STEM CELLS IN REGENERATIVE MEDICINE

by

JENNIFER LYNN MUMAW

(Under direction of Dr. Steve L. Stice)

Stem cell therapeutics offer novel treatments for disease and tissue injury that cannot be achieved by other means. As these therapeutic are developed, validating correct developmental progression, optimizing patient compatibility and applicability for use in clinical use are vital for success. Here we show the progression of two developing therapies, one for neural deficits and one for bone formation, in their various stages of development. Both of these therapeutics offer hope for very different disorders, and progression to the clinic presents unique hurdles due to the complex nature of their role in healing the patient's body. We present some of the aspects of these potential therapies as they progress to becoming a viable treatment.

Pluripotent cell derived neural cell therapy offers the potential aide in the treatment of multiple neurodegenerative disorders, however complexities involved in treating these diseases require thorough examination and testing before cellular therapies can be used. ESC derived neural progenitor differentiation into neurons showed structural changes similar to what is seen in the developing nervous system *in vivo.* Additionally, using neural cells for therapy requires that the cells integrate into the patient's body to treat the disease, and generating cells that are compatible with the patient's immune system will prevent the need for lifelong

immunosupressive treatments. Here we show that blood cells transduced with POU5F1/OCT4 were capable of generating cells of all germ layers and differentiated neurons.

In a vastly different therapeutic for bone regeneration, genetically modified mesenchymal stem encapsulated in a poly (ethylene glycol) allows the use of a biological barrier to prevent the cells from migrating away from the site of injury and protect the encapsulated cells from the patient's immune system. Here we develop a method for cryopreserving this therapeutic in a "ready to use" format that can facilitate the distribution to centers for use in animal models and in the future, to the clinic.

Stem cell therapies offer the opportunity to provide unique treatments for many needed diseases. Optimizing efficacy and safety will allow for the best potential outcome as the stem cells cross the bridge between a potential therapeutic and effective treatment.

INDEX WORDS: Embryonic stem cells, induced pluripotent stem cells, ultrastructure, partially reprogrammed stem cells, differentiation, neural development, mesenchymal stem cells, bone formation, poly (ethylene glycol) encapsulation, cryopreservation, BMP-2, POU5F1/OCT4

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DEDICATION

I would like to dedicate this work to my parents, Brian and Carol Mumaw, my brother William Bradley Mumaw and to my grandparents Dorothy and Maynard Mumaw, and Helen and William Earl Redfern. Thank you all for indulging me in my desire to pursue research.

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CHAPTER 1

INTRODUCTION

Cells are the essence of life and make a person who and what they are. Personality, behavior and physical appearance can all be attributed to the unique characteristics of cells and connections between cells. Every life begins with a single totipotent cell that is capable of developing into the trillions of cells that are found in the adult body. In normal daily life cells are lost through programmed cell death and replaced through regeneration of proliferating nascent stem cells. The loss of this normal balance in the cellular life cycle is implicated in many disorders ranging from Parkinson's Disease, Amyotrophic lateral sclerosis, Alzheimer's disease (recently reviewed by [1]), autoimmune disorders [2], to diabetes [3-4]. The belief behind this piece of work is that therapy designed using cells or through the transplantation of cells can generate more effective treatments and cures for debilitating disorders resulting from cellular deficits caused by disease and injury.

Cellular therapy holds new promise for disease treatments as is seen with the sensational headlines in mainstream news circles. Stem cell discoveries have been acclaimed to be able to cure hearing problems [5], restore vision [6], replace burned skin [7], treat spinal cord injury [8] and alleviate the effects of macular degeneration [9]. The words "stem cells" provide hope for a better life for many individuals, and the media touts the phrase to ellicit an enthusiastic response from the public about progression in regenerative medicine. However, most discoveries

presented by the media are far from ready for clinical use and still require careful testing for safety and effectiveness. As these therapies develop, stringent validation must be employed before taking these cells into the clinic.

Currently the NIH has close to 3,500 listed stem cell therapies in clinical trials (clinicaltrials.gov). The majority of these clinical trials involve multipotent/adult stem cells. These stem cells are formed during development and are retained from fetal to adult life. While they are more lineage restricted than embryonic stem cells, in that they can only form a few cell types when differentiated, these cells have great potential in regenerative medicine. Hematopoietic stem cells and mesenchymal stem cells (MSCs) are two of the most commonly used multipotent stem cells for regenerative medicine. Hematopoeitic stem cells, or blood precursor stem cells, have become common place in hospitals for the treatment of blood and immunodeficiency disorders [10-13]. Increased interest in MSCs has led to multiple clinical trials testing their ability to be an immune modulator as well as a regenerative cell type [14-16]. Additionally MSCs also are potential vehicles for genetic engineering with their allogeneic tolerability [17] and ability to be easily manipulated by viral transduction [18]. Combining the lineage differentiation capacity of MSCs to form bone, cartilage and adipose, with genetic engineering can provide new therapeutics for treating skeletal and cartilage deficits. Multipotent stem cells hold great promise in regenerative medicine; however, their lineage restricted development and limited supply, both within the human body and for cellular treatments, puts restrictions on the potential of these cells for treating many diseases. Using pluripotent cell types which have greater expansion and differentiation potential can provide additional treatments which are unavailable through use of multipotent stem cells

While adult stem cells are the most common stem cells in clinical trials, three clinical trials using human embryonic stem cell derived cells for spinal cord injury and two trials for macular degeneration have been approved [19]. The embryonic stem cells [20] and the genetically engineered induced pluripotent stem cells [21-23] are capable of prolonged expansion and generating all cell types found within the body. Induced pluripotent stems are the most recently discovered pluripotent cell type and provide the unique opportunity to generate an unlimited source of cells genetically identical to the person from which the cells were harvested. The established capabilities of embryonic stem cells as an *in vitro* model for human development will continue to provide a standard that all pluripotent cell types can be compared against. While this discovery of induced pluripotent stem cells is still in the early stages, significant strides are being made in this field. The impact of this discovery on regenerative research is evident with nearly 19,000 publications arising using the key word "induced pluripotent stem cells" within the last 5 years. As future strides in this field are made, the most important goal is to make the cells as representative of cells found in the body during normal development. This will provide the safest and most efficacious treatments as possible. This work is composed of three separate sections devoted to the progression of regenerative therapeutic to the clinic. Each section will focus on a different aspect of a developing therapeutic treatment in promoting its progression to becoming a viable therapeutic.

Chapter 3 compares human embryonic stem cells and derived developing neural cells to *in vivo* counterparts, establishing that *in vitro* culture conditions for neural development can replicate *in vivo* development. Using embryonic stem cells and their derived cells for *in vitro* experiments and transplantation requires that they closely resemble the corresponding cells in the

body and this chapter looks at similarities and differences of the *in vitro* culture system and developing embryo.

Chapter 4 will further the efforts of the previous chapter in the pursuit of methods to generate neural cells, but it will focus on generating neural cells from alternate cell sources. Peripheral blood mononuclear cells are the most attractive cell type for generating patient specific stem cells with regards to patient discomfort and safety. This study will look at the use of genetic engineering of cells isolated from peripheral blood using only POU5F1/OCT4 to create partially reprogrammed cells. It will also show that derived cells were capable of giving rise to all three germ layers, including differentiated neurons.

Chapter 5 will examine a method for making a developing treatment applicable for clinical settings. The combination of stem cell technologies, genetic modifications and biomedical engineering allows for the rapid generation of bone. The use of a potential osteoinductive protein, BMP-2, to illicit rapid bone formation when produced by MSCs in a biocompatible gel provides a unique ways to treat bone defects. However, one roadblock in progressing to the clinical setting is making the therapeutic readily available. This chapter describes the use of cryopreservation to preserve the genetically modified encapsulated cells for immediate availability in clinical settings.

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CHAPTER 2:

LITERATURE REVIEW

Stem cells offer hope to many people suffering from debilitating diseases that currently have no effective cure. The usefulness of these cells is dynamic with the ability to be a cellular replacement therapy and a model for human development and disease. Stem cells come in multiple different types, but this review will focus on embryonic stem cells (ESCs), pluripotent stem cells (iPSCs) and multipotent stem cells: ESCs are pluripotent cells that have been isolated from the blastocyst and can give rise to all 220 cell types found in the human body; iPSCs are somatic cells that have been turned into ESC like cells through genetic manipulation, also capable of giving rise to all 220 cell types in the body; Multipotent stem cells, or adult stem cells, are found throughout the body after embryonic development, often in stem cell niches, and are important in tissue regeneration. Each of these types of stem cells offers great opportunities for scientific research and therapeutic development and this review will cover the contributions that these cells have made thus far to regenerative medicine and the methods employed for increasing their therapeutic potential.

Embryonic stem cells:

The isolation and culture of the pluripotent cells from embryos remained an elusive feat until 1981 when two groups successfully designed a culture system to support these enigmatic

cells from the mouse embryo [1-2]. To establish the mouse embryonic stem cell (mESC) lines, cells were isolated from the inner cell mass (ICM) in blastocyst stage embryos and cultured on mitotically inactivated mouse embryonic fibroblast (iMEF) feeder cells with serum [1-2]. Understanding the pluripotent state began with the discovery of factors that allowed for the maintenance of an undifferentiated phenotype in continued culture. The first factor discovered to have a pivotal role for maintaining mouse pluripotency was mouse Leukemia Inhibitory Factor (LIF) [3-4]. As researchers advanced to isolating human embryonic stem cells (hESCs) it was quite perplexing that factors initially recognized as a pivotal point in the maintenance of mECS showed no beneficial effect in generating hESCs from the ICM of human blastocysts [5-7]. The lack of knowledge of which factors could maintain pluripotency in hESC delayed the establishment of cell lines and is still a perplexing issue for researchers in generating cell lines from additional species.

The first mESCs and future derived lines demonstrated the characteristics that have come to define embryonic stem cells; they are capable of giving rise to all three germ layers: endoderm, ectoderm, and mesoderm both *in vitro* in embryoid bodies and in teratomas when injected into immune compromised mice, they can be cultured long term while maintaining a normal karyotype [1-2] through expression of telomerase [5], and in nonhuman lines can form chimeras with germ line transmission [8]. These key features of mECSs have allowed for their integral role in studying human diseases.

The use of animals in research for modeling human diseases began in 1916 [9] and the use of animals as model organisms for development and disease has been crucial for the progression of research. The discovery of mouse embryonic stem cells in combination with new mammalian gene altering technologies [10-12] led to the first gene knockout mice [13-15]. These

advances in biomedical research allowed for the formation of mice which modeled human diseases including atherosclerosis [16-18], hypertension [19], Lesh-Nyhan [20], elevated cholesterol levels [17], cardio myopathy [21] and cystic fibrosis [22-23] which were significant in generating groundbreaking discoveries in the role of genetics behind these diseases. These mice have come to be indispensible models for finding novel treatments and testing possible treatments, such as novel compounds and cell therapy, to ameliorate disease symptoms.

Isolation of Human Embryonic Stem Cells:

Despite the groundbreaking discoveries in mice, it amazingly took another 18 years after the isolation of mESCs for the first isolation of hESCs in 1998 [5]. This lag in the time between the discovery of mESC and hESC highlights the intricate differences between species in early development with the need to tailor culturing methods to suit hESCs. The first hESCs were also cultured on iMEFs, but they required the addition of a different growth factor to maintain pluripotency than that of mESCs, Basic Fibroblast Growth Factor (bFGF) [5].

As described by Thompson et al. 1998, the use of bFGF proved to be vital in establishing culture conditions for hESCs. Unlike the mouse, LIF did not help to maintain pluripotency of hESCs and the addition of BMPs initiated differentiation to the trophoblast [24]. Additional signaling factors activin and Nodal have also been recognized as being involved in the maintenance of pluripotency in humans [25] and playing a role in mice in mESC propagation [26]. Even though the factors and pathways involved in the maintenance of pluripotency have exhibited some differences (recently reviewed [27]) the core pluripotency genes activated by these pathways are the same.

Generating Neural Cell Types From Embryonic Stem Cells:

While there are numerous diseases that can be treated with cell replacement therapy (see below), neural replacement therapy holds promise for degenerative diseases that have no cure such as Parkinson's disease, Huntington's disease, amyotrophic lateral sclerosis, Alzheimer's disease, multiple sclerosis, stroke, and spinal cord injury, both as a cell therapeutic and a model for human neural development. ESCs derived neural cells can provide an important system for understanding and preventing the progression of these diseases (recently reviewed [28]). As with many organs, the regeneration capacity of the brain is limited despite the presence of proliferative stem cell populations [29-30]; Embryonic stem cells can provides an unlimited source for generating neural cells to overcome the lack of available primary tissue.

Differentiation of ESCs to neural cell types requires that the cells progress through a neural stem cell state [31]. Neural cells first begin developing in rosette structures, resembling the developing neural tube *in vivo* [32-33]. The cells isolated from neural rosettes can be expanded through growth factor mediated propagation allowing generation of sizable populations without differentiation [34]. This population expresses markers that distinguish them from ESCs and neurons. Embryonic stem cells express pluripotent transcription factors Oct4, Sox2 and Nanog [35], neural stem cells retain Sox2 expression [36] but also express Musashi [37], and the immature marker Nestin [38], while maturing neural cells express Tuj [39], and MAP2[40]. Analysis of cells examining protein and gene expression is valuable in understanding the differentiation state of the cells, but when it come to functionality of mature neurons, other methods need to be employed. Structural analysis can provide a unique technique for analyzing cultures.

Neural cells have specific structures that allow communication between cells. Functional neurons are capable of sending and receiving signals through specialized structures called synapses [41]. Recording neural transmission using patch clamp [42] or microelectrode arrays [43] coupled with fine structure analysis [44] can provide detailed information in understanding the signal transmission of these cells. Fine structure analysis can also verify that the structural formation of the pre and post synaptic clefts are present and that vesicles are available for neural transmission[44]. As low functionality or lack of transmission may be due to lack of factors necessary for synaptic development (recently reviewed [45]), ultrastucture can also be used to examine the effects of growth factors and morphogens on organelle development in order to examine the progression of neural differentiation. The development of neurons can be characterized through formation of neurofilaments, high ribosomal presence and mature mitochondria [46-47] allowing for comparisons between synaptic structure formation and intracellular development. While standard ultrastructure analysis is time consuming and low throughput, recently described techniques such as neurocartography [48] and array tomographic immunoflourescence [49] enable larger, more efficient sample analysis. Combining functionality testing, ultrastructure, protein expression and gene analysis provides a more complete picture of the neural development from ESCs.

Progression of Embryonic Stem Cells in Cell Therapy:

The ability of ESCs to generate all the tissues found in the body makes them an attractive method for therapeutics involving cellular regeneration. The cells can be differentiated to ectoderm which makes up the skin [50] and nervous system [32], to the endoderm that consists of the visceral organ such as pancreas [51] and liver [52] and to the mesoderm that makes up the

muscle, bone and blood [53]. There is currently a large discrepancy between the number of people that need organs and the number of organs that are available [54], with 18 people dying every day while waiting for an organ [55] making alternatives for treatments of high priority.

ESCs have long been acknowledged for their potential to treat many diseases through cell transplantation. Table 1 lists some of the possible treatments that can become available through cells derived from pluripotent cells. These treatments have shown potential in initial pre-clinical trials by establishing their ability to exhibit function *in vitro* or generate partial recovery in *in vivo* models. While the development of most of these potential therapeutics is still a long way from being applicable in humans, clinical trials of ESC trials have started with the first patient receiving ESC derived oligodendrocytes in a spinal injury [56-57] and an additional two approvals using ESCs derived retinal pigment epithelial cells will begin in clinical trials for macular degeneration [58-59]. Now that the first ESC trials have begun, the world waits: beginning this new form of treatment will answer some of the lingering questions scientists have about the safety and efficacy of creating implants of cells derived from pluripotent cells. If these first trials prove that ESC derived cells can integrate into the patient's body without forming aberrant growth or tumor formation it could open the door to the realm of possibilities that ESCs offer.

Isolation of Embryonic Stem Cells from Species Important in Biomedical Research:

The use of animals in biomedical research is vital to the understanding of diseases through the use of gene knockouts and generation of tissues for xenotransplantation (recently reviewed [81]), however the progression of ESC culture has proven to be limited

Table 1: Progression of human embryonic stem cell therapies *in vitro* and *in vivo:*

Disease	Cell Type Generated	Reference
Insulin Dependent Diabetes	Insulin Responsive Pancreatic Cells	[60-61]
Crushing Spinal Cord Injury	Oligodendrocytes	[62-63]
Severed Spinal Cord Injury	Motor Neurons/ Oligodendrocytes	[64]
Alzheimer's Disease	Cholinergic Neurons	[65]
Deafness	Sensory Hair Cells	[66]
Liver Disease	Hepatocytes	[67]
Blood Transfusion	Red Blood Cells	[68-69]
Skin Damage	Keratinocytes	[70-71]
Stroke	Neural Cells	[72]
Transfusion	Platelets (Megakaryocytes)	[73]
Cardiac Infarction Injury	Cardiomyocytes	[74]
Lung Damage	Lung Epithelium	[75]
Joint Damage	Cartilage	[76]
Macular Degeneration	Retinal Pigment Epithelial Cells	[77]
Cancer	Natural Killer Cells	[78]
HIV-1	Natural Killer Cells	[79]
Memory due to radiation damage	Undifferentiated hESC Cells	[80]

in many species; currently bona fide ESC lines have only been established in primates and rodents [5, 82-84]. While there has been progression in multiple species such as sheep [85-86], pig [87-88], cow [89-92], horse [93-96], dog [97-99], cat [100-101], rabbit [102-105], hamster [106], mink [107], and chicken [108-110], they all fail to meet the hallmarks of embryonic stem cells. Reports in these lines show only partial characteristics that define ESCs. The cells isolated from the ICM of various species are often lacking the evidence they are capable of forming chimeras with germ line transmission. This limits their use in creating biomedical knockout models and for generating xenotherapeutic tissues. While there are many facets both to individual species isolation of ESCs and culture conditions, further understanding of the transcription factors involved in the establishment and maintenance of these cells will aid in the development of proper technique for developing ESCs from these elusive species.

Induced Pluripotent Stem Cells:

Differentiation once started during development was believed to be a one way process except in the case of cell fusion and somatic cell nuclear transfer [111-113]. Discoveries by Yamanaka were able to shift this paradigm. Starting with 24 prospective genes known to play a role in pluripotency maintenance or induced long term proliferation (see below), 4 factors were isolated that when transduced into a somatic fibroblast could initiate an ESC like state [111]. These factors, POU5F1/OCT4, SOX2, KLF4, and C-MYC were the mouse cellular proverbial fountain of youth. Further research showed that the same effect could be applied to human fibroblasts using POU5F1/OCT4, SOX2, KLF4, and C-MYC [114], or POU5F1/OCT4, SOX2 NANOG and LIN28 [115] to generate induced pluripotent stem cells (iPSCs). As more and more cells came to the state of pluripotency it was discovered that cells which maintain an endogenous

expression of the pluripotency genes could be reprogrammed with less exogenous factors [116]; Cells can be reprogrammed with only POU5F1/OCT4 and SOX2 being added [114-115, 117-120] and neural stem cells, which endogenously express KLF4 and high levels of SOX2 and c-MYC, can be reprogrammed with only POU5F1/OCT4 [121]. These studies report that while the additional factors are not necessary they increased the efficiency of the process.

While NANOG is one of the "core" pluripotent transcription factors, NANOG has been found to be dispensable for the initiation of the reprogramming process; however, it is vital for the maintenance of pluripotency [122] and has been proven to play a vital role in reprogramming when doing somatic-ES cell fusion [123]. While NANOG may not be important for initiation, it is vital for completion of a pluripotent state in reprogrammed cells and in its absence cells can only be partially reprogrammed [111, 124]. Selecting for NANOG expression has been shown to allow for more efficient chimerism with pluripotent cells and increases the incident of germline transmission [125]. This requirement for NANOG further underscores this gene as a "core" member of pluripotency factors.

This method for generating iPSCs allows for the creation of patient specific pluripotent stem cells without the complications of ESC and the moral issues regarding embryo destruction, but these cells currently still fall short of ESCs. While some variation between lines of ESCs has been noted, greater variation between iPSC lines in respect to their characterization[126] and differentiation potential [127] has been reported, with some derived cells being at risk for early senescence [128]. Chromosomal instability has been noted in iPSCs with a belief that selective pressure in reprogramming increases the risk of aneuploidy [129]. Additionally, iPSCs are subject to incomplete epigenetic reprogramming [130], mutations in proto-oncogenes [131], and tumor suppression genes [132] with increased risk of copy number variations, particularly at

early passages [133]. While these discrepancies will require that all lines are thoroughly examined before the cells head toward the clinic, their current use in therapeutic discoveries is still bringing progress.

Core Transcription Factors Involved in Maintaining Pluripotency:

The remarkable discovery of induced pluripotent stem cells resulted from information gathered through the regulation of pluripotency from embryonic stem cells. Through transcriptional profiling, transcription factors that are involved in the maintenance of pluripotency can be examined with LIN28, KLF4, POU5F1/OCT4, SOX 2, NANOG, c-MYC and NODAL being actively transcribed in ESCs [134]. Three of these transcription factors have been identified as "core": NANOG, SOX 2 and POU5F1/OCT4 demonstrate both regional and temporal expression in the totipotent cells of the ICM [135-139]. These "core" transcription factors are kept in a careful balance of expression though the positive synergistic and/or autoregulation of the "core" pluripotent factors [140-141]. POU5F1/OCT4 and SOX2 and NANOG are capable of regulating pluripotency through gene transcription by binding to the promoters of developmentally important homeodomain proteins [140]. Although, while all three factors are required for pluripotency, NANOG has been identified as the key factor regulating the establishment of the pluripotent epigenome [123]. Additionally numerous transcription factors beyond the "core" factors have been shown to play a role in pluripotency either through promotion of an undifferentiated state or through the inhibition of lineage differentiation.

