$\pm 3.26 \times 10^4$	$\pm 2.12 \times 10^4$	$\pm 0.1$	$\pm 0.3$	$\pm 0.1$	$\pm 1.27 \times 10^4$	$\pm 1.03 \times 10^6$



**Figure A.0.2 Method for analyzing CFU-F** A) Example of the colony forming unit fibroblast plate after staining cells with three different concentrations of cells per well: 50, 100, and 500. (B) Diagram of a magnified colony