Although KLF4 is not given the "core" designation, KLF4 plays a role in pluripotency maintenance. Klf-4 has been shown to be important in establishing integration of pluripotent cells into mouse chimeras. Stem cells from the epiblast express the "core" team with NANOG,

SOX2 and POU5F1/OCT4 but lack KLF4. These cells are capable of pluripotent lineage differentiation but do not integrate into chimeras; however with the exogenous expression of KLF4, these cells are capable of integrating into developing blastocysts and contributing to the germ line [142].

The remaining identified factors from the transcriptional screening c-MYC, LIN28 and NODAL have roles in preventing differentiation to the separate germ lineages and/or have a role in proliferation. c-MYC is well known for inducing increased proliferative effects as a proto-oncogene, but it has also been shown to have a role in maintaining pluripotency and self renewal in the mouse [143-144]. It can also repress differentiation to the endoderm in hESCs [145]. LIN28 has been shown to regulate microRNAs that are involved in deciding between pluripotency and differentiation [146-147], and NODAL is capable of attenuating the neuroectodermal differentiation of hESCs [148]. As the understanding of pluripotency progresses the importance of these factors and their synergistic roles will undoubtedly become clearer. Still, these pluripotent genes' role in generating induced pluripotent stem cells (see above) have proven to have a vital impact on the future of stem cell research.

iPSCs in Modeling Diseases:

Disease specific iPSCs are still in the early stages and an increasing numbers of cell lines are being generated from diseased individuals. Cells from an elderly patient with Amyloid Lateral Sclerosis have been reprogrammed and used to generate motor neurons [149] and cells isolated from 5 Parkinson's patients were capable of generating dopaminergic neurons [150] showing that tissue specific differentiation is possible from affected individuals. The generation of these cells can provide a diverse genetic background for screening drugs and toxins that may

be harmful to these cell types. One of the most remarkable discoveries is the fact that the reprogrammed cells from diseased individuals are showing that the reprogramming process does not affect the ability of the generated cells to reproduce symptoms of the disease. This opens the door for drug screening studies examining how to prevent the progressive deterioration of the diseased cells.

Cardiac diseases have been recapitulated with iPSC technology; mutations in ion channels that cause heart arrhythmias that can be life threatening and the iPSCs derived from affected individuals with these diseases can provide an excellent proof of principle for these generated cells as well as a model for the disease in a dish. Cardiomyocytes have been derived from iPSCs in two patients with long QT syndrome, one with a mutation in the gene encoding the KCNH2 potassium channel [151] and one with a mutation in the gene CACNA1C, a gene encoding $Ca_v1.2$ calcium channel [152]. Both disorders were recapitulated by the cells through arrhythmic beating of the derived cardiomyocytes. These arrhythmias could be ameliorated though the use of drugs, showing that these cells respond in a typical manner as expected in vivo [151-152]. These studies have shown the power of iPSCs to mimic the disease from the person which they were derived.

Additionally numerous studies have looked at the ability of this technology to increase the understanding of neural diseases. Fibroblasts from a patient with Spinal Muscular Atrophy once reprogrammed and differentiated in culture have been shown to be defective in motor neural survival and show some amelioration of defect through addition of the drug that increases the *survival neuron protein*, the protein whose lowered expression results in the disease [153]. Generated neural cells from iPSCs in a person with Familial Dysautonomia generated fewer of the affected peripheral nervous cells and were responsive to the exposure with a candidate drug

that reduced the expression of the aberrantly spliced *IFBKAP*, gene known to be a key player in the disease [154]. Neurons have also been generated from iPSCs of four patients with a form of Autism Spectrum Disorder called Retts syndrome, a disease with a complex phenotype that results from a single genetic mutation. The neurons generated demonstrated multiple differences to normal controls such as decreased soma size, decreased spine density, and reduced synaptic formation. These effects could be rescued with IGF2 and induced in the wild type through missense mutations in the gene responsible for Retts syndrome, MeCP2 [155]. These initial studies provide promise for creating "dish patients" with neurodegenerative diseases allowing a new avenue to examine pharmaceutics in ameliorating the disease symptoms and generate tissue for transplantation lacking the defective characteristics.

Induced Pluripotent Stem Cell in Biomedical Research Species:

Recent advances in iPSCs may overcome the roadblocks to establishing pluripotent lines in previously unobtainable species and demonstrate that the factors of pluripotency can cross lines of phylogenics. Human factors can be used on pig mesenchymal stem cells to generate pluripotent cells capable of generating chimeras with germline transmission [156] and human pluripotent factors in chickens can reprogram cells that are capable of forming chimeras and differentiating into all three germ layers (Liu et al. manuscript in preparation) demonstrating that these factors have experienced conservation though evolution. The list of creatures that now have induced pluripotent cell lines sounds like an elaborate petting zoo with species such as sheep [86, 157], rat [158], pig [156, 159-161], rabbit [162], horse [96], and monkey [163] entering the mix. While these discoveries provide hope for the future with these species in regenerative medicine, only one publication showed chimerism with possible germline transmission in a non-rodent

[156]. Future use of this technology in biomedical research is dependent on the efficient generation of reproducing strains of animals with genetic modification, thus further emphasis should be placed on producing viable, reproducing offspring from these generated cells.

Use of Multipotent Stem Cells in Cell Therapy:

Multipotent stem cells are present in niches throughout the body, with neural stem cells being documented in the subventricular zone [164], and in the olfactory regions [165] [166]. Intestinal stem cells have been found in the crypts of the intestine [167]. Epithelial stem cells have been identified in the bulb of the hair follicle [168] and in the cornea [169]. Bronchioalveolar stem cells have been found in the lung [170], and dental pulp stem cells in the tooth [171]. Also found were spermatagonial stem cells in the testis [172], germ line stem cells in the ovary [173]. Satellite stem cells can be found in the muscle [174], and cardiac stem cells in the heart [175]. Hematopoietic stem cells are present in the bone marrow [176], fetal liver [177] and circulating in the blood. [178]. Mesenchymal stem cells are present in bone marrow [179], adipose tissue [180], fetal tissues [181], peripheral blood [182] and cord blood [182]. Even though these stem cells reside in multiple tissues within the body their therapeutic uses are limited. As with the availability of organs, the prevalence of these cells is not equivalent to the need, with only a few of these cell types permitting sizable expansion *in vitro.* Currently the multipotent stem cells that offer the most ubiquitous clinical applications are the hematopoietic stem cells and mesenchymal stem cells with both already being used in the clinic.

Transplantation of hematopoietic stem cells has become commonplace in hospitals with the ability to treat multiple blood [183-184] and immunodeficiency disorders [185-186]; however, these cells are limited to only minimal expansion in vitro [187]. Hematopoietic

transplantation can also initiate graft vs. host disease (GVHD). This disease is potentially life threatening with the cells in the transplant attacking the diseased individual. These reactions are a result of activation of the T cells in the transplant [188] and ablation of these cells prior to transplant helps to reduce this life threatening complication, but it does not completely eliminate the possibility of patients getting this reaction [189-190]. While the use of mesenchymal stem cells in the clinic is notas prevalent as hematopoietic stem cells, emerging discoveries are providing hopes that these cells can have extensive therapeutic applications. They are even being explored providing aid in preventing GVHD with hematopoietic stem cell transplantations [191].

Mesenchymal stem cells get to the heart of the matter with injections into a damaged human heart showing ability to reverse prior damage done to the heart [192] and improve cardiac symptoms [193]. In addition to the applications as a heart healer, these cells have been proposed for treatment in numerous diseases including cancer therapy [194], graft vs host disease (GVHD) [195], intestinal disease[196], lung injury[197], wound healing [198] and multiple sclerosis [199]. One of the most attractive aspects of MSCs for clinical application is the ability of these cells to modulate the immune response over a broad range of immunogenic cell types.

MSCs have been shown to have effects on multiple cell types involved in inflammation and tissue rejection. Preclinical studies have shown that MSCs can evade alloreactive T-cells by the lack of expression of B7-1, B7-2 CD40, and CD-40 ligand, inflammatory co-stimulatory proteins involved in initiating cell-based immune response [200]. It has been proposed that the effects of MSCs include modulating the response of mature monocyte dendritic cells with the down regulation of TNF-alpha and regulating IL-10 in mature plasmacytoid dendritic cells which reduces the immune-inflammatory response initiated by the two dendritic cell types [200-201].

In addition, the response of T-cells shifts from a TH1 to a TH2 response with decreases of INF-gamma secretion from TH1 cells and an increase in IL-4 secretion from TH2 cells in the presence of MSC, which further induces TH-2 differentiation inducing a more humoral immunity (TH2) rather than cellular immunity (TH1) [200]. MSCs also induced an increase in the CD4+ and CD25+ T-regulatory cells [200, 202-203] which are responsible for creating a more tolerant environment by down regulating the inflammatory T-cell response. Additionally MSCs can increase healing potential by inducing the expression of IL-6, IL-8, vascular endothelial growth factor and prostoglandin E2 [200]. All these molecules have been shown to have a role in regeneration in injury models [204-207]. These key features of MSCs make them an attractive cell type to be used with transplanted tissues to promote healing and have resulted in approval of Phase I/II clinical trials using these cells for treating multiple diseases.

Recent clinical data had shown that these cells are well tolerated by the recipients and may help reduce the effects of stroke [208], reduce the severity of GVHD [209], improve the symptoms of lupus [210], help with symptoms of multiple sclerosis [211], and decrease the severity of limb ischemia [212]. While these trials implicate a success, additional trials indicate that MSCs may not be the best option in every application. In a trial for Crohn's diease there was an improvement in the symptoms experienced by half of the cohort, however the other half of the cohort had their condition worsen to the point of requiring surgical intervention [213]. Additionally, a clinical trial for spinal cord injury did not provide any benefit to the patients and may have increased the prevalence of neuropathic pain [214]. Currently, the clinical trials using MSCs are still in the early phases and the only valid result that can be concluded is that these patients did or did not experience any adverse reactions; the reported improvement at this time

can only be deemed anecdotal. The potential for these cells to fail in larger Phase III clinical trials is still possible.

This potential for possible failure could come from reports that the immunomodulatory characteristics of the MSCs are lost following differentiation and result in adverse affects to the recipient. These studies also indicate that the initial recovery achieved by these cells will deteriorate with time. The beneficial effect in a model for cardiac repair was eventually lost in a rat model [215] and the MSCs produced pro-inflammatory factors and expressed co-stimulatory molecules following longer times of differentiation in vivo [215-216]. Even with the great potential for regeneration with MSCs, their use in the clinic should be done carefully knowing that the cells, once they lose their stem cell phenotype, could potentially create an inflammatory response in the therapeutic environment. As pluripotent and multipotent stem cell therapy progresses it will become even more vital to have adequate animal models for testing these potential treatments. Generating transgenic species in addition to rodents will allow for better prediction of adverse effects and also help to satisfy the requirements of the FDA to test products in non-rodent species [217].

Using Microencapsulation to Prevent Graft Failure:

With every therapy there comes inherent risks, and reducing those risks is integral for maintaining the quality of lifestyle of patients. While most cellular therapies (other than hematopoietic stem cell transplants) are depleted of cells which initiate GVHD [218], the outcome of successful treatment relies on the successful acceptance of the therapeutic cells. All allogeneic cellular therapies run the risk of rejection with the patient recognizing the therapeutic cell as foreign. Thus, patients receiving these cells will be required to be on immunosuppressive

treatment to prevent rejection. Developing strategies to keep cells separated from the humoral based and adaptive immune system that could reduce or eliminate the need for immunosuppressives is an ongoing process. One method for accomplishing this task is to isolate the therapeutic cells with a barrier that allows for the diffusion of molecules but also prevents the passage of cells and larger proteins such as IgG antibodies through the membrane [219].

Cellular encapsulation is a viable method that would allow this barrier to be created. These capsules provide a suitable environment for the cells while allowing them to perform therapeutic tasks. Currently there are multiple available biomaterials for creating cellular encapsulation with alginate [220], poly(ethylene glycol) (PEG) [221], and polyethersulfone [222] already having approval for clinical trials. These capsules provide a unique opportunity to introduce stem cells for therapeutic use without allowing the cells to come in contact with the patient's immune system. Additionally, this developing technology allows for these cells to be modified in ways that would normally initiate their rejection. Using genetically engineered cells in microencapsulation can allow for the localization and perseverance of these cells once implanted.

Gene therapy is attractive in therapeutics: it allows cells transduced with genes of interest to produce therapeutic proteins in a site specific manner. This facilitates high levels of the protein to be produced only where it is needed, thus preventing potential deleterious effects of the protein systemically. The incorporation of MSCs in this therapeutic method has the potential to introduce the immunomodulatory effect while producing the therapeutic protein [223]. When encapsulated, MSCs have been shown to retain the ability to differentiate into chondrocytes, adipocyces and osteocytes [224-225] making them an attractive source for cartilage and bone regeneration therapeutics. MSCs can also increase the function of co-encapsulated cells [226]

which would allow for optimal production from cells of low availability, such as pancreatic islets and hepatocytes, reducing the number of cells needed to achieve the desired response. MSCs have also shown the ability to maintain their immunomodulatory potential after encapsulation through reduction in response to the encapsulated cells with lowered IL-1β and TNF-α mRNA expression in lymph nodes from treated animals [225]. These characteristics of MSCs make them an attractive cell type for encapsulated therapies in addition to their ability to produce genetically engineered proteins [225]. Cellular therapy using encapsulation for producing gene therapeutics is attractive in treating cancer [225, 227-228], heart conditions [229] and neurodegenerative disorders [230-232] with pre-clinical trials showing positive results; however, despite the conception of encapsulation in the 1960s, a viable treatment has still failed to make it through clinical trials.

While this therapy has high potential for disease treatments, the reality has fallen short of expectations [233]. Before these treatments can be viable, multiple caveats need to be overcome including the initial foreign body response following implantation which results in the release of pro-inflammatory molecules that reduce the effectiveness [234] or kill the encapsulated cells [235]. Preventing the overgrowth of the capsules by endogenous cells is important to prevent suffocating the encapsulated cells [236]. As further research progresses to provide long term survival of these cells, their potential therapeutic application in gene therapy will permit the use of unique and effective treatments for diseases.

Microencapsulation for Bone Regeneration:

Bone is a valuable tissue for regenerative medicine in fracture healing with 550,000 people receiving bone grafts per year [237]. While autologous bone grafting is the gold standard for

bone regeneration, graft harvesting is not without risk. During surgery patients receiving autologous bone grafts have higher surgical risks due to increased surgical times and blood loss [238]. Following surgery 33% of patients still report having pain two years after the transplant at the site of harvest [239] and the patients are at increased risk for fractures at the donor site and often experience hip instability [240].

Multiple members of the BMP family, a part of the TGF-β superfamily, have been shown to induce ossification [241], and while they are named for their ability to form bone [242], BMPs have additional roles in development [243]. BMPs are involved in the establishment of dorsal ventral patterning and left right symmetry [244], in cell fate determination though specification of ectoderm [245] and limb bud [246], in modulating proliferation[247], and in apoptosis [248]. These versatile BMP signaling factors are secreted in an immature form and require cleavage to yield the active protein [249]. This cleavage allows for the BMPs to bind as heterodimers and homodimers through di-sulfide linkage [250], and for bone formation the heterodimer BMP2/BMP7 is the most effective combination in inducing ossification [251].

The binding of BMPs to their receptors is one of the mechanisms for such different functions throughout development [244]. BMPs bind to serine/threonine kinase type I and type II BMP receptors when in their active form. After binding their receptors SMAD 1/5/8- co-SMAD 4 signaling cascades are activated and translocated to the nucleus [252]. JNK, p38 [253], and pI3 kinase [254] are also involved with osteogenic signaling; in osteoblasts reduction of either of these pathways reduces osteogenic marker expression and it is believed that cooperation between these pathways are involved in bone formation [252].

BMPs have been shown to induce ossification [241] and aide in bone matrix maturation and mineralization [255] making them a potential substitute for bone grafts. Of the BMPs, BMP-

2 and BMP-7 have been used in humans to promote spinal fusion, fracture healing and oral defects with differing reports of success [256]. One drawback to BMPs is their rapid clearance if injected in solution [257] and animal studies have shown that sustained exposure is required for efficient bone generation [258]. Finding methods to produce constant amounts of BMPs at in situ sites of are of importance. Transducing cells with BMPs for injection have shown increased rate of ossification [259-261]. Encapsulating BMP-2 transduced cells allows for more localized delivery and evasion of immune mediated clearance of the engineered cells [262-264]. This technology combined with BMP-2 offers a new means for producing BMPs at a site of injury.

Conclusions:

Diseases and injuries steal the life from many people and stem cell therapy offers hope that no other treatment can match. As these derived cells cross over the line from the bench to the bedside further research in generating efficacious cells will be of the utmost importance. As the understanding of pluripotency and development progresses, the prospect of "the bionic man" may become more than a possibility. One day hearts and livers may be grown in a dish and all this will be based on the discoveries made from pluripotent cells, but the reality is that pluripotent cells will deliver more than artificial organs. They will deliver therapeutics in addition to knowledge and understanding that current medical technology has been unable to obtain.

The power of pluripotent cells in generating knockout and genetically engineered animals will allow for better understanding of human diseases. The ability to generate transgenic models in large animals will provide better prediction of how derived cells will perform in a body that is

150-3000 times larger in body mass than the current rodent models. This is vital for testing therapeutics for stroke and nerve injury in the spinal cord. With neurons only having to travel distance of a few centimeters in mouse, larger distances and larger structure from animals more physiologically relevant to humans will predict the potential of treatments.

To ensure that patients have the most successful recovery as possible, patient derived cells should continue to be developed. In addition to being a better source of cells for promoting integration, patient derived cells can alleviate the need for long term immunosuppressive treatment. Making these new therapeutics safe will be a continual challenge for researchers, but with the remarkable progress in the short time since discovery the outlook is good.

Combining biotechnological advances such as microencapsulation with stem cell research can provide new efficient treatments for disorders such as bone nonunion. The ability to engineer MSCs to produce high quantities of therapeutic proteins makes them an attractive vehicle for gene therapy. This combined with microencapsulation can provide novel treatments with minimal risks to patients.

Whether stem cells are directly injected or involved in drug development, they are going to play an intricate role in the medicine of the future. From the discovery of pluripotent and multipotent stem cells at the turn of the 20th century [265-266] to their first use in clinical trials at the turn of the 21st stem cells are making their mark. These cells are the future of medicine and can provide therapy to individuals in need.

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CHAPTER 3

NEURAL DIFFERENTIATION OF HUMAN EMBRYONIC STEM CELLS AT THE ULTRASTRUCTURAL LEVEL

Jennifer L. Mumaw, Dave Machacek, John P. Shields, Mahesh C. Dodla, Sujoy Dhara, and Steve L. Stice. Reprinted with permission of *Microscopy and Microanalysis*. 16, 80–90, 2010.

Abstract:

Neurodegerative disorders affect millions of people worldwide. Neural cells derived from human embryonic stem cells (hESC) have the potential for cell therapies and/or compound screening for treating affected individuals. While both protein and gene expression indicative of a neural phenotype has been exhibited in these differentiated cells, ultrastuctural studies thus far have been lacking. The objective of this study was to correlate hESC to neural differentiation culture conditions with ultrastructural changes observed in the treated cells. We demonstrate here that in basic culture conditions without growth factors or serum we obtain neural morphology. The addition of Brain Derived Neurotrophic Factor (BDNF) and serum to cultures resulted in more robust neural differentiation. In addition to providing cues such as cell survival or lineage specification, additional factors also altered the intracellular structures and cell morphologies. Even though the addition of BDNF and serum did not increase synaptic formation, altered cellular structures such as abundant polyribosomes and more developed endoplasmic reticulum indicate a potential increase in protein production.

Introduction:

Human embryonic stem cells (hESCs) and cell populations derived from these pluripotent cells provide promise for therapeutic treatments for many debilitating diseases. In addition, hESCs also provide an *in vitro* human developmental model that offers a unique opportunity for investigating key factors which are impossible to study *in vivo* [1-2]. Characterization of hESCs' gene and protein expression has provided researchers with

information about development and key cell signaling events necessary for directed differentiation. For example, Shin et al. (2005) used basic fibroblast growth factor (bFGF) to induce cholinergic neurons and the combination of retinoic acid, sonic hedgehog, and bFGF to induce motor neuron differentiation from hESCs. In addition to the biochemical characterization of these cells, physical analysis is also important to understand the viability and differentiation status of hESCs and hESC derived cultures [3]. The use of hESCs are of interest in part because they are thought to developmentally resemble endogenous cell populations as they differentiate *in vitro*; therefore, it is important to determine the ultrastructural characteristics of hESCs and their derivative cell types. The ultrastructure of the hESCs demonstrate similarities to those of the early embryo and the inner cell mass [4] in respect to the mitochondria [5], endoplasmic reticulum, high nucleus to cytoplasmic ratio, and golgi [6]. As the biosynthesis needs of the cells alter when differentiation begins there is a marked change in organelle morphology [6-7] indicating changing roles of these organelles as the cell phenotype changes. Greater understanding of the alterations the cells undergo as they differentiate will allow for optimized integration and cell function for therapeutic approaches in addition to providing the most phenotypically normal cells for *in vitro* studies. It is therefore vital to analyze detailed ultrastructure at various stages of differentiation.

The objective of this study was to investigate directed neural differentiation of hESCs at an ultrastructural level. More specifically, to determine ultrastructural changes of neural cells when differentiated in minimal neural differentiation media and in complex neural differentiation media containing BDNF and serum. The directed differentiation was conducted at three levels: undifferentiated hESCs, neural progenitor cells (hNPs), and neural cultures. At the progenitor stage the neural cultures were separated into two groups: one differentiated under basal culture

conditions without growth factors and serum and the other under more complex culture conditions with the addition of BDNF and serum. The *in vitro* conditions used here influenced the ultrastructure of cells differentiated from hESCs. Neuronal cells progressively exhibited more differentiated phenotype with a defined soma and refined process extending from the cell body. The intracellular development also showed alterations of organelles such as the appearance of well formed rough endoplasmic reticulum, prominent golgi apparatus, extensive filaments organized around the cell body and through the processes. While both the basal and complex culture conditions resulted in developing neuronal morphology, the addition of BDNF and 5% fetal bovine serum (complex) to the cultures produced cells that were more robust in the organelles for protein synthesis. In complex culture condition neural cultures had higher protein synthesis organelles such as abundant polyribosomes, well formed rough endoplasmic reticulum, and mitochondria maturation (increased density, a more elongated shape and apparent cristae) were observed. Ultrastructural analysis of hESCs can reveal the effects of culture condition and thus provide another level of information for optimization of hESC differentiation for future hESC therapies.

Materials and Methods:

hESC Maintenance:

WA09 hESCs derived from excess in vitro fertilized human embryos were obtained from WiCell. hESCs were cultured in Dulbecco's minimal essential medium/Ham's F12 medium (DMEM/F12), 2 m*M* L-glutamine, 0.1 m*M* minimal essential medium (MEM) nonessential amino acids, 50 U/ml penicillin, 50 µg/ml streptomycin (all from Invitrogen, Inc.), 4 ng/ml basic

fibroblast growth factor (bFGF; R&D Systems) and 5% Serum (Invitrogen, Inc.). Cells were cultured on mitomycin-C (Sigma, Inc.) mitotically inactivated murine embryonic fibroblasts, manually dissociated, and passaged to new feeder layers every 3-4 days. The cells were maintained at 37 degrees Celsius and 5%CO_2.

hNP Maintenance:

ENStem-A™ (Millipore Inc.) hNP cells were derived from WA09 hESCs and maintained as described previously [8]. Briefly, cells were grown on poly-ornithine (20 µg/mL)/laminin (Sigma-Aldrich, Inc.) (5 µg/mL) coated plates in ArunA Expansion and Maintenance Media (maintenance media) with 2mM L-Glutamine and 20 ng/mL b-FGF (all from Millipore, Inc.). Cells were passaged approximately every 48 hours and split 1:2 following manual dissociation.

Differentiation of hNP into Neurons:

hNP cells were differentiated into neurons on poly-ornithine and laminin coated plates under two conditions: 1) in maintenance media without bFGF or 2) complex media: maintenance media with BDNF and 5% fetal bovine serum (Hyclone, Inc.). hNP cells were allowed to differentiate under these two conditions for 6 weeks and 7 weeks respectively. For immunocytochemistry neural progenitor cells were cultured on polyornithine/laminin coated glass slides. For neuronal differentiation, cells were differentiated using maintenance media without growth factors and stained after 0, 3, and 5 weeks of differentiation.

Immunocytochemistry:

For immunostaining, after removing the culture media, the cells were washed once with phosphate buffer saline (PBS) and fixed with 2% paraformaldehyde in PBS for 20 minutes. Fixed cells were washed twice with PBS. Cells were permeabilized with 0.1% triton, 6% donkey serum in Tris buffer for 20 minutes followed by incubation in blocking buffer consisting of 6% donkey serum in Tris buffer for 45 minutes. For cell surface labeling, permeabilization was excluded. Primary antibodies were diluted in blocking buffer for 1 hour at room temperature and washed 2 times with Tris buffer before secondary antibody application. Secondary antibodies were diluted in blocking buffer and applied for 1 hour at room temperature. The cells were then washed four times, five minutes for each wash, with Tris buffer. The slides were mounted with ProLong Gold antifade reagent with DAPI (4',6-diamidino-2-phenylindole, Molecular Probes, Inc.) for cell nuclear staining. Primary antibodies and dilutions used included the following: Mouse anti-Sox2 (1:100; R&D Systems, Inc.), mouse anti-Nestin (1:200; Neuromics, Inc.), chicken anti-beta III tubulin (1:500; Chemicon, Inc.), rabbit anti-MAP2 (1:500; Chemicon, Inc.), and mouse anti-GFAP (1:500; Sigma, Inc.), mouse anti-Tra-1-81 (1:100 Chemicon, Inc.). Secondary antibodies and dilutions used were: Donkey anti-mouse IgG Alexa Fluor® 488 (1:1000, Molecular Probes, Inc.), donkey anti-rabbit Alexa Fluor® 488 (1:1000, Molecular Probes, Inc.), donkey anti-rabbit Alexa Fluor® 594 (1:1000, Molecular Probes, Inc.), and donkey anti-chicken IgY Texas red (1:250, Jackson ImmunoResearch Laboratories, Inc.). Images collected with Olympus IX81 DSU imaging system (Olympus, Inc.).

Scanning Electron Microscopy:

Cells were plated on glass coverslips and immersion fixed using 2.0% gluteraldehyde in PBS for one hour. The cells were washed three times with PBS and postfixed in 1% osmium tetroxide diluted in 5% sucrose and PBS for 45 minutes. The cells were washed three times with distilled water and then carried though an alcohol dehydration series(35%, 50%, 75%, 85%, 95% and 100%). Each step of the series was processed for 15 minutes and the 100% EtOH step was repeated 3 times. The cells were critical point dried using a Samdri model 780-A (Tousimis). A 153 Å thick coating of gold was placed on the samples using SPI Module Sputter Coater (Structure Probe, Inc.). The images were taken on a LEO 982 Field emission scanning electron microscope (Carl Zeiss, Inc.).

Transmission Electron Microscopy:

hESCs:

Colonies of WA09 hESCs were manually harvested to minimize mouse embryonic fibroblast (MEF) contamination and immediately immersion fixed in 2.5% glutaraldehyde in PBS for one hour. The cells were washed three times with PBS and postfixed in 1% osmium tetroxide diluted in 5% sucrose and PBS for 45 minutes. The cells were washed three times with distilled water, and then carried through an ethanol dehydration series (35%, 50%, 75%, 85%, 95% and 100% EtOH). Each step of the series was processed for 15 minutes and the 100% EtOH was repeated 3 times. The cells were transitioned into propylene oxide (PO) with two exchanges 30 minutes each. The cultures were infiltrated and embedded in Epon 812 using Luft's Ratio [9]. The sample was transferred into resin/PO mixture at 1:4 ratio for one hour, then 1:1 ratio for one

hour, and 4:1 ratio for one hour and 100% resin for one hour and then left overnight in fresh resin. The following morning the resin was again changed and the samples were polymerized at 60 degrees Celsius overnight.. The samples were cut on a RMC MT-X ultramicrotome (Boeckeler Instruments, Inc.) to approx. 60 nm thickness using a diamond knife. The sections were viewed on JEOL 100 CX transmission electron microscope (JEOL, Inc.).

hNP and Differentiating Neurons:

hNPs and differentiating hNPs were grown in suspension culture. The cells were immersion fixed in 2.5% gluteraldehyde in PBS at 37 degrees Celsius and immediately placed on ice for 45 minutes. The cells were processed as described above for hESCs with the exception that they were enblock stained with 2% aqueous uranyl acetate.

Results and Discussion:

Phenotype characterization of the states of differentiation from WA09 hESCs to neurons was completed using immunological staining methods (Fig. 3.1). hESCs express the pluripotant cell surface marker Tra-1-81 (Fig. 3.1A). Following derivation the hNP cells express the neural stem cell marker SRY (sex determining region Y)-box 2 (Sox 2) (Fig. 3.1B) and the immature neural marker, Nestin (Fig. 3.1C). The hNP cultures were Glial Fibrillary Acidic Protein (GFAP) negative (Fig. 3.1C) indicating the lack of radial glia progenitors. Following 3 weeks of differentiation the cells express the intermediate differentiation marker β III-tubulin, which labels fetal and postnatal neural cells, but do not show expression of the mature neural marker Microtubule-associated protein 2 (MAP2) which labels dendritic processes (Fig. 3.1D). As the

cells continue to mature they begin to show loss of expression of β III-tubulin at 5 weeks corresponding to an up regulation of MAP2 (Fig. 3.1E). However, cells still do not show expression of GFAP after 5 weeks of differentiation (Fig. 3.1E). These results are consistent with the finding that even in multiple replicates differentiating ENStem-A™ hNPs cultures have a high neural purity.

Scanning electron microscopy (SEM) images of WA09 hESCs grown on a MEF feeder layer depict hESCs forming organized colonies. The hESC colony is circular in shape and has a clear border with the MEF cells (Fig. 3.2A). While there are areas of differing morphology within the colony, there are no structures indicative of differentiation. The TEM images depict hESCs with large nuclei while other organelles, such as Golgi and endoplasmic reticulum in particular, are not readily observed (Fig. 3.2C, 3.2D). The hESCs have an abundant amount of ribosomes and polyribosomes (Fig. 3.2C) and have electron translucent, immature round mitochondria exhibiting few cristae (Fig. 3.2C) consistent with the findings made by others [5-6]. There are also phagocytotic vesicles (Fig. 3.2D) in the hESCs which is a result of autophagocytosis, a process by which cellular organelles are processed for degradation [4, 10]. All these features are indicative of stem cells which are in an undifferentiated state.

The incongruous cell morphology within the hESC colony can be due to differing stages of the cell cycle or could result from conservation of surface area where the cells express more microvilli on the apical surface to retain the same amount of exposed surface area as a cell which has a more flattened morphology [11]. Many cells have extensive microvilli (Fig. 3.2B), cellular structures whose role in hESCs is poorly understood. The presence of microvilli on hESCs is consistent with rhesus monkey embryos [12] and cells in the inner cell mass at the blastocyst stage of development [13]. The microvilli are in close apposition and intertwine with

the microvilli from neighboring cells (Fig. 3.3A), which could indicate cell-cell communication through the microvilli. These microvilli extensions allow cells to physically contact non-bordering cells and can possibly aid in signaling and preferential migration of cells within the colony. Microvilli play important roles in metabolic regulation, calcium signaling [14-15], and establishment of polarity in embryonic epithelium; however, there have been no documented reports, to our knowledge, of cell-cell signaling or migration cue transmission between cells though microvilli in hESC. The morphology of the hESC colony also indicates that the stem cells aggregate more tightly with other hESCs as opposed to the MEFs (Fig. 3.2A).

The TEM images also depict cells that an undifferentiated state. hESCs exibited large nuclei while other organelles, such as Golgi and endoplasmic reticulum in particular, are not readily observed (Fig. 3.2C, 3.2D). The hESCs have an abundant amount of ribosomes and polyribosomes (Fig. 3.2C) and have electron translucent, immature round mitochondria exhibiting few cristae (Fig. 3.2C) consistent with the findings made by others [5-6]. There are also phagocytotic vesicles (Fig. 3.2D) in the hESCs which is a result of autophagocytosis, a process by which cellular organelles are processed for degradation [4, 10]. All these features are indicative of stem cells which are in an undifferentiated state. The clear boundary observed between the hESC colony and the MEF layer (Fig. 3.2A) is likely a result of the tight junctions between the hESCs (Fig. 3.3B). The preference for hESCs to form gap junctions between cells within the colony and only minimally with the MEFs alter the adherence between the cell types [16]. hESC have distinct separation between the apical surface and the basal surface of the colony. There are cell junctions lateral to the apical membranes which express microvilli (Fig. 3.2D, 3.3B). These junctions are indicative of tight junctions [17] and are present between all hESCs with a surface exposed to the culture media (Fig. 3.2D). hESCs appear to sequester

themselves within the culture environment. The presence of tight junctions between the outer layer of the colony allows for the cells to tightly control the environment within the colony (Fig. 3.2D, 3.3B). This control would allow for the cells to successfully allow intercellular signaling and maintain controlled contact for signal transduction in the presence of endogenous signaling cues. Tight junctions within the hESC colony can influence the passage of factors from outside the colony into the colony while still remaining capable of responding to developmental cues in the environment through the increased surface area provided by the microvilli. Once the colony forming hESCs begin neural development these features are lost. We have previously demonstrated that hESCs derived hNP cells are motile [18].

The EM images indicate that differentiation of hESCs into hNP cells produce a multitude of structural changes. hNPs no longer grow in a static colony like hESCs and tight junctions are no longer observed. The hNPs have undergone morphological developmental changes consisting of developing extensive lamellipodia, a flattened appearance, and an increase in size (Fig. 3.4C). The cells are in close opposition to the substrate and have interactions with multiple cells (Fig. 3.4A, 3.4B). This indicates that both the extracellular matrix (ECM) and the cell-cell communication are important for the maintenance and survival of the hNPs. A subset of the hNP cells are morphologically similar to the appearance of neural crest cells [19] with both cells types being flat and demonstrating neurite extensions that permit contact with multiple neighboring cells and the ECM. The cells also exhibit differing morphologies as some cells are flattened and others are more raised (Fig. 3.4B). While this could indicate a multitude of different populations of neuronal precursors, it can also be an effect of cell cycle. During cell division the neural cells retract the lamellipodia and filopodia resulting in a spherical morphology prior to dividing [20] as do these cells which can be demonstrated with time-lapse [18]. The TEM images of the cells

indicate that the cells still are in a progenitor state, like the hESCs, by maintaining large nuclei and sparse endoplasmic reticulum and Golgi; however, hNPs have an extensive increase in the number of ribosomes and polyribosomes relative to hESCs (Fig. 3.4D). Mitochondria also have more prominent cristae (Fig. 3.4D) indicating an increase in biosynthesis following derivation from hESCs [5].

Differentiation of hNPs results in cultures of more mature neuronal phenotype. SEM micrographs depicted neuron-like cells which have well defined somas (Fig. 3.5B) and network like formations (Fig. 3.5A). The cells possess multiple phenotypes, but due to the network formations, individual cells were not easily distinguished. Unipolar (Fig. 3.5C) and bipolar cells (Fig. 3.5B), as indicated by neurites extending from one side of the soma or from two sides of the soma respectively, were observed in the cultures. Other cell types were not definitively recognized in these micrographs; however they have been shown to exist in these cultures [8, 21].

The cells also generate growth cone like structures at the end of processes (Fig. 3.5D): these structures are integral components in path finding, migration and synaptogenesis in neurons. Unlike the hNPs, neurons had very little surface area contact with the substrate. Alternatively, the neurons retained contact with other neuronal cells, but have only marginal adhesions to the substrate leaving the somas unattached to the ECM. These areas of adhesion occur along the processes (Fig. 3.4E, 3.4F) and at the growth cone like structures (Fig. 3.5B, 3.5E) with few somas making contact with the substrate (Fig. 3.5B); This indicates that the interactions with the ECM are reduced during neuronal *in vitro* differentiation, allowing permissive conditions for motile neural structures . Unlike other non invasive cell types, neurons are capable of autoregulating adhesion through modulation of ECM binding integrins [22]. This

capability allows for the cells to alter adhesion due to migratory cues and modulate growth cone motility. Neural crest cells also alter adhesion strength prior to migration [23] which allows the cells to change affinity for a given substrate and increase their capacity to migrate toward/away from cues.

Following differentiation neural processes changed structure, and the neurites developed organized bundles of microtubules which were evident in the lateral and horizontal TEM cross-sections (Fig. 3.5H). There are differences in the composition of some of these processes with most falling into two types of neurites. Neurites with a prominent presence of ribosomes and no mitochondria and neurites with a low presence of ribosomes but have mitochondria. These particular structural differences are indicative of cells that could possibly be polarizing and differentiating between axons and dendrites [24]. The mitochondria observed in both the neurites and the somas of the differentiated cells and were more elongated than in the hESCs (Fig. 3.5H). The mitochondria had developmentally altered becoming more elongated and containing more cristae as the cells matured into neurons. These morphological changes were consistent with the changes observed in development of human embryos. As the embryo changes from being anaerobic after fertilization to aerobic following implantation there is an increased need for functional mitochondria [25]. These developmental changes are visualized through the increased development of cristae and the increased in the size and mainly length of the mitochondria [25]. Under our basal differentiation conditions there were no obvious synaptic junctions and limited endoplasmic reticulum and Golgi apparatus were observed. The cells appear to have organelle changes consistent with embryonic development [25] to resemble more neuronal ultrastructure [26]; however, the lack of synaptogenesis and the sparse presence of

endoplasmic reticulum and Golgi indicate that additional factors may be necessary to make the cultures form synaptic connections and become functional neuronal cultures.

Analysis though electron microscopy can help ascertain the most beneficial culturing conditions and effects of growth factors on cellular organelles. Neural progenitor differentiation in basal media supplemented with BDNF and 5% serum induced more complex structures (Fig. 3.6A). Both of these factors are known to increase the viability and differentiation of neural cells [27]. The cells differentiated in these conditions formed neural cell structures tethered to the substrate through long extensions composed of multiple cells exhibiting differing phenotypes (Fig. 3.6A, 3.6B, 3.6C, 3.6D). These structures bore resemblance to the columnar organization seen during cellular migration from the ventricular plate [20]. This morphological resemblance indicated that the differentiated hNPs were capable of following migratory cues and forming three dimensional structures that may resemble brain tissue organization. The cells not involved in forming the large structures were also composed of many phenotypes which layer upon each other (Fig. 3.6E). The cells nearest to the substrate were thinner and flatter in appearance with short processes, the middle layer had cells with larger cell bodies and longer, thicker processes, and the top layer was composed of cells with web-like processes (Fig. 3.6E) again indicating preferential structural formations within the culture and potential organization. The TEM images showed cells with reticulated mitochondria and Golgi apparatus (Fig. 3.6F, 3.6G). The endoplasmic reticulum was well formed and extensively studded with ribosomes (Fig. 3.6H). The neurites also contained microtubules with many also containing mitochondria (Fig. 3.6F). While synapses were not readily encountered using TEM, ongoing studies in our lab suggest that these cells do possess functional ion channels and therefore may be capable of forming synapses under the appropriate conditions (unpublished). The addition of glial conditioned media has been shown to robustly increase the synaptic activity [28] and glial conditioned media or glial cell co-culture may enhance synaptic activity in our cultures.

Conclusions:

The work shown here demonstrated that the development of neurons through hNPs from hESCs does follow a similar progression in organelle and ultrastructural development as neural development from a blastocyst stage embryo *in vivo* [12, 19-20, 25, 29]. The addition of BDNF and serum induced the cells to form more complex structures resembling structural formations found in the developing nervous system.

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Figure 3.1: Immunocytochemical Characterization of Differentiation to Neural Cells from Embryonic Stem Cells A) WA09 hESCs display surface expression of pluripotant marker TRA-1-81(green) scale = 50 microns. B) hNPs are Sox-2 positive (green) scale = 10 microns. C)hNPs are positive for immature neural marker nestin (red), and negative for radial glia marker GFAP (green) scale = 10 microns. D) hNPs differentiated for three weeks are positive for maturing neural marker ß III-tubulin (red), and negative for mature neural marker MAP2 (green) scale = 10 microns. E) hNPs differentiated for five weeks are positive for mature neural marker MAP2 (green) and have reduced expression of maturing neural marker ß III-tubulin (red) scale = 10 microns. F) hNPS following 5 weeks of differentiation do not show expression of glial marker GFAP (green) with majority of cells expressing mature neural marker MAP2 (red) scale = 10 microns. All images are stained with DAPI for cell nuclear staining.

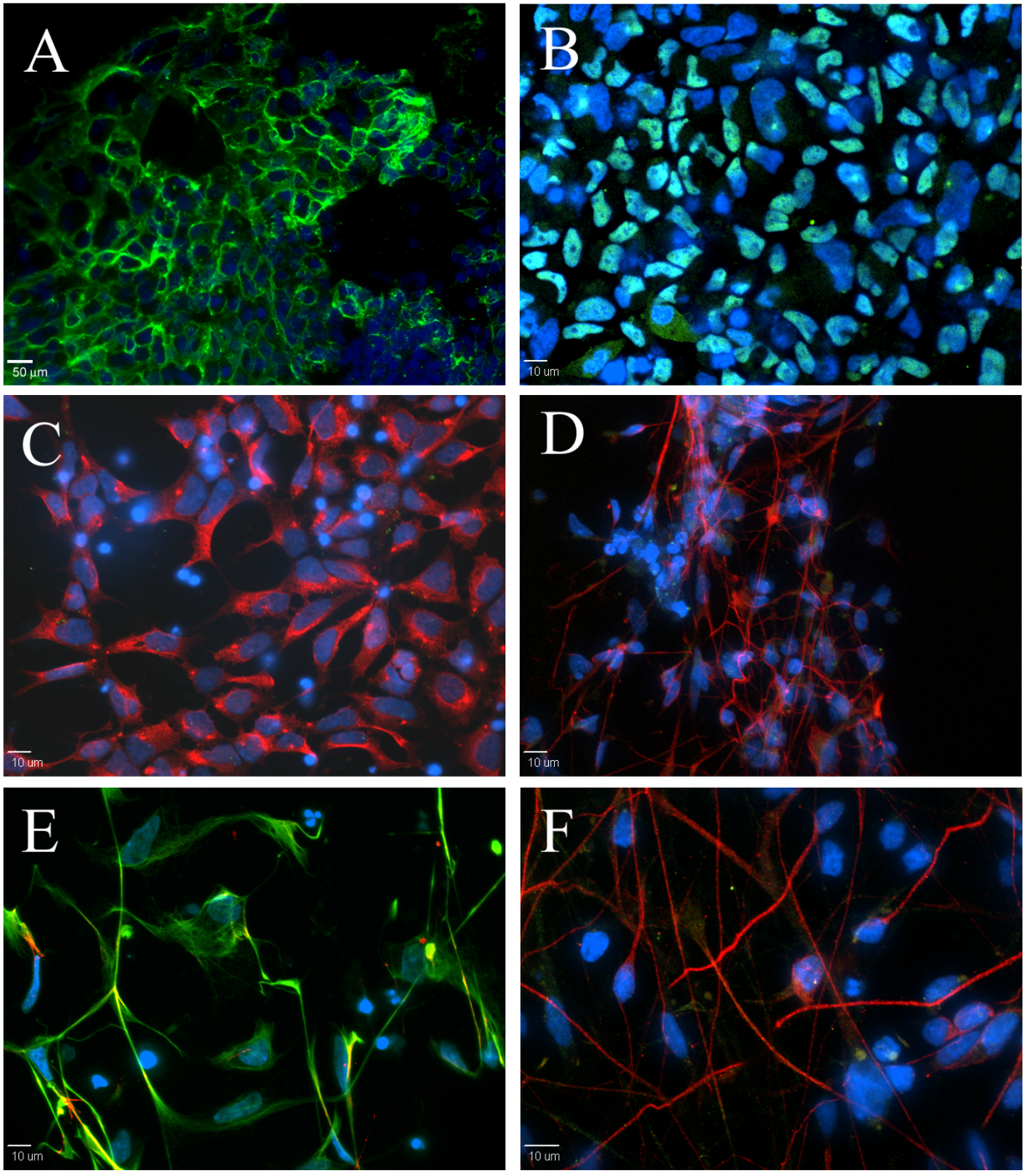
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Figure 3.2: Human Embryonic Stem Cells. A) hESC colony on a MEF feeder layer scale = 100 microns. B) hESC have different cell surface morphologies (center of the colony) scale = 5 microns. C) hESC TEM; image nuclei, (N), with condensed heterochromatin, mitochondria with few cristae (M) and polyribosomes (arrow) scale = 1 micron. D) Phagocytotic vesicles (asterisk) indicative of autophagocytosis, and cross sections of microvilli (double arrow) and cell-cell junctions (arrow) scale = 0.5 micron.

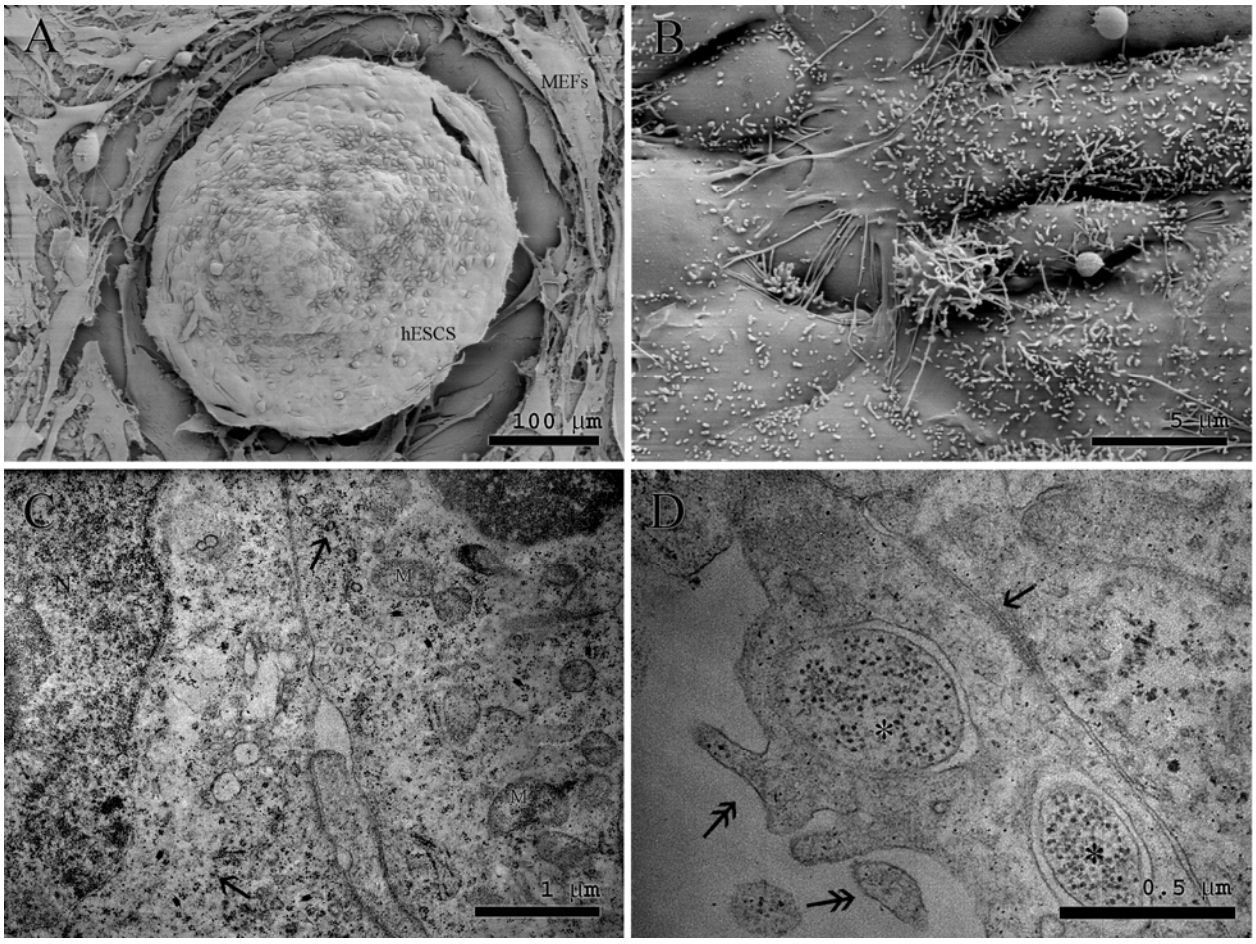
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Figure 3.3: Microvilli on Human Embryonic Stem Cells. A) Microvilli (arrow) on neighboring hESC intermingle and intertwine scale = 1 micron. B) At the edge of the hESC colony the number of tight junctions increase (arrow) and microvilli (double arrow) extending from the cells scale = 1 micron

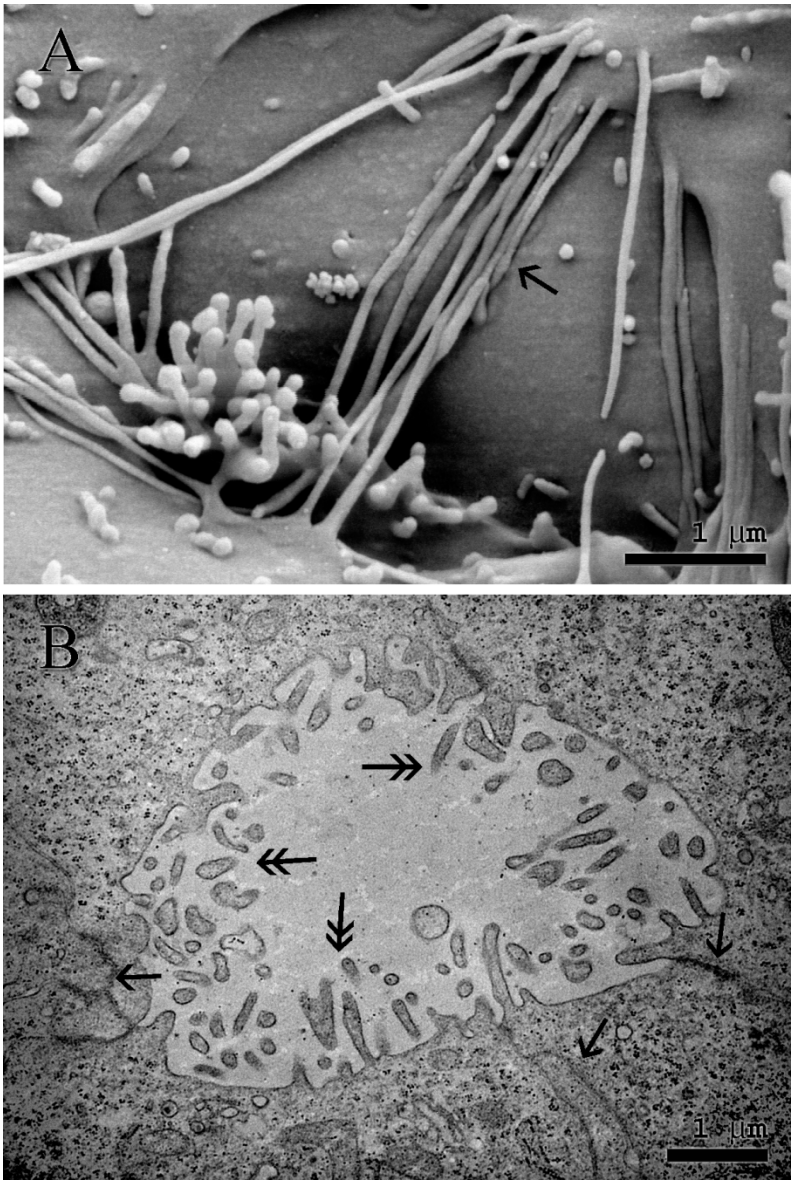
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Figure 3.4: Human Neural Precursor Cells Differentiated from Human Embryonic Stem Cells. A) hNPs have extensive cell to cell contact (arrow) and increased surface area following differentiation from hESCs scale = 50 microns. B) hNP cell morphology differs among hNPs (from flattened (arrow) to slightly rounded (double arrow) scale = 20 microns. C) hNPs (arrow) exhibit very close contact with the substrate scale = 20 microns. D) hNPs with extensively expressed polyribosomes (arrow) and mitochondria (M) which have limited number of cristea scale = 1 micron.

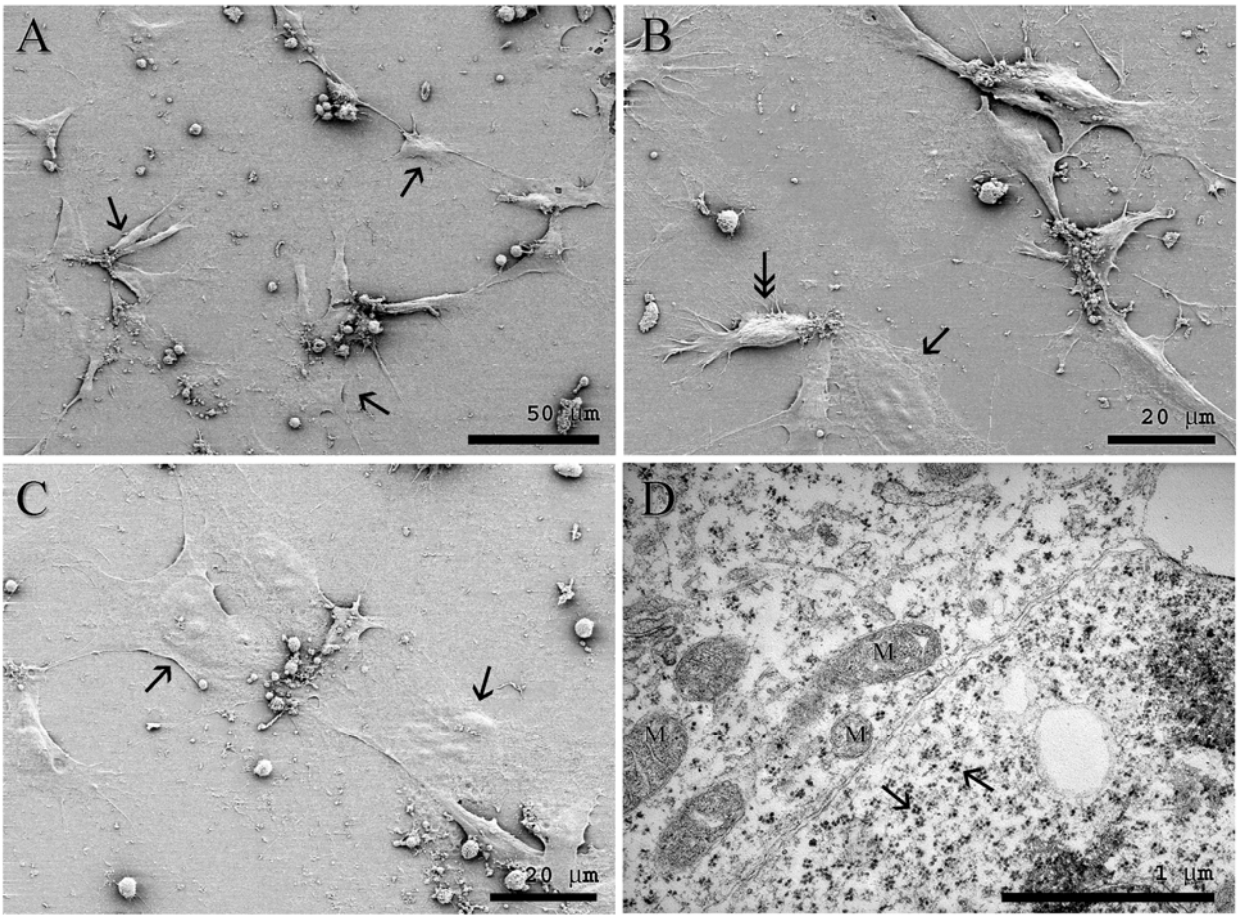
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Figure 3.5: Adherent hNPs Undergoing In Vitro Differentiation with No Additional Growth Factors in the Medium. A) Differentiated neuronal morphology with the refined neurite extensions scale = 100 microns. B) Somas (asterisk) of three cells. One cell (polygon) can be distinguished as a bipolar cell (bidirectional neurite extension) scale = 20 microns. C) A unipolar neuronal cell (arrow) scale = 50 microns. D) Growth cone formations with clear filopodia (arrow) and lamellipodia (astrisk) scale = 10 microns. E) Cells have minimal contact on the substrate. One point of adhesion (arrow) is clearly visible along a process near the soma scale = 20 microns. F) Areas of adhesion are also present along processes near growth cones scale = 5 microns. G) Cell junctions between the cells (darkened membranes) can be visualized scale = 2 microns H) Mitochondria have few cristae (M). Neurites can be found that have mitochondria (*) and others that lack mitochondria (polygon), indicating cell polarization scale = 2 microns.

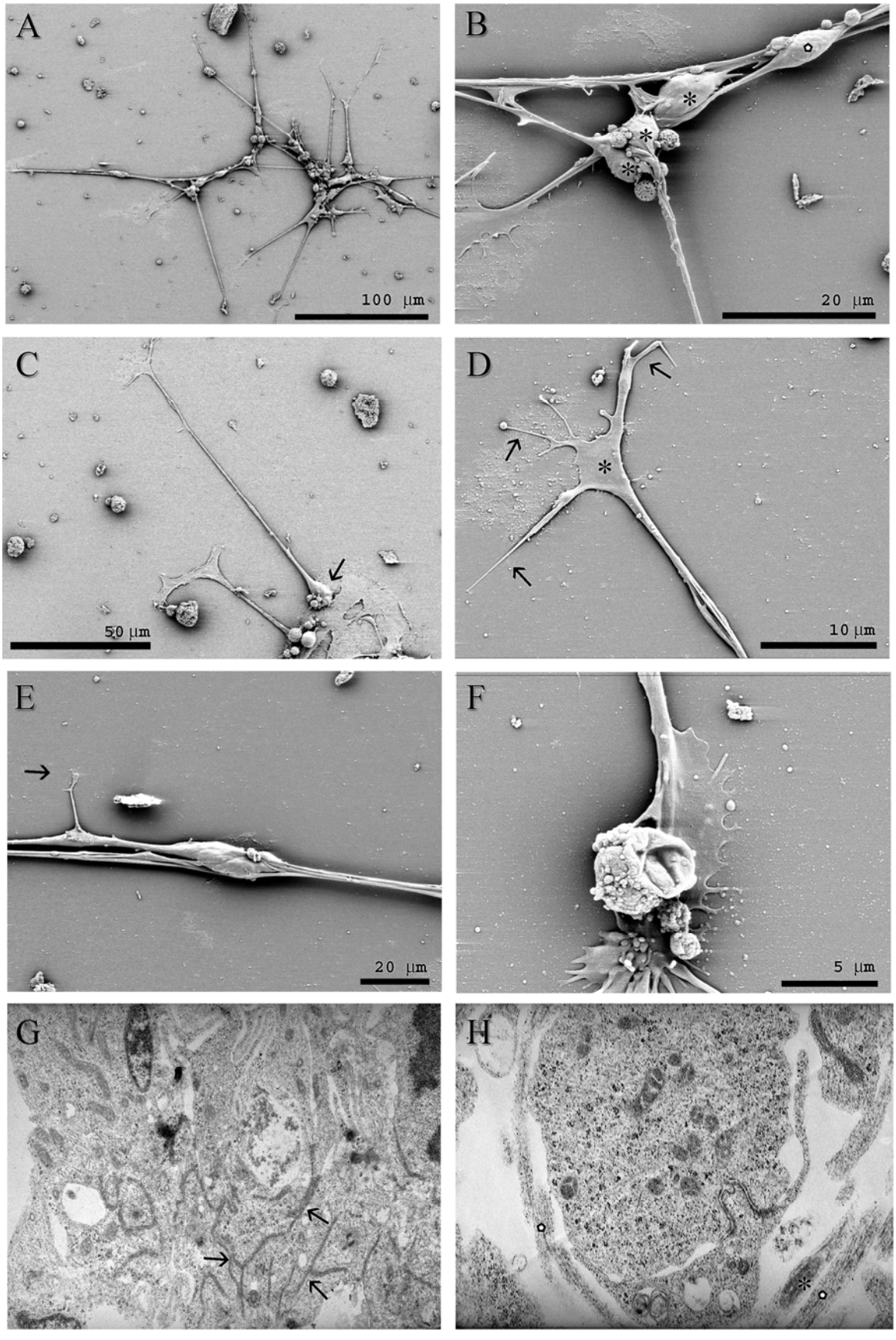
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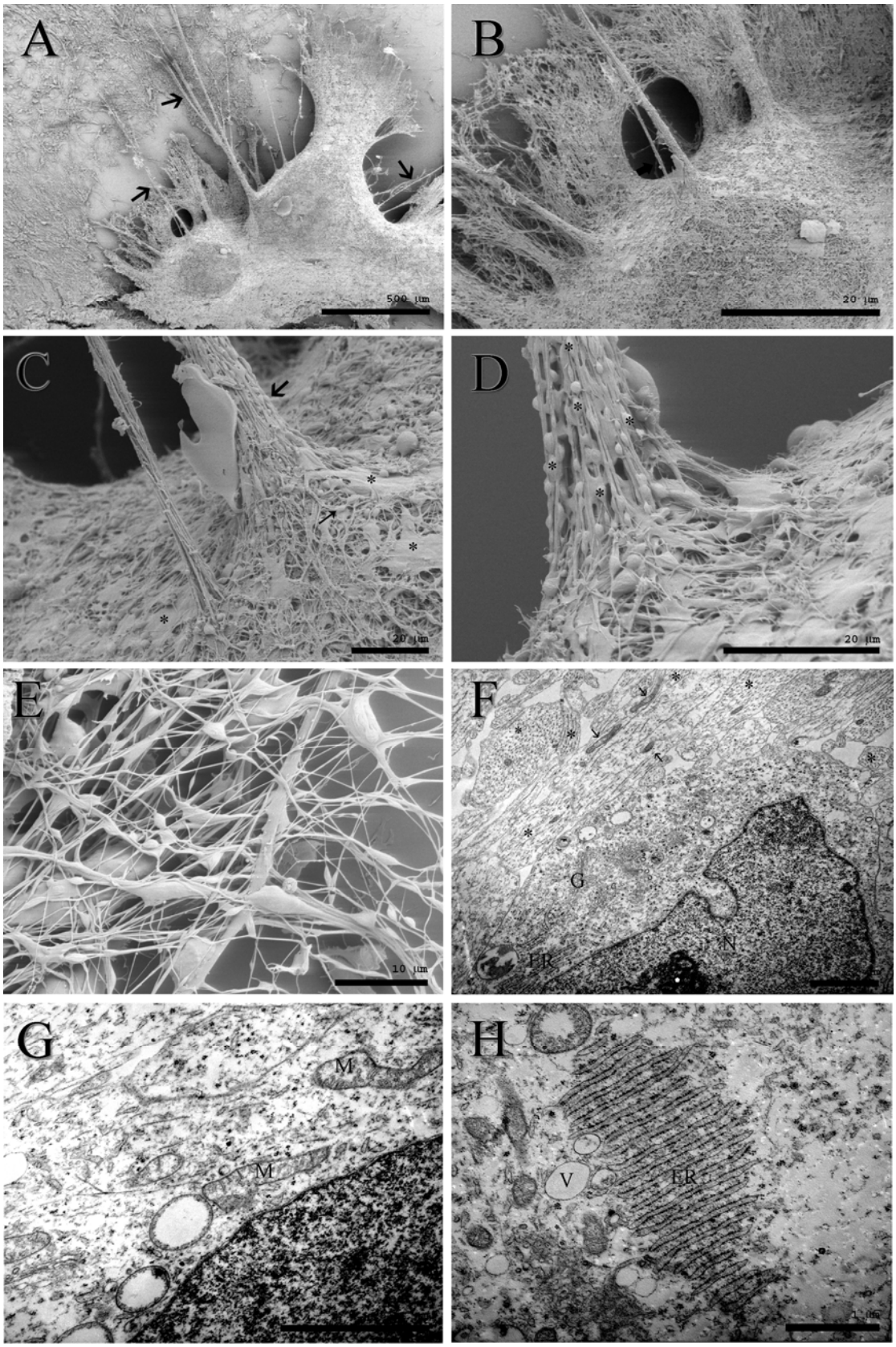
Figure 3.6: hNPs Differentiated in Complex Medium (serum and BDNF) A) Organized neural cell structure tethered to the substrate (arrow) scale = 500 microns. B) Mesh like formation of the cells near the substrate and more tightly organized tethers (arrow) scale = 20 microns. C) Multiple cell morphologies are visible at higher magnification: Broad flat cells with minimal extensions (asterisk) and smaller cells with fine extensions (arrow) scale = 20 microns. D) Multiple cell bodies visible within the tethered structure (asterisk) scale = 20 microns. E) These 3 dimensional structures demonstrated some organization, with broader flatter cells proximal to the substrate and smaller cells with fine processes distal to the substrate scale = 10 microns. F) TEM image showing clear rough endoplasmic reticulum (ER), nucleus (N) with condensed heterochromatin (polygon), golgi apparatus (G), and neurites (asterisk, arrow). The neurites have visible mitochondria (arrow) and microtubules (asterisk). Microtubules are organized in a parallel fashion. Mitochondria (M) are more mature, having an increased number of cristea scale = 1 micron. H) Well formed endoplasmic reticulum and vacuoles (V) scale = 1 micron.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CHAPTER 4

PARTIAL REPROGRAMMING OF PERIPHERAL BLOOD MONONUCLEAR CELLS WITH POU5F1/OCT4

To be submitted: Jennifer L. Mumaw, Erin T. Jordan, Jamie Chilton, Yangqing Lu, Kim J. Kelly, Franklin D. West, Deanne King, Aniriban Majumdar, and Steven L Stice

Abstract:

Cellular reprogramming offers the ability to make patient specific stem cells and has potential applications in regenerative medicine and drug discovery. Of the potential cell types available for harvest, peripheral blood is an attractive source due to the ease of collection and minimal risk to donors. However, peripheral blood mononuclear cells (PBMCs) have been difficult to reprogram using conventional methods making their use in induced pluripotent stem cell research limited. Here we show that complete reprogramming of PBMCs is not required for these cells to become pluripotent. Integration of POU5F1/OCT4 in PBMCs with and without KLF4 was capable of producing proliferative colonies when grown in pluripotent culture conditions resembling cell types described in previous reports as "partially reprogrammed" cells. These lines were negative for pluripotent markers alkaline phosphatase, TRA-1-81, TRA-1-60, SSEA4, and e-cadherin indicating lack of full reprogramming to induced pluripotent stem cells (iPSCs). However, after 15 days of differentiation, cultures formed all three germ lineages as seen through expression of markers for mesoderm (desmin), endoderm (alpha fetoprotein) and ectoderm (nestin/tuj). Extended neural differentiation for 5 weeks resulted in the expression of neural/ oligodendrocyte markers PDGR, NeruoD, calbindin, HuB, HuC, HuD, and MAP-2. These results from partially reprogrammed cells provide a new method for generating cells of all three lineages, circumventing the need for complete reprogramming.

Introduction:

The discovery that somatic cells in the adult body could be reprogrammed into cells resembling embryonic stem cells using transcription factors [1] opened up new possibilities in biomedical research. Initial studies have validated the use of induced pluripotent stem cells (iPSCs) for modeling monogenetic diseases that affect the nervous system including Spinal Muscular Atrophy [2] Familial Dysautonomia [3] and Retts Syndrome [4]. These pilot studies have validated the potential for iPSCs to recapitulate the symptoms from diseased donor and react to therapeutic treatments. However, it has been shown that iPSC differentiation potential to neural lineages is less efficient than human embryonic stem cells even though they follow the same pattern of development [5] which may put limit the potential neural applications of iPSC cells. While much progress has been made in understanding iPSCs and the role of the transcription factors, further research is required to optimize protocols for use in disease models or therapeutics.

Initially 4 transcription factors, POU5F1/OCT4, SOX2, c-MYC and KLF4 were used to reprogram mouse somatic cells [1]. These 4 factors [6] and additional factors LIN28 and NANOG are capable of reprogramming human cells; LIN28 and NANOG can be used in place c-MYC and KLF4 [7], or in combination (to include all 6 factors) which increase reprogramming efficiency [8]. As more cell types are reprogrammed the roles of endogenously expressed genes becomes clearer. Keratinocytes which endogenously express higher levels of KLF4 and c-MYC are more efficient at generating iPSCs than fibroblasts [9]. Additionally, neural stem cells with endogenous expression of SOX2, KLF4 and c-MYC can be reprogrammed with only exogenous POU5f1/OCT4 [10]. Using cells with higher levels of "reprogramming" gene expression

increases efficiency and reduces the time for colonies to arise [9, 11]. Using small molecules has also been shown to increase efficiency in reprogramming by affecting chromatin structure [12-14]. Additionally, small molecules can be used to mimic the effect of the reprogramming factors, OCT4 [11], SOX2 [14-18] or KLF4 [19] replacing the need to have these exogenously expressed. Thus far the reprogramming of cells requires that the transcription factors POU5F1/OCT4, SOX2, c-MYC and KLF4 be either added virally, mimicked with small molecules, or already endogenously present in the cell.

Reducing the number of factors required for reprogramming is attractive for minimizing the presence of aberrant proto oncogenes within the genome and limit the need for overexpression of the reprogramming factors. Unfortunately, cells that are efficiently reprogrammed with less factors, such as keratinocytes, are rare in adults [20] and harvesting neural stem cells is a more involved medical procedure [21] with potentially high risk to the donor. While fibroblasts can also be transduced with reduced factors, these cells can harbor genetic mutations that are transmitted to derived iPSCs [22] and are associated with the potential for skin infection and post harvest pain in donors [23]. Blood is an attractive alternative that poses minimal risk and pain to donors and is routinely done by medical professionals. However reprogramming of unmobilized peripheral blood mononuclear cells (PBMCs) has proven to be more difficult than other cell types [24-27] and only isolated T-cells, have been shown to reprogram with efficiency similar to fibroblasts [28]. Since T-cells may potentially have undergone T-cell gene rearrangements [28] reprogrammed T cells may introduce variability depending on the level of rearrangements [29], and should be used with caution when using these cells for reprogramming.

Here we show that transducing PBMCs cells with reprogramming factors results in colony formation resembling what is described in the literature as "partially reprogrammed" [1, 7, 30-33]. While partially reprogrammed cells have been described in both retroviral [1, 7, 30-31] and protein [32-33] reprogramming, little is known of the potency of these cells. Here we show that in PBMCs successfully transduced with POU5F1/ OCT4 alone or with KLF4 generated cell lines in 2/4 donors which formed colonies under hESC culture conditions but lacked both alkaline phosphate activity and pluripotent cell surface markers expression. Despite the partial reprogrammed phenotype these derived lines were capable of differentiation into all three germ layers and could be differentiated into neural cells expressing markers of maturing neural phenotypes, even in the absence of SOX2. SOX2 is known to be expressed in neural epithelium during development [34],and plays a vital role is maintaining neural cell number [35]; however it is dispensable for specification of neural cells and generation of neural stem cells as seen in conditional knockout studies in mouse [35]. These findings underline the importance of OCT4 in lineage specification and demonstrate that partially reprogrammed cells could potentially provide a safer method for generating patient specific cell therapeutics than fully reprogrammed iPSCs.

Material and Methods:

Isolation and culture of human peripheral blood monocytes:

All experiments were performed according to University of Georgia institutional approval from the Office of Human Subjects. 80 mls of blood was harvested via venipuncture from 4 individuals, 2 female and 2 male ranging in ages from 28-35, and collected into a syringe

containing 250 ul of Acid Citric Dextrose per ml of blood collected. The syringes were inverted 3-4 times following collection to ensure proper mixing. The blood was then layered on Histopaque 1077 (Sigma Aldrich). The tubes containing the blood and histopaque 1077 were spun at 400 x g for 30 minutes. The plasma was then removed and discarded and the peripheral blood mononuclear cells (PBMC) were collected from the buffy coat layer. The cells were resuspended in phosphate buffered saline (Hyclone) and spun at 250 x g for 10 minutes, repeating this step 4 times. The cells were finally resuspended in PBMC media, 10% Defined Fetal Bovine Serum (Hyclone), 50 U/ml penicillin, 50 µg/ml streptomycin (Invitrogen), 80 µg/ml Endothelial Cell Growth Supplement (BD Biosiences), in Dulbecco's minimal essential medium (Sigma Aldrich) and plated at 5 X 10^5 cells per cm^2 on plates coated with 100 µg/ml fibronectin (Sigma Aldrich). Cell counts were performed using trypan blue (Sigma) exclusion staining and counted using a hemocytometer. 48 hours after plating the plates were washed with PBS and fresh media was added. For replating, cells were harvested with 0.05% Trypsin (Gibco). All cells were kept at 37° C and 5% CO_2.

Optimization of Transduction:

Optimization was done on three donors, 1 female and 2 male donors ranging in age from 28-35 years old. To optimize the density of transduction cells PBMCs were plated in 96 well plates at 1.37 x 10^5, 2.15 x 10^5, and 2.93 x 10^5 cells per cm^2. These values corresponded to 30-40% confluency, 50-60% confluency and 70-80% confluency respectively. The cells were plated in triplicate of each density from all three donors and transduced 24 hours following plating at a 10 multiplicity of infection (MOI) with the lentivirus EF1α-e GFP from the viPSC kit (Thermo Scientific). The cells were stained with 10 µM Hoest Dye 33342 (Sigma Aldrich) 72

hours following transduction and fixed with 4% Paraformaldehyde (Electron Microscopy Sciences). The plates were imaged with an Eclipse 2000TE-S Inverted Microscope (Nikon). Three images of each well were taken with corresponding UV and GFP filters (Nikon). Cells were counted using Image J software (National Institutes of Health) with an average of 623 cells counted per image. For optimization of day transduction cells were plated at 2.15 x 10^5 cells per cm^2 at 3 and 5 days post harvest in 96 well plates. Cells were transduced, imaged and quantified as described above. For analysis of the effect of MOI on transduction efficiency cells were plated at 2.15 x 10^5 cells per cm^2 and transduced with 0,1,5,10,30 or 50 MOI. Cells were imaged and quantified as described above. For optimization of transduction reagents cells were plated at 2.15 x 10^5 cells per cm^2 and transduced at 10 MOI with the addition of one of the following transduction reagents: Genejammer: 3% Genejammer (Aligent) was mixed in base media lacking antibiotics and serum for 10 minutes and then combined with virus and incubated another 10 minutes and added to wells with complete media; Polybrene (Sigma Aldrich): 8 μg/ml was added to media with virus and incubated 10 minutes. Media was replaced with transduction mix on the plate and centrifugated at 10,000 RPM for 10 minutes; Polybrene and Chondroitin sulfate C (CSC) (Sigma Aldrich): 80 μg/ml of each were added to the media with virus and incubated 10 minutes. Media was replaced on the plate and centrifuged at 10,000 RPM for 10 minutes; Transdux (System Biosciences): Media was mixed with virus and 1X Transdux and then added to the plate. Cells were imaged and quantified as described above.

Reprogramming transduction:

Due to low transduction efficiency of the female donor used in the optimization, a second age/sex/race matched donor was added for reprogramming transductions. PBMC were plated at

2.15 x 10^5 cells per cm^2 in 48 well plates and transduced with the lentiviral vectors from the viPSC kit (Thermo Scientific). The kit contains six factors: POU5F1/OCT4, SOX-2, C-MYC, KLF4, NANOG and LIN 28 (OSCKNL) and a separate eGFP all driven by the EF1α promoter. Transductions were carried out with Transdux as described above at 10 MOI with a ratio of 1:1:1:1:1:1. Transduced cells were replated on feeder layers of mitomycin C (Sigma Aldrich) mitotically inactivated murine embryonic fibroblasts (MEFs) 3 days following transduction and switched to hESC/ PT-RPs medium: Dulbecco's minimal essential medium/Ham's F12 medium (DMEM/F12), 2 m*M* L-glutamine, 0.1 mM minimal essential medium (MEM) nonessential amino acids, 50 U/ml penicillin, 50 µg/ml streptomycin (all from Invitrogen), basic fibroblast growth factor (hESC: 4 ng/ml; PT-RPs: 10 ng/ml) (bFGF; R&D Systems) and 20% KnockOut Serum Replacement (Invitrogen) the day after plating.

Human embryonic stem cell (hESC) culture and partially reprogrammed cells (PT-RPs) culture:

WA09 hESCs derived from excess in vitro fertilized human embryos were obtained from WiCell and PT-RPs cells were obtained following transduction with OSCKNL factors were cultured on MEFs with daily media changes. Colonies were manually dissociated and passaged to new feeder layers every 3-4 days.

PT-RPs Directed Neural Differentiation:

PT-RPs were cultured in neural induction media with 1X N2 Supplement (Invitrogen) 2mM L-Glutamine, 50 U/ml penicillin, 50 µg/ml streptomycin and 4 ng/mL bFGF on MEFs for 7 days and then replated onto plates coated with Matrigel (BD biosciences) at 0.0123 mg per cm^2 surface area of culture vessel. Cells were maintained in neural maintenance media with 1X ANS

(ArunA Biomedical), 2mM L-Glutamine (Gibco), 50 U/ml penicillin, 50 µg/ml streptomycin (Invitrogen) and 20 ng/mL bFGF (Millipore) in AB2 medium (ArunA Biomedical). Cultures were passaged 1:2 with manual dissociation every 72 hours and plated on fresh matrigel coated plates. Maturation was performed by removing bFGF from the culture with media changes every 2 days for 5 weeks.

hNP Maintenance and Differentiation:

hNP1 ™ (ArunA Biomedical Inc.) cells were derived from WA09 hESCs and maintained as described previously [36]. Briefly, cells were grown on plated coated with Matrigel (BD Biosciences) at 0.0123 mg per cm^2 surface area of culture vessel and cultured in neural maintenance media. Cells were passaged approximately every 48 hours and split 1:2 following manual dissociation. Cells were differentiated with the same conditions as described for PT-RPs.

Differentiation:

hESCs and PT-RPs were switched to ESC media lacking bFGF with media exchanges every other day for 15 days. 15 day differentiated and undifferentiated hESC and PT-RPs were fixed with 4% Paraformaldehyde (Electron Microscopy Sciences).

RNA/DNA isolation RT-PCR and PCR:

hESCs and PT-RPs cells were manually isolated from MEF layers and neural differentiation cultures were directly harvested: mRNA was isolated from cultures of hESC at passage 68 and PT-RPs at passage 12. RNA was extracted using the RNeasy kit (Qiagen)

following the manufacturer's instructions. cDNA was synthesized using iScript cDNA synthesis kit (Bio-Rad). Polymerase chain reaction was performed using primers listed in Supplementary Table 1. The reaction was completed using GoTaq Green Master Mix (Promega) and run on 1.5% agarose (Bio-Rad) gels.

Immunocytochemistry:

For intracellular markers cells were permeabilized with 0.1% triton, 6% goat serum in Tris buffer for 20 minutes followed by incubation in blocking buffer consisting of 6% goat serum in Tris buffer for 45 minutes. For cell surface labeling, permeabilization was excluded. Primary antibodies were diluted in blocking buffer for 1 hour at room temperature and washed 2 times with Tris buffer before secondary antibody application. Secondary antibodies were diluted in blocking buffer and applied for 1 hour at room temperature. For extracellular markers the procedure was the same as described above without permeabilization. The cells were then extensively washed with Tris buffer. The slides were mounted with ProLong Gold antifade reagent with DAPI (4',6-diamidino-2-phenylindole, Molecular Probes) for cell nuclear staining. Primary antibodies and dilutions used included the following: Mouse anti-Nestin (1:200; Neuromics, Inc.), mouse anti-beta III tubulin(Tuj) (1:250; Neuromics),mouse anti-SSEA4 (1: 200; Developmental Studies Hybridoma Bank) mouse anti-Desmin (1:100 BD Biosciences), and mouse anti-alpha fetoprotein(1:50; Santa Cruz Biotechnology). Conjugated primary antibodies and dilutions include the following: Mouse Alexa fluor anti-Tra-1-81 (1:200; BD Biosciences), Alexa fluor anti-Tra-1-60 (1:200; BD Biosciences) and FITC anti- E-Cadherin (1:200; BD

Biosciences). Secondary antibodies and dilutions used included the following: Goat anti-mouse IgG Alexa Fluor® 488 (1:1000; Invitrogen)

Results:

PBMCs have a low transduction potential: Optimization of transduction with lentiviral EF1α-eGFP was performed examining the effects of plating density, day of transduction post harvest, MOI and transduction reagents. Plating density affected transduction efficiency, 50-60% confluency (2.15 x 10^5cells per cm^2) had higher percent GFP positive cells than plating density of 30-40% (1.37 x 10^5 cells per cm^2) ($p<0.05$)(Supplemental Figure 4.1A) and transduction percentages did not change based on whether the cells were transduced 3 or 5 days post harvest (Supplemental Figure 1B). All future transductions were performed on day 5 post harvest. Increasing MOI showed that cells isolated from all donors had higher GFP percent positive at higher MOI transductions ($p<0.001$) (Figure 4.1A). Differences between MOI effects in each donor were also noted ($p<0.05$) (Figure 4.1A). The use of transduction regents was able to increase transduction efficiency in 2/3 donors (donor 2 and 3). Donor 1 showed no increase in transduction efficiency. In donor 2, Transdux increased transduction efficiency over all other conditions ($p<0.05$) and in donor 3 both Transdux and Polybrene showed an increase in GFP positive cells from other conditions ($p<0.05$) but were not different from each other. Transdux was used for the remainder of the experiments. In comparison to previous reports of 44% transduction efficiency with 20 MOI in cord blood [26], even under optimal conditions of Transdux at 50-60% confluency, PBMC transduction efficiency was lower as in Donor 2 (Figure 4.1C).

Transduction of PBMCs with OSCKNL did not result in AP positive colonies: Transduction with all 6 factors, OSCKNL, resulted in colony formation from 2/4 donors, one from a male donor and one from a female donor (Figure 4.2A, 4.2C). Colonies were first apparent on day 5 and day 7 post transduction for PT-RP line 1 and PT-RP line 2 respectively. The colonies from PMBCs (Figure 4.2A, 4.2C) were less compacted than the hESC (Figure 4.2E). Alkaline phosphatase (AP) staining was performed to examine the amount of AP activity, a marker for pluripotency. The partially reprogrammed (PT-RP) colonies from the PBMCs did not show positive staining with AP (Figure 4.2B, 4.2D) when compared to the hESCs (Figure 4.2F). PBMCs that were not transduced did not from colonies when cultured in hESC medium for 40 days and were indistinguishable from MEFs after 14 days of culture.

Integration of pluripotency genes in PT-RPs: PCR analysis was done on DNA from the two lines to examine which factors were integrated into the PT-RP lines through use of a forward primer in the EF1α promoter and a reverse primer in each of the 6 factors, OSCKNL (All primers are listed in Supplemental Table 4.1). Both lines had integration of POU5F1/OCT4 and PT-RP line 2 also had integration of KLF4 (Figure 4.2G). The positive cell lines used were from animal lines transduced with all six factors from the viPSC kit which had previously been verified for gene integration from the viral vectors (Liu et al., PNAS submitted).

Expression of pluripotency genes in PT-RPs: To examine which exogenous reprogramming factors were being expressed in the PT-RP colonies, RT-PCR was done on the cDNA isolated from PT-RP Line 1, PT-RP line 2, non-transduced PBMCs and control hESCs. Both PT-RP lines

expressed pluripotency markers POU5F1/OCT4, c-MYC, and KLF4, but not other hESC pluripotency markers SOX2, NANOG, and LIN28 (Figure 4.2F).The non transduced PBMCs expressed endogenous POU5F1/OCT4 C-MYC and KLF4 (Figure 4.2F).

PT-RP colonies do not express cell surface pluripotency markers: Pluripotency markers TRA-1-81, TRA-1-60 and SSEA4 can be used to distinguish pluripotent cells from differentiated cells. The PT-RPs showed no expression of TRA-1-80 (Figure 4.3A, 4.3B), TRA-1-60 (Figure 4.3E, 4.3F) SSEA4 (Figure 3I,3J) or E-caherin (E-CAD) (Figure 4.3L, 4.3M) that were present in hESC cultures and marked cells that are undifferentiated (Figure 4.3C, 4.3D, 4.3G, 4.3H, 4.3K, 4.3L, 4.3N, 4.3O).

In vitro differentiation of PT-RPs altered cell morphology and expressed markers of all three germ layers: Random differentiation by removing bFGF from the medium resulted in the differentiation of the PT-RP cells. Cells with a flattened mesodermal morphology (Figure 4.4A, arrows), cells with a compacted endodermal morphology (Figure 4.4B, in circle), and cells with neural morphology of the ectoderm (Figure 4.4C, arrows) were present by 10 days of differentiation. At 15 days of differentiation PT-RPs expressed the mesodermal marker desmin (Figure 4.4D, 4.4E), endodermal marker alpha fetoprotein (Figure 4.4H, 4.4I), and in 5 weeks of neural enhancing differentiation conditions expressed early neural marker Nestin (Figure 4.4L, 4M) and differentiating neural marker Tuj (Figure 4.4P, 4.4Q). PT-RPs retained a rounder cellular morphology following differentiation when compared to hESCs for mesodermal (Figure 4F, 4G), endodermal (Figure 4.4J, 4.4K) and neural enhancing differentiation(Figure 4.4N, 4.4O; 4.4R, 4.4S).

PT-PR in enhanced neural conditions express PAX6 before differentiation and markers of more advanced neural types following extended differentiation: Neural genes were analyzed with RT-PCR from undifferentiated colonies and cultures differentiated for 5 weeks in enhanced neural differentiation conditions. When grown in hESC culture conditions hESCs and PT-RP cells expressed platelet derived growth factor receptor (PDGFR) , and PT-RPs also expressed an early neural marker, PAX6, which was absent in hESCs (Figure 4.5A). Following 5 weeks of differentiation with removal of bFGF, when grown in neural enhancing conditions known to induce neuronal differentiation [37], PT-PR expressed markers of neural lineage NeuroD and oligodendrocyte marker PDGFR, and markers of more differentiated neural types including calcium binding protein, Calbindin, differentiated microtube assembly protein, microtubule associated protien2 (MAP-2), post mitotic neural markers HuB, HuC, and HuD (Figure 4.5B).

Discussion:

Despite their reduced propensity to reprogram [24-27] PBMCs are an attractive cell source for generating pluripotent cells. The epigenetics of human blood CD34+ and mononuclear cells were shown to be closer to ESCs and iPSCs than other examined cell types [24], and the epigenetic marks in mouse blood derived iPSCs resembled the marks in ESCs more than iPSCs generated from other tissues [38]. These epigenetic signatures of PBMC might also have been permissive for pluripotency seen in the PT-RP cells generated here. While we cannot rule out the possibility of T-cells being partially reprogrammed in the current experiment, the culture

conditions were not permissive for T-cell growth and further examination of the T- cell rearrangement region will determine if PT-RPs are of T-cell origin.

PBMCs also showed expression of three pluripotent factors, c-MYC, KLF4, and POU5F1/OCT4, before reprogramming, similar to findings in cord blood [39]. PBMCs failed to generate iPSC or partially reprogrammed colonies when cultured in hESC conditions, suggesting that culture conditions alone did not reprogram the cells and exogenous factors were required to obtain the PT-RPs pluripotent cell type. In other cell types expressing endogenous c-MYC and KLF4, transduction with only POU5F1/OCT4 was unable to generate proliferate colonies [9] further indicating that epigentics of PBMCs make them more amenable to partial reprogramming.

Partially reprogrammed cells generated by increased POU5F1/OCT4 may present unique pluripotent characteristics over other types of partially reprogrammed cells. POU5F1/OCT4 has been shown to be a master regulator of lineage development with concentration dependent specification of mesoderm, endoderm and ectoderm from POU5F1/OCT4 in ESCs [40]. Additionally, over expression of POU5F1/OCT4 in mesenchymal stem cells (MSCs), a cell type with high levels of plasticity as seen through expression of pluripotency genes NANOG, SOX2 and REX-1 [41], resulted in increased survival signaling, telomerase activity, down regulation apoptotic signaling and enhanced neurosphere formation and presence of all three germ layers in teratoma formation [42]. Additionally, POU5F1/OCT4 over expression in skin derived fibroblasts induced *in vitro* formation of multipotent blood cells [43]. Similar to results in the current study,POU5F1/OCT4 transduced fibroblasts could generate colonies, however the fibroblast derived colonies were only capable of proliferating for approximately 7 passages [43]. Here PT-RPs were still proliferative at passage 15 and were not senescing at that stage.

POU5F1/OCT4 transduced fibroblasts expressed both SOX2 and NANOG [43], but neither gene was expressed in our PT-RPs cultures. POU5F1/OCT4 over expression in MSCs [40], fibroblasts[43], and PBMCs in this study, indicated that increases of POU5F1/OCT4 over endogenous levels can promote lineage conversions. However the resulting alternate lineages derived from POU5F1/OCT4 over expression may be dependent on the starting population. POU5F1/OCT4 over expression in fibroblasts was unable to generate teratomas in immune compromised mice, showing limited lineage differentiation potential [43]. However, POU5F1/OCT4 over expressed in MSCs did form teratomas, indicating these cells could differentiate into all three germ layers [42]. PBMC derived PT-RPs like MSCs had the capacity to differentiate into all three germ lineages, indicating that features shared between these two cell types permitted greater plasticity than seen in fibroblasts.

The findings in this project support the hypothesis that POU5F1/OCT4 is binding to sites that are known to be bound by POU2F1/OCT1 and through this binding, POU5F1/OCT4 is capable of inducing the expression of developmental genes involved in promoting a conversion to partially reprogrammed cells [43]; The expression of PAX6 has been shown to be regulated by POU2F1/OCT1 and SOX2 in neural development [44], and we observed that PAX6 was expressed in both lines that expressed POU5F1/OCT4 in the absence of SOX2 expression. Given PAX6 role as a master regulator gene of neural specification in humans [45], our results also indicate that induced POU5F1/OCT4 expression might influence PAX 6 and resulting neural potency without involving SOX2. In contrast co-binding of SOX2 and POU5F1/OCT4 is believed essential for neural lineage differentiation in the developing mouse ICM [46]. Our studies suggest that POU5F1/OCT4 alone may have regulatory effects independent of SOX2 in human neural development.

Continued expression of POU5F1/OCT4 was seen both in PT-RPs and the differentiated cultures. This lack of silencing of POU5F1/OCT4 upon differentiation is a major drawback if these cells are to be used in the future. Despite continued expression of POU5F1/OCT4 PT-RPs in the absence of bFGF, a portion of the cells differentiated into the three germ lineages. We believe that our culture may be heterogeneous, that some PT- RPs cells were still expressing POU5F1/OCT4 while in others the expression POU5F1/OCT4 was silenced and permissive of differentiation. Examining the differentiating cells for individual expression for POU5F1/OCT4 and markers of differentiation will help to determine if the differentiating cells are still expressing POU5F1/OCT4 or if this expression is due to populations of undifferentiated cells. To further circumvent the continued expression of POU5F1/OCT4 from transduced genes, combing these findings with alternate reprogramming methods can be used. Partially reprogrammed cells have been reported to arise during the generation of iPSCs with recombinant proteins [32-33] and we believe that introducing POU5F1/OCT4 proteins in a conducive cell type such as PBMCs may be more effective in generating uniform partially reprogrammed populations by avoiding the potential problems associated with integrating and continued expression viral POU5F1/OCT4.

Conclusions:

PT-RPs provide a method of lineage conversion that does not go through a fully reprogrammed iPSC state [1] or use master regulatory genes for lineage conversion[47-48]. These cells provide the first report of all three germ cell lineage conversion without the endogenous or exogenous SOX-2 expression indicating that SOX-2 is required for iPSCs but not

for generating partially reprogrammed pluripotent cell capable of differentiating into all three germ lineages.

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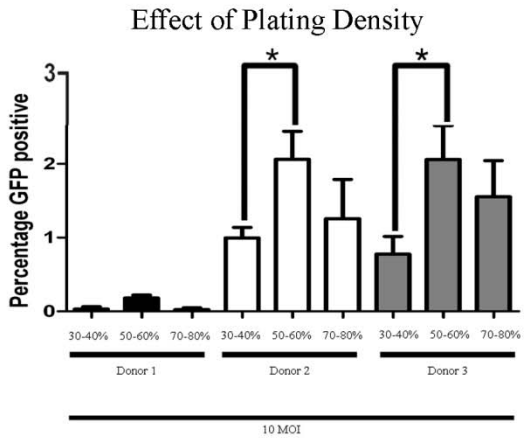
Supplementary Table 1: RT-PCR and PCR primers for pluripotent transcription factors and neural markers:

Viral Specific Primers		5'-3' Sequence
EF1α Forward		TGGAATTTGCCCTTTTTGAG
POU5F1 Reverse		CCTTGGAAGCTTAGCCAGGT
SOX2 Reverse		GAGGCAAACTGGAATCAGGA
c-MYC Reverse		TGCCTCTCGCTGGAATTACT
KLF4 Reverse		ACTCCGGTGAGTTGTGTGG
NANOG Reverse		CAGATCCATGGAGGAAGGAA
LIN28 Reverse		TCTGGTGCACAAAGACATCC
Pluripotent Gene Expression Primers		5'-3' Sequence
POU5F1	Forward	GAGAAGGAGAAGCTGGAGCA
	Reverse	TCGGACCACATCCTTCTCG
SOX2	Forward	GGGGGAAAGTAGTTTGCTGCCTCT
	Reverse	TCCGCCGGGGCCGGTATTTA
c-MYC	Forward	TGCATCGACCCCTCGGTGGT
	Reverse	TCGGTGTCCGAGGACCTGGG
KLF4	Forward	GCGGGCTGCGGCAAAACCTA
	Reverse	AAGGCGAGGTGGTCCGACCTG
NANOG	Forward	AGGAAGACAAGGTCCCGGTCAA
	Reverse	GGCCTTCCCCAGCAGCTTCC
LIN28	Forward	GGCTCCGTGTCCAACCA
	Reverse	CAGGAGCCTGCCTCTTTT
GAPDH	Forward	TTGATTTTGGAGGGATCTCG
	Reverse	GAGTCAACGGATTTGGTCGT
Neural Lineage Gene		5'-3' Sequence
NEUROD	Forward	TGCCCAGTCCGCCTTACGGT
	Reverse	AACTCGGCGGACGGTTCGTG
PDGRR-α	Forward	GGGCCGTGTGACTTTCGCCA
	Reverse	CCCCAAGACCCGACCAAGCA
PAX6	Forward	ACCGTGGCTCGGCCTCATTTC
	Reverse	CACTCCGCTGTGACTGTTCTGC
CALB1	Forward	CGCTGACGGAAGTGGTTACCTGG
	Reverse	TAGCAAGTGGTTGCGGCCACC
ELAVL2	Forward	TGGACTTTGGGGTCGAAAGCGT
	Reverse	GCCATATCCCAAGCTCTGCCCTGT
ELAVL3	Forward	TGGTGGACCAGGTCACAGGTGTC
	Reverse	TCGATGGCGATCGGCGAGAA
ELAVL4	Forward	CAAGTCACAGGAGTGTCCAGAGGGG
	Reverse	GGTAATTGGGGAGAACCTGGGGG
MAP2	Forward	CCATCACTCCTGGCACCCG
	Reverse	CACCTGGCCTGTGGCGGAT

Figure Supplementary 4.1: PBMC transduction optimization of MOI and day of transduction post harvest using lentiviral EF1α-eGFP A) PBMCs from three donors were plated at three densities 1.37 x 10^5, 2.15 x 10^5, and 2.93 x 10^5 cells per cm^2 and transduced with 10 MOI (* $p<0.05$). The plating densities corresponded to 30-40% confluency, 50-60% confluency and 70-80% confluency respectively B) To test the effect of say of transduction the PBMCs were plated at 2.15 x 10^5 cells per cm^2 and transduced at 10 MOI with Transdux

A


Effect of Plating Density
Percentage GFP positive
3
2
1
0
*
*
30-40%
50-60%
70-80%
30-40%
50-60%
70-80%
30-40%
50-60%
70-80%
Donor 1
Donor 2
Donor 3
10 MOI

B

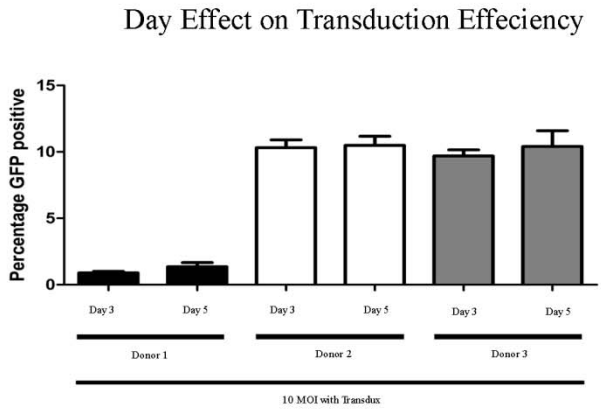

Day Effect on Transduction Effeciency
Percentage GFP positive
15
10
5
0
Day 3
Day 5
Day 3
Day 5
Day 3
Day 5
Donor 1
Donor 2
Donor 3
10 MOI with Transdux

Figure 4.1: PBMCs transduction optimization of MOI and transduction reagent with EF1α-eGFP: A) PBMCs from 3 donors were transduced with increasing MOIs from 0,1,5,10,30 to 50 without transduction reagents. (*$p<0.05$) B) PBMCs were transduced in the presence of transduction reagents, Genejammer, Transdux, Polybrene and Polybrene plus CSC. Transductions were performed at 10 MOI. (*$p<0.05$) C) Phase, GFP and Phase/GFP/Hoechst dye staining overlay of donor2, 50-60% confluent at 10 MOI with Transdux 72 hours after transduction.

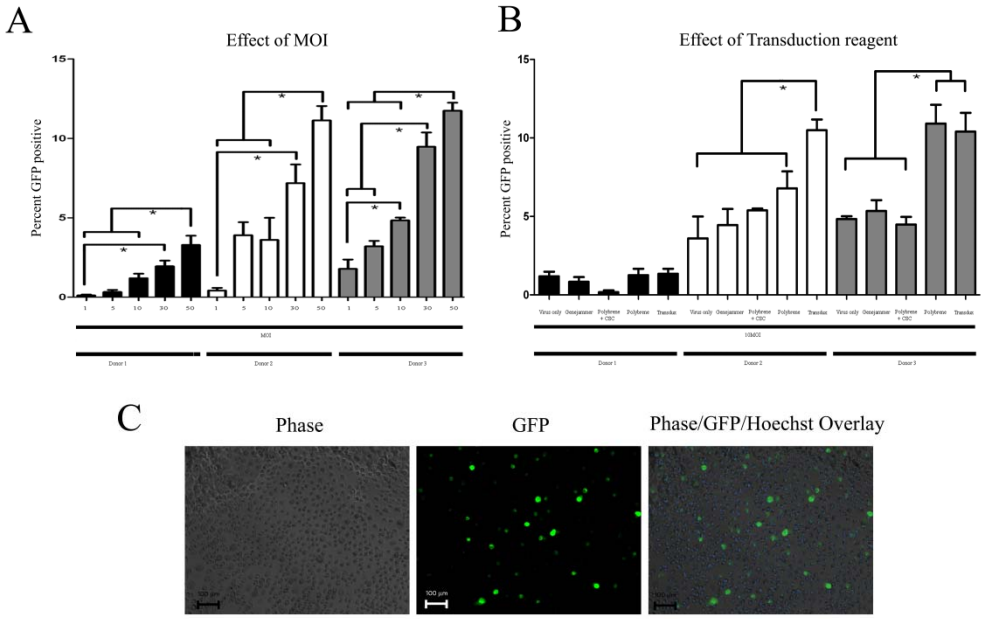
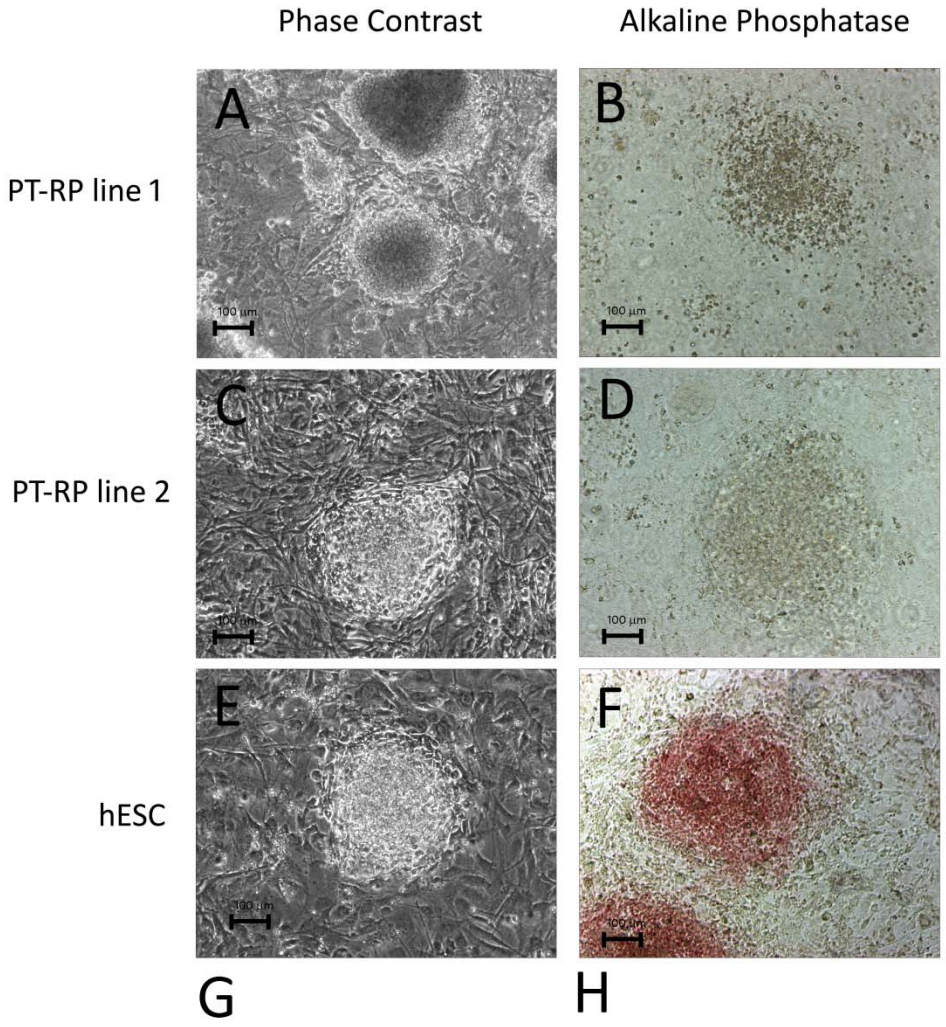
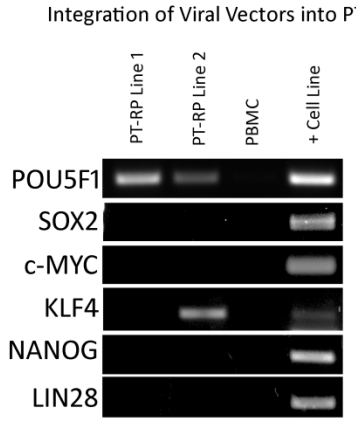

A
Effect of MOI
Percent GFP positive
15
10
5
0
1
5
10
30
50
MOI
Donor 1
Donor 2
Donor 3
B
Effect of Transduction reagent
Percent GFP positive
Virus only
Ostojaimmer
Polybrene + CBC
Polybrene
Transduc
VSMOI
C
Phase
GFP
Phase/GFP/Hoechst Overlay
100 µm

Figure 4.2: Colony morphology; Pluripotency marker, Alkaline Phosphatase (AP) staining; Viral pluripotency gene integration; Pluripotent transcription factor expression: PT-RP line 1 A) Phase contrast of colonies on MEF layer B) AP staining of colonies; PT-RP line 2 C) Phase contrast of colonies on MEF layer D) AP staining of colonies; hESCs E) Phase of colonies F) AP staining of colonies. G) PCR for integration of transcription factors into PT-RP, PBMCs and positive cell line (+ cell line). The positive cell line was taken from a transduced animal cell line with verified integration of viPSC genes. H) RT-PCR of expression of pluripotent genes POU5F1/OCT4, c-MYC , and KLF4 in PR-PR line1, PT-RP line 2, PBMCs and hESCs.


Phase Contrast
Alkaline Phosphatase
PT-RP line 1
PT-RP line 2
hESC
A
B
C
D
E
F
100 µm
G
H


Integration of Viral Vectors into PT-RP
PT-RP Line 1
PT-RP Line 2
PBMC
+ Cell Line
POU5F1
SOX2
c-MYC
KLF4
NANOG
LIN28

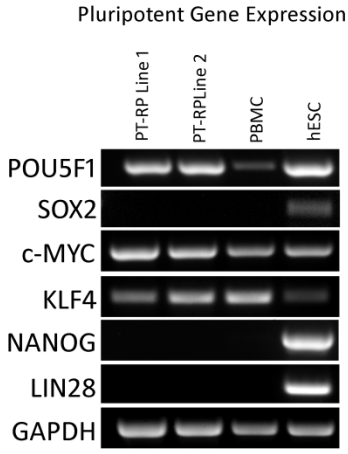

Pluripotent Gene Expression
PT-RP Line 1
PT-RPLine 2
PBMC
hESC
POU5F1
SOX2
c-MYC
KLF4
NANOG
LIN28
GAPDH

Figure 4.3: Pluripotent cell surface marker expression in PT-RP and hESCs. A-D) TRA-1-81, E-H) TRA-1-60, I-L) SSEA-4, and M-P) Blue- nuclear stain for Dapi.

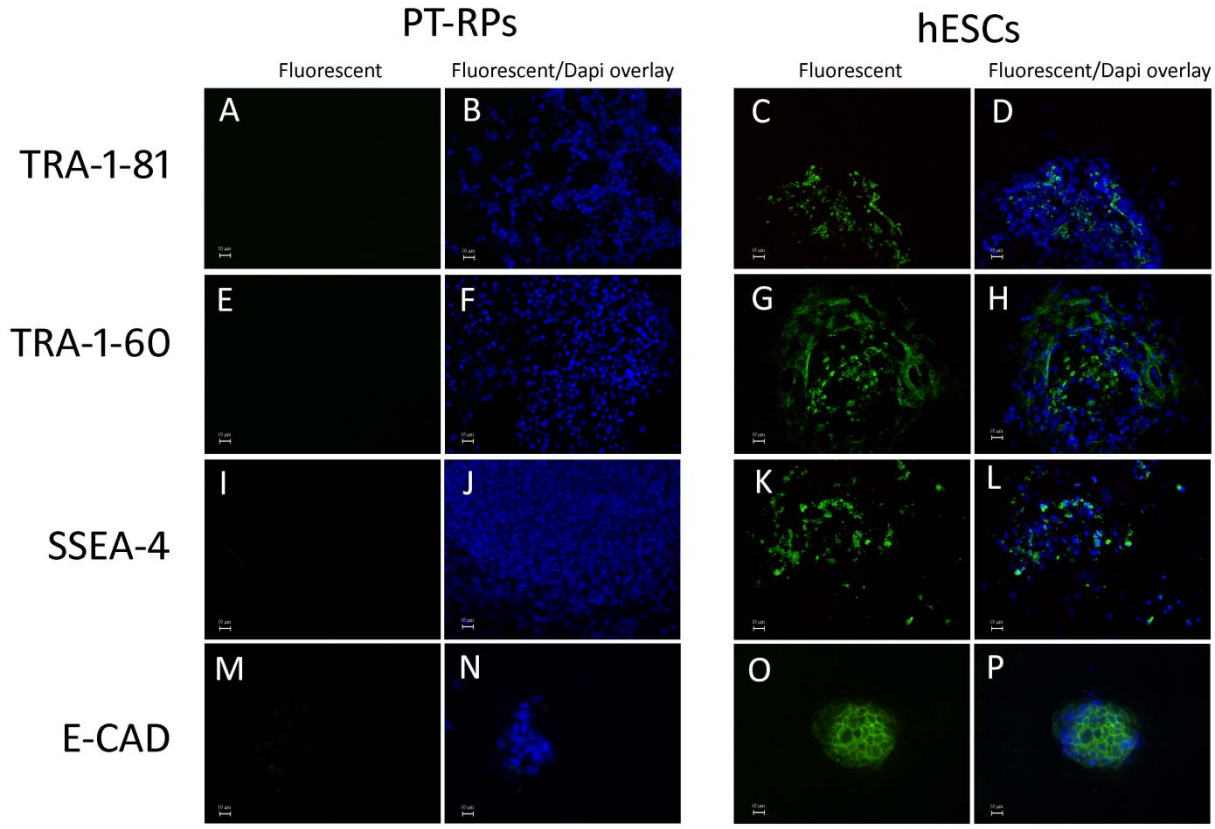

PT-RPs
hESCs
Fluorescent
Fluorescent/Dapi overlay
Fluorescent
Fluorescent/Dapi overlay
TRA-1-81
TRA-1-60
SSEA-4
E-CAD
A
B
C
D
E
F
G
H
I
J
K
L
M
N
O
P

Figure 4.4: 10 days of differentiation following differentiation induction by removing bFGF. PT-RP phase contrast showed A) cells with flattened morphology indicative of mesodermal lineage (arrows) B) tightly packed groups of cells with morphology of the endodermal lineage and C) cells with defined somas and finer processes representing the ectodermal lineage. Staining of PT-RPs and hESC 15 days of differentiation by removing bFGF, for Endodermal marker alpha fetoprotein (D-G), Mesodermal marker Desmin (E-H), and Ectodermal early neural differentiation marker Nestin (I-L) and maturing marker Tuj (M-P).

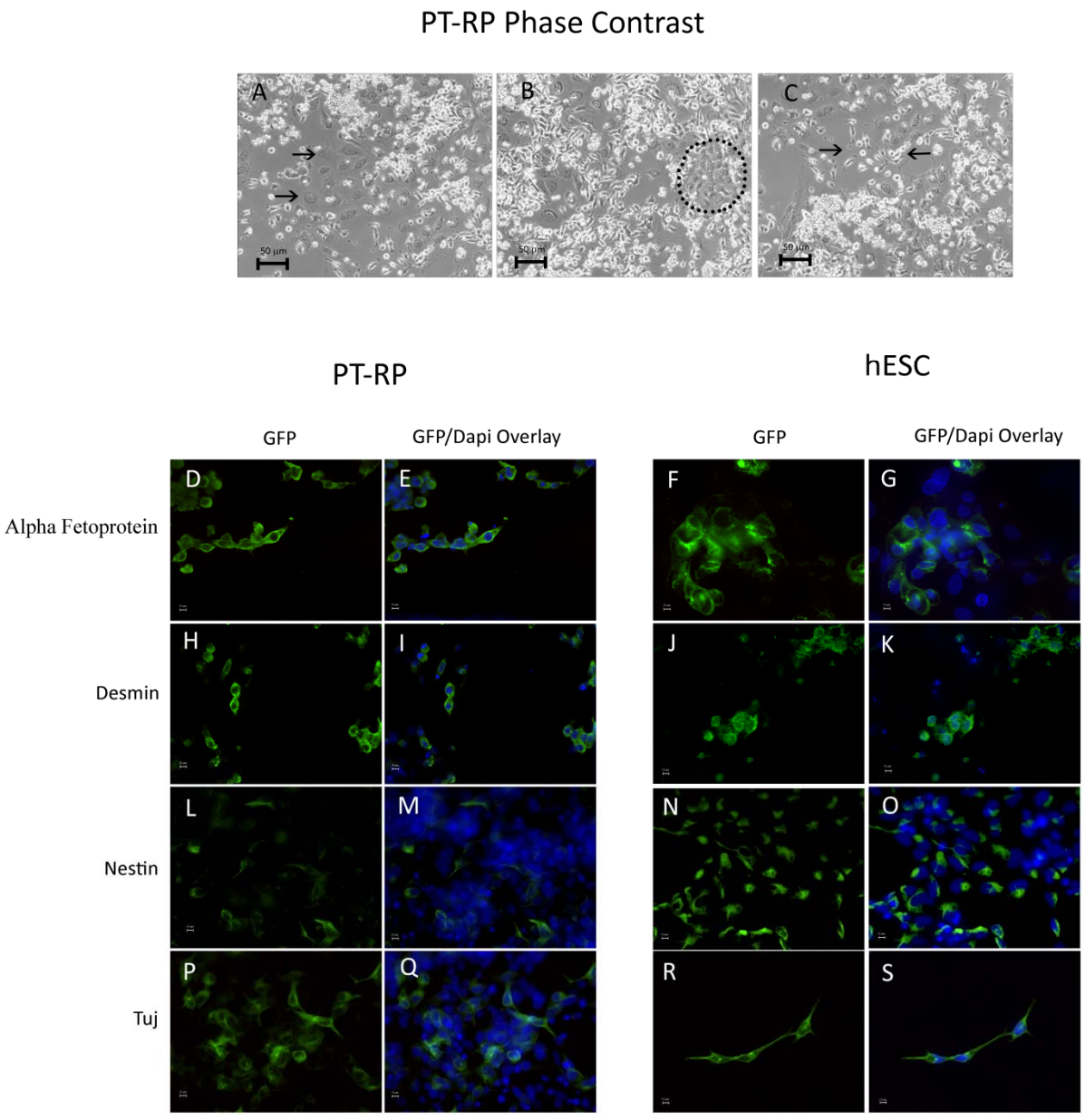
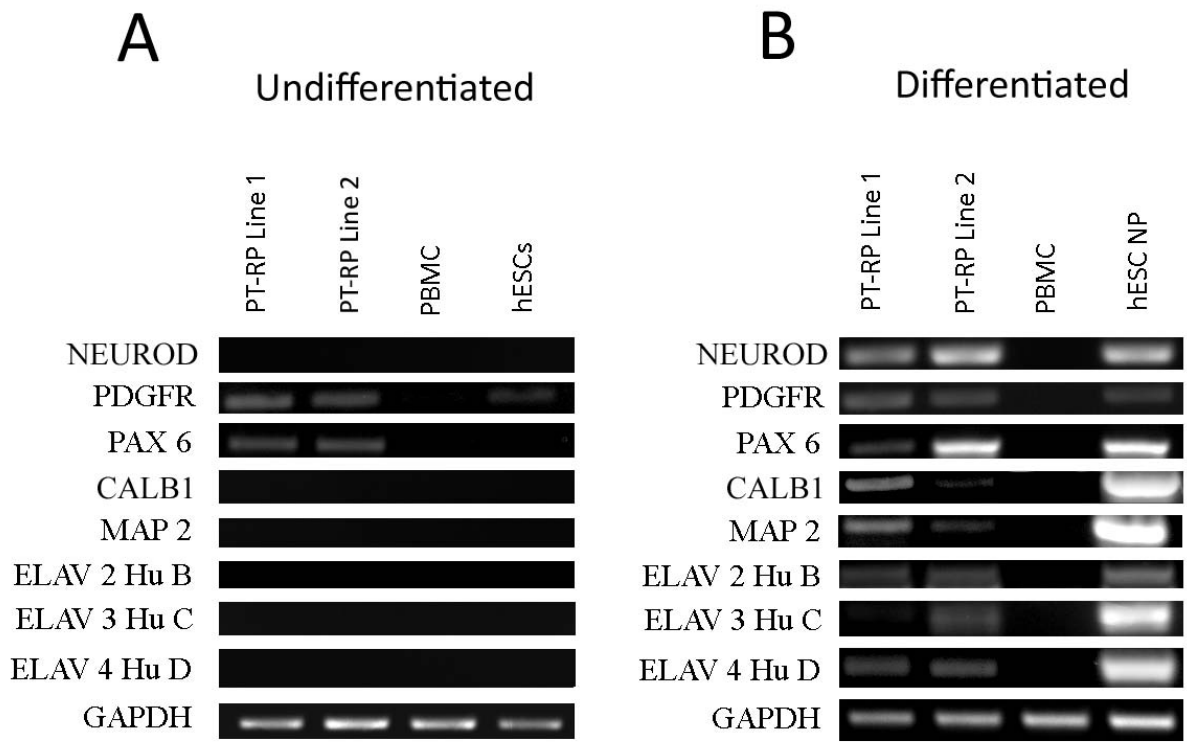

PT-RP Phase Contrast
A
B
C
50 µm
50 µm
50 µm
PT-RP
hESC
GFP
GFP/Dapi Overlay
GFP
GFP/Dapi Overlay
Alpha Fetoprotein
Desmin
Nestin
Tuj
D
E
F
G
H
I
J
K
L
M
N
O
P
Q
R
S

Figure 4.5: RT-PCR of neural lineage marker NEUROD, oligodendorcyte marker PDGFRα, calcium binding protein CALB1, neural differentiation marker MAP2, and post mitotic markers HuB, HuC, and HuD. A) before differentiation and B) following 5 weeks of neural differentiation.


A
Undifferentiated
PT-RP Line 1
PT-RP Line 2
PBMC
hESCs
NEUROD
PDGFR
PAX 6
CALB1
MAP 2
ELAV 2 Hu B
ELAV 3 Hu C
ELAV 4 Hu D
GAPDH
B
Differentiated
PT-RP Line 1
PT-RP Line 2
PBMC
hESC NP
NEUROD
PDGFR
PAX 6
CALB1
MAP 2
ELAV 2 Hu B
ELAV 3 Hu C
ELAV 4 Hu D
GAPDH

CHAPTER 5

CRYOPRESERVATION OF BMP-2 GENETICALLY MODIFIED PEG ENCAPSULATED MSCS

To be Submitted: Jennifer L. Mumaw, Erin T. Jordan, Corinne Sonnet, Franklin D. West, Yangqing Lu, Elizabeth Davis, Alan Davis, Jennifer West, and Steve L. Stice

Abstract:

Autologous bone grafting is currently the most effective treatment for long bone nonunion fractures; however, it is not without considerable risks to donors necessitating the development of alternative therapeutics. Currently poly (ethylene glycol) microencapsulation technology and BMP-2 transgene delivery systems are being developed together as an effective method for inducing rapid bone formation. With the promise of these technologies, methods to make these treatments available for clinical application are presently lacking. In this study we used mesenchymal stem cells (MSCs) as a vector for transgene production. MSCs were chosen due to their ease of harvest, replication potential and immunomodulatory capabilities. MSCs used were from sheep and pig due to their use as large animal models for bone nonunion. We demonstrated that the cryopreservation of these microencapsulated therapeutic MSC did not affect their cell viability, transgene BMP-2 production or ability to initiate bone formation in a mouse model for heterotopic ossification when compared to freshly prepared samples. Additionally, microspheres showed no appreciable damage from cryopreservation when examined with light and electron microscopy. While the viability of MSCs in all conditions were reduced by 4 days following encapsulation when compared to the day of encapsulation, genetically modified encapsulated MSCs at 4 days post encapsulation also showed additional reduction in viability. These results validate the use of cryopreservation in preserving the viability and functionality of PEG encapsulated BMP-2 transduced MSCs, but reduced viability by day 4 post encapsulation indicates that further optimization of encapsulation protocols are required for prolonged MSC survival in order to be an effective therapeutic.

Introduction:

Bone is the second most transplanted tissue behind blood transfusions[1]. Autologous bone grafting is currently considered the gold standard for treating nonunions [2], but multiple features make it less than ideal for long bone nonunion treatment. The most promising graft donor site, the iliac crest, is available in limited quantity [3]. As long bone nonunions can require up to 30 mls of marrow, the amount harvested from the iliac crest can be insufficient [4]. Bone grafting presents considerable risks to patients by increased surgical times and blood loss [5], with 1/3 of patients experiencing chronic pain 24 months post-transplant [6] and recipients being at increased risk for donor site instability and fractures [7]. Additionally, large bone defects like those received by soldiers injured in combat [8-9] often do not heal without surgical intervention ending often in an undesirable outcome, amputation [10].

Bone morphogenetic protein 2 (BMP2) is a potential therapeutic that can fill the need for bone healing. Recombinant BMP2 can induce rapid ossification in orthopedic applications [11-12] but has a relatively short half life and is administered at high dosages or continually maintained to promote extensive and expedited bone regeneration [13-15]. Having a fast and maintained release/production of BMP-2 as an off the shelf therapeutic might be used without the morbidity associated with bone grafting, reduce recovery time and minimize future surgeries. Mesenchymal stem cells (MSCs) could be a vector for delivering BMP2, MSCs have several potential advantages: they can be easily harvested from adult bone marrow [16] and adipose tissue [17-18], are immunomodulatory [19-21], have allogeneic tolerability [20], are easily expanded in vitro and differentiate into bone even after long term culture [18, 22].

Cellular encapsulation with genetically engineered cells producing BMP2 in a PEG polymer for bone regeneration was developed to extend expression of BMP-2 *in vivo* [23]. PEG is an attractive material for biomedical applications with biocompatibility in multiple tissues [24-28]. Additionally, the mechanical properties of PEG can be altered to replicate that of soft tissue through the incorporation of extracellular matrix proteins [29] and copolymers such as poly (propylene fumarate) [30]. As soft tissue injury often occurs at the same time as long bone injury healing involves the regeneration of both tissues[31]. Mimicking *in vivo* soft tissue has been shown to be more permissive for physiological healing in creating an environment permissive for angiogenesis [32], a vital component for correct bone healing [33]. PEG-DA can be biodegradable in tissues through manipulations of the peptide sequences linking PEG moieties which makes the structure cleavable through proteolytic processes [34-37] allowing the polymer and encapsulated cells to be removed by the body during the healing process. Initial studies with BMP-2 transduced cells encapsulated in PEG have been shown to be superior to unencapsulated cells through the extended presence at the site of treatment and increased induction of heterotopic ossification in the mouse [38]. This therapy has applications in human medicine for replacing or use in conjunction with current technologies for increasing the rate of bone healing; however, processes to make the PEG encapsulated cells available for immediate use are lacking.

Cryopresered cells can be stored as "ready to use" prior to the therapeutic application [39], and cryopreservation of PEG-DA cell encapsulation preparation would also enhance and widen their therapeutic uses because the encapsulation and testing of preparations could be conducted well in advance in controlled good manufacturing practices (GMP) facilities for distribution to the clinical setting. Cryopreservation allows for thorough testing of the encapsulated MSCs with the ability to thaw samples for validating cell viability, therapeutic

production, sterility, and microbead integrity to ensure that the highest quality production had been preformed. However the post thaw affects of PEG encapsulation on cell survival, transgene expression and biological activity has not been previously investigated.

Pig and sheep are a suitable models for human bone studies with long bone dimensions [40-41] and structure [42-43] that are similar to man. Pig has been shown to have similarities in bone remodeling [42] while sheep provide a comparable model for bone in growth into osteoconductive biomaterials [44]. Using MSCs isolated from both pig and sheep we have explored the possibility of cryopreserving PEG encapsulated MSCs expressing BMP-2. The cryopreservation of the cells within the polymers showed no reduction in viability in comparison to non-preserved encapsulated MSCs, and the encapsulated cellular spheres showed no physical damage resulting from cryopreservation. It was also found that cell lines from various donors may have different potentials in genetic modification and transgene production. Using this process genetically modified cryopreserved MSCs producing BMP-2 maintained function as seen through initiation of bone formation in an *in vivo* model for heterotopic ossification. While optimization for increasing cell viability in PEG are still required, these results demonstrate PEG microbeads have the potential to be manufactured for "off the shelf" therapeutic use.

Material and Methods:

MSC isolation and culture:

Porcine MSCs were isolated previously [45] and ovine MSCs were isolated with the same plate adherency techniques from healthy female ewes as previously described. Briefly, MSCs were isolated from bone marrow aspirates with 0.25 mls ACD per ml of bone marrow.

MSCs were plated by mixing in a 3/5 ratio with MSC culture medium: Alpha-Minimun Essential Medium (Gibco), 10% defined fetal bovine serum (Hyclone), 2 mM L-glutamine, 50 U/ml penicillin, 50 μg/ml streptomycin (all from Gibco/Invitrogen) and plating on tissue culture flasks. Cultures were maintained at 37° C and at 5% CO2. MSCs were harvested using 0.05% trypsin (Gibco) and replated at 5,000 cells/cm^2 upon reaching 80-90% confluency (60,000-75,000 cells/cm^2).

Lineage differentiation:

Differentiation was performed using previously established protocols [45] with moderate alterations: for adipogenic and osteogenic differentiation, 36,000 cells/cm^2 were plated in 6 well plates. MSCs were allowed to reach confluency and then switched to adipogenic or osteogenic medium: adipogenic differentiation was initiated in induction medium: Dulbecco's Modified Eagle Medium (DMEM) high glucose (Invitrogen), Pen/Strep (Gibco), 1 μM dexamethasone, 10 μg/mL insulin, 200 μM indomethacin, 500 μM 3-isobutyl-1-methyl-xanthine (Sigma), and 10% FBS (Hyclone) for 3 days followed by 14 days in differentiation medium: DMEM high glucose, Pen/Strep, 10 μg/mL insulin, and 10% FBS. Differentitiated plates were stained with 0.7% Oil Red O. Osteogenic differentiation was performed using HyClone Advance STEM Osteogenic Differentiation kit (Thermo Scientific) with medium changes every third day for 21 days and samples were stained with Von Kossa. For chondrogenic differentiation potential of MSCs, 3 x 10^6 cells were pelleted in 15 ml conical tubes and then changed to chondrogenic medium DMEM (high glucose), 100 nM dexamethasone, Pen/Strep, 50 μg/mL ascorbic acid, 40 μg/mL L-proline, 1 × ITS + 1 supplement, 1 mM sodium pyruvate (Sigma), and 10 ng/mL TGF-β3 (R&D Systems). Medium was changed every third day for 14 days. Micromasses were stained with Alcian Blue.

Proliferation:

Proliferation was determined using manual cell counts with 0.4% trypan blue (Sigma) live/dead exclusion staining, and only live MSCs were counted. MSCs were plated at 6,000 cells/cm^2 and harvested for counts 12 hours following plating. This initial count was deemed time 0 and MSCs were harvested and counted at 12, 24, 36, 48 and 60 hours after the initial count. Counts were performed in triplicates.

Microencapsulation:

Using techniques previously described[38], MSCs were harvested using 0.05% trypsin and counted on a hemocytometer using 0.04% Trypan Blue (Sigma) staining for live/dead exclusion. 3.5 x 10^4 MSCs/ul were suspended in aqueous hydrogel solution containing 0.1 g/mL 10 kDa PEG-DA, 1.5% (v/v) triethanolamine/ HEPES buffered saline, 37mM 1-vinyl-2-pyrrolidinone, 0.1 mM eosin y, 9 mM pluronic acid. For photo initiation, 1.17M 2,2-dimethoxy-2-phenyl acetophenone was dissolved in 1-vinyl-2-pyrrolidinone and 3 uL of this solution was added per mL of sterile mineral oil (Sigma-Aldrich). Hydrogel/cell suspension was mixed with mineral oil containing the photoinitiator and vortexed for 2 seconds while being exposed to white light followed by another 18 second exposure with mild mixing. Microencapsulated MSCs were separated from the oil with four washes in MSC culture medium with 5 minute centrifugation at 1350 RPM and decanting between washes.

Viability assays:

Cell viability was assessed using theLIVE⁄DEAD Viability⁄Cytotoxicity Kit for Mammalian MSCs (Invitrogen). Images were taken using TCS SP5 Spectral Confocal Microscope (Leica). 3 sets of images were taken per condition with 30 images in each set with an average of 87,210 cells being counted in each treatment using Image J (NIH).

Cryopreservation and thawing:

MSCs and microspheres were frozen in MSC culture medium containing 10% DMSO. The MSCs were frozen in controlled rate freezing containers, Mr. Frostys (Nalgene labware) for 4-24 hours at -80° C and then transferred to liquid nitrogen. Vials were thawed in a 37° C waterbath with constant swirling. The MSCs were resuspended with medium immediately following loss of ice from cell/microbead suspension. To limit confounding factors microbeads were thawed using a ratio of twenty percent physical cell loss. This number was established on the percentage of cells lost during cryopreservation and thawing processes.

Adenoviral Transduction optimization and BMP-2 quantification:

First generation human type 5 adenoviruses containing the E1-E3 deletion were constructed with human cDNA for BMP-2 inserted in the E1 region. MSCs were harvested and plated one day prior to transductions. Transductions were preformed as described previously [45] with minor changes. Upon reaching a density of 36,000 cells /cm^2 the MSCs were prepared for transduction. To increase cell-viral interactions transductions were performed in reduced medium volumes. Medium was changed with replacement of 32% of normal culture volume of MSC culture medium. Transduction medium was made equaling 20% of normal culture volume

with Alpha MEM medium with 2mM L-glutamine and mixed with 0.72% Genejammer (Agilent Technologies) and allowed to incubate for 5 minutes at room temperature. The virus was then added to the transduction medium and allowed to incubate for 10 minutes at room temperature. For optimization of BMP-2 transduction, transductions were performed using 5,000, 7,500, 10,000 and 15,000 vp/cell (Supplementary Figure 5.1). The remainder of the experiments were performed with 15,000 vp/cell. The transduction mixture was then added to the cell culture drop wise around the plate. After four hours the culture volume was brought up to normal volume with MSC culture medium. MSCs were harvested 24 hours after the transduction. The MSCs were harvested and replated at 36,000 cells/cm^2 or encapsulated then replated at 36,000 cells/cm^2.BMP-2 was quantified from harvested medium using a BMP-2 elisa (R&D systems).

Scanning electron microscopy and light microscopy:

Both freshly prepared and cryopreserved microbeads containing ovine MSCs were immersion fixed using 2.0% gluteraldehyde in PBS for one hour. The MSCs were washed three times with PBS and postfixed in 1% osmium tetroxide diluted in 5% sucrose and PBS for 45 minutes. The microbeads were washed three times with distilled water and then carried though an alcohol dehydration series. The MSCs were critically point dried using a Samdri model 780-A (Tousimis). A 153 Å thick coating of gold was placed on the samples using SPI Module Sputter Coater (Structure Probe). The images were taken on 1450EP environmental Scanning Electron Microscope (Carl Zeiss).

Heterotopic bone assay:

All animal studies were performed with Baylor College of Medicine Institutional Animal Care and Use Committee approval. Female non-obese diabetic/ severely compromised immunodeficient mice (NOD/SCID; 8-12 weeks old; Charles River Laboratories) were injected with 3 x 10^6 microencapsulated MSCs either freshly prepared or cryopreserved and thawed from ovine A MSCs and ovine B MSCs. Microbeads were injected into the hind limb quadriceps of 3 mice per group (n = 12). Animals were euthanized at 2 weeks and x-rayed. The tissue was then harvested and fixed in formalin.

Graphical Representation and Statistics:

Graphs were made in Prism (Graphpad) and all statistics were also done in Prism. Statistics comparing BMP-2 production were performed using 2-way ANOVA with Bonferonni post test. Viability comparisons were done with 1-way ANOVA using Tukeys post test. Doubling times were calculated using the exponential growth equation in Prism and comparison of doubling times were done with 1-way ANOVA with Bonferroni post test.

Results:

Isolated Ovine MSCs are Capable of Adipogenic, Chondrogenic and Osteogenic Differentiation: Lineage differentiation of Porcine MSCs used in this study were previously validated [45]. To determine the potential of ovine MSCs to produces MSCs of the adipogenic, chondrogenic and osteogenic lineages MSCs were differentiated using previously developed protocols. Ovine MSCs isolated through plate adherence from bone marrow aspirates were

capable of adipogenic, chondrogenic and osteogenic differentiation (Figure 5.1A, 5.1B, 5.1C). Ovine MSCs underwent 21 days of osteogenic differentiation and showed evidence of calcium deposition as seen through Von Kossa silver nitrate staining (Figure 5.1A). After 14 days of condrogenic differentiation the micromasses exhibited sulfate proteoglycans as seen through Alcian Blue staining (Figure 5.1B), indicating the presence of condrocytes. At 17 days of adipogenic differentiation, lipid droplets were visible within MSCs through Oil Red O staining (Figure 5.1C), validating the capacity of these derived MSCs to differentiate into all three mesenchymal stem cell lineages.

Cell lines exhibited proliferation differences with and without adenoviral transduction: During the expansion phase it was noted that the ovine A MSCs reached confluency faster than the ovine B MSCs and porcine MSCs. Using 5 counts at 12 hour intervals proliferation rates were determined. Ovine A MSCs had a doubling time of 15.19 (+/- 0.705) hours (R^2=0.9813), Ovine B MSCs had a doubling time of 19.65 (+/-1.545) hours (R^2=0.9867) and porcine MSCs had a doubling time of 34.54 (+/- 3.175) hours (R^2=0.9755). The doubling times from each line were all statistically different ($p<0.05$) (Figure 5.1D). To understand the effect transduction had on the proliferation rates, doubling times of ovine A and ovine B MSCs were determined by plating the MSCs 24 hours after transduction and counting as described for the nontransduced cells. Adenoviral BMP-2 transduced ovine A and ovine B MSCs showed a significant reduction in the proliferation rates from the non transduced MSCs ($p < 0.05$) (Figure 5.1E) with a doubling time of 24.07 (+/- 2.065) hours (R^2= 0.9827) and 25.37 (+/- 2.16) hours (R^2= 0.9820) respectively.

BMP-2 Adenoviral Transduction of MSCs Had a Significant Donor Effect: To determine the ability of the MSC to produce BMP-2 following adenoviral transduction and the effect of

cryopreservation on BMP-2 production, monolayers of MSCs were transduced with 15,000 viral particles/cell. 15,000 vp/cell was chosen based on the highest BMP-2 production from optimization of 5,000, 7,500,10,000 and 15,000 vp/cell ($p<0.05$) (supplementary Figure 5.1). The MSCs were replated 24 hours after transduction or cryopreserved. Medium was harvested from cultures every 24 hours for 72 hours and quantified for BMP-2 expression (Figure 5.1F). The lines showed a significant donor effect ($P<0.001$) with ovine A MSCs producing the most BMP-2. Ovine B MSCs had a significant increase in BMP-2 expression from cryopreserved samples at 48 and 72 hours ($P<0.001$).

Cryopreserved Encapsulated MSCs Demonstrate High Levels of Cell Viability and Sustain BMP-2 Production: To examine the effect of cryopreservation on the survival of encapsulated MSCs, the viability of MSCs encapsulated in PEG-DA were assessed using a live/dead assay which stains the cytoplasm of live MSCs with Calcein AM (Figure 5.2A, 5.2E, 5.2I, 5.2L) and the dead MSCs DNA with Ethidium Homodimer (Figure 5.2B, 5.2F, 5.2J, 5.2M). No statistical difference was seen in the cell viability between the freshly prepared MSCs and the cryopreserved MSCs , but a significant reduction in cell viability was observed between day 0 and day 4 post-encapsulation in both freshly prepared and cryopreserved microspheres ($p<0.0001$) (Figure 5.2D, 5.2H). When encapsulated, BMP-2 transduced MSCs produced a reduced quantity of BMP-2 (Figure 5.3A) when compared to monolayer BMP-2 transduced MSCs (Figure 5.1B) at 72 and 96 hours post-transduction ($p < 0.05$). Porcine encapsulated BMP-2 producing MSCs showed an increase in BMP-2 production at 72 ($p<0.01$) and 96 hours ($p<0.001$) post transduction, and cryopreserved ovine B MSCs had a reduction in the quantity of BMP-2 produced at 72 and 96 hours post transduction ($p < 0.01$) (Figure 5.3A). Ovine A MSCs had no difference between the cryopreserved and freshly prepared encapsulated MSC BMP-2;

Within the ovine lines, ovine A MSCs produced significantly more BMP-2 than ovine B MSCs at 96 hours post transduction ($P<0.01$) under both conditions (Figure 5.1B, 5.3A). BMP-2 transduction has no effect on viability immediately following encapsulation (Figure 5.3B), but the BMP-2 encapsulated MSCs did have reduced viability at day 4 ($p < 0.05$) when compared to the non-modified MSCs (Figure 5.3C).

Microspheres Do Not Show Surface Damage Resulting from Cryopreservation: The integrity of the microspheres was examined following cryopreservation though scanning electron microscopy and light microscopy. The light microscopy images (Figure 5.4A, 5.4D) show the perimeter of the bead containing encapsulated ovine MSCs as being one contiguous surface with no rough edges. Additionally high magnification images of the encapsulated ovine microbeads demonstrated that the spheres possess contiguous surface with no loss of integrity (Figures 5.4B, 5.4E). Cryopreservation did not result in any changes in MSC encapsulated surface morphology (Figure 5.4C, 5.4F).

BMP-2 Transduced MSCs Produce Bone in Murine Models Following Cryopreservation: In an animal model for heterotopic ossification, BMP-2 transduced encapsulated microbeads produce similar quantities of bone. Two weeks following injection into NOD/SCID , when viewed by X-ray analysis, both with and without cryopreservation (Figure 5.5A and 5.5B) and the no treatment control (Figure 5.5C).

Discussion:

A major hurdle in making clinical treatments for these diseases is finding a way to make the therapeutics widely applicable and readily available for medicinal uses. The recipient of a

cell therapy, like organ transplantation, is at risk for graft rejection and cell encapsulation is widely used to attempt to modulate this immunological process. In this study we demonstrated for the first time that primary MSCs could successfully be cryopreserved in PEG microbeads. This is a valuable progression in the movement of PEG encapsulation procedures from the bench top to the bedside. The combined PEG-DA microbead encapsulation and cryopreservation method that yields high MSC viability post-thaw, similar to alginate and sodium cellulose sulfate cell encapsulation techniques [46-49]. However, unlike previous reports of damage in alginate capsules during the cryopreservation process [10, 50-51], PEG micropheres did not show any appreciable damage upon removal from cryopreservation when examined by both light and scanning electron micropscopy. Compromises in the integrity of the microbead can result in exposure of the encapsulated MSCs and initiation of an immune rejection [52]. Cryopreserved encapsulated BMP2 transduced MSCs maintained their potential to form bone in a mouse model for heterotopic ossification, indicating that these preparations can be stored with no adverse effects on quality of the treatment, eventually allowing for production of a human based product at GMP facilities with distribution to clinics.The viability of the primary MSCs was adversely affected by adeno genetic modification and stressors in transduction and encapsulation process. For encapsulated BMP-2 transduced MSCs to be a viable treatment for long bone injury extended BMP-2 production will be required [15]. Here we show that the viability of the genetically modified encapsulated MSCs was reduced to less than 40% by day 4 which may severely limit their therapeutic potential. To increase the viability it will be necessary to incorporate additional methods for promoting cell viability such as inclusion of extracellular matrix proteins [53] or choosing cell lines with more substantial viability [38].

The MSC line used for adenoviral BMP-2 transduction can significantly impact amount of BMP-2 expressed. Since limited numbers of MSC lines between and within species were used here, specific conclusion among individual MSC lines would be premature; however in general we found significant donor variation which affected both the rates of proliferation and BMP-2 production from the MSC lines. This suggests that cell line selection may have an impact on time required to expand cells in culture and the quantity of therapeutic BMP-2 produced. Since the amount of BMP-2 expression and rate of proliferation followed the same trend, shorter cell cycle time may be an indicator of cell lines which are more amenable to higher rates of transduction. As adenovirus is most effective at transducing cells in the S phase [54], cells with a shorter doubling time would be more likely to pass through S phase in the presence of active virus. There was less difference in BMP-2 production between all lines following encapsulation, but a difference between the ovine lines was still observed. This again indicates that MSC line to line variability significantly impacts the amount of BMP-2 produced, and that BMP2 expression optimization may need to be conducted for each batch or lot of MSC collected regardless of prior experience. Cryopreservation will potentially facilitate the storage of large lots of characterized product.

Conclusions:

Microencapsulation of MSCs holds much promise for therapeutics in diseases without current effective treatments. To move these treatments forward, methods for preserving and long term storage of encapsulated MSCs to allow for "off the shelf" therapeutics is necessary. The cryopreservation of PEG encapsulated MSCs did not reduce cell viability between the

cryopreserved and freshly prepared MSCs both with and without genetic modification and did not demonstrate any physical damage resulting from the cryopreservation process. Cryopreservation does not induce any negative effects on the encapsulated MSCs both with and without transduction and has no effect on the ability of the transduced cells to form bone; however, the encapsulated MSCs did have reduced viability following adenoviral transduction indicating a need for incorporating methods that increase viability of encapsulated MSCs to prolong protein production. Donor to donor variability results in significant transgene production making cell line choice important for optimizing gene expression. This demonstrates that PEG encapsulated MSCs have potential for being used as a treatment method for clinical applications.

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Supplmental Figure 5.1: Transductions of cells at 36,000 cells/ cm^2 were optimized with various amounts of vp/cell: 5,000; 75,000; 10.000; and 15,000 vp/cell were used in transductions. Media samples were quantified at 24 hours with a BMP-2 ELISA. (*) represent statisitcal difference from other samples ($p<0.05$).

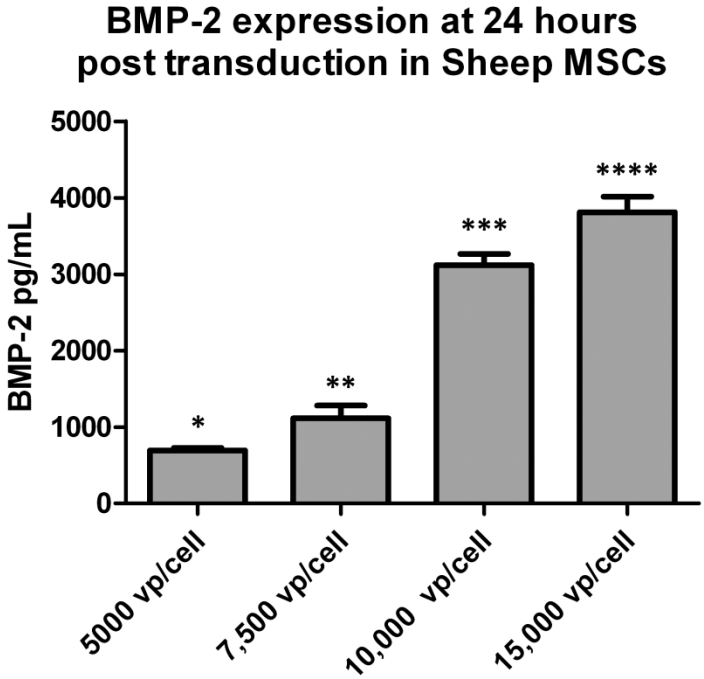

BMP-2 expression at 24 hours
post transduction in Sheep MSCs
BMP-2 pg/mL
5000
4000
3000
2000
1000
0
*
**
***
****
5000 vp/cell
7,500 vp/cell
10,000 vp/cell
15,000 vp/cell

Figure 5.1 Characterization of ovine MSCs and proliferation and BMP-2 transduction of ovine and porcine MSCs: Ovine MSCs A) osteogenic differentiation is seen through dark staining of calcium depositions by Von Kossa silver nitrate staining. B) condrogenic differentiation with chondroitin sulfate proteoglycans stained blue with Alcian Blue staining, and C) adipogenic differentiation as seen through intracellular lipid staining with Oil Red O. D) Ovine A, ovine B, and porcine cell line proliferation rates E) Proliferation rates of ovine A and ovine B following transduction with 15,000 vp/cell of adenoviral BMP-2 F) Transduction with 15,000 vp/cell adenoviral BMP-2 production from ovine and porcine MSCs both with and without cryopreservation.

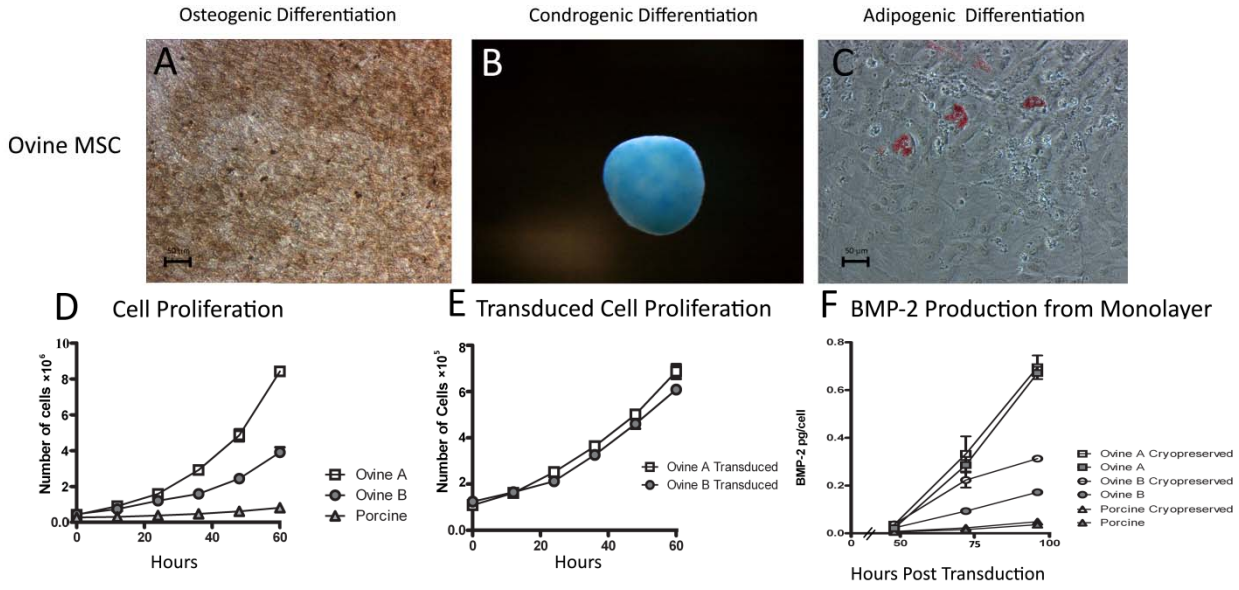

Osteogenic Differentiation
Condrogenic Differentiation
Adipogenic Differentiation
Ovine MSC
A
B
C
D Cell Proliferation
Number of cells ×10⁵
Hours
Ovine A
Ovine B
Porcine
E Transduced Cell Proliferation
Number of Cells ×10⁵
Hours
Ovine A Transduced
Ovine B Transduced
F BMP-2 Production from Monolayer
BMP-2 pg/cell
Hours Post Transduction
Ovine A Cryopreserved
Ovine A
Ovine B Cryopreserved
Ovine B
Porcine Cryopreserved
Porcine

Figure 5.2 Viability of cells on day 0 and 4 post encapsulation with and without cryopreservation: Cells were stained with calcien AM for live (green) and ethidium homodimer for dead (red). On the day of encapsulation ovine A MSCs stained for A) Live, B) Dead and C) overlay. And on day 4 post encapsulation E) ovine A MSCs stained for F) Live, H) Dead and G) overlay. D) Graphical representation of counts of 90 images. Cryopreserved ovine A encapsulated MSCs on day of thaw I) Live, J) Dead and K) overlay. Day 4 post thaw ovine A MSCs stained for L) Live, M) Dead and N) overlay. H) Graphical representation of counts of 90 images (averaging 87,000 cells per image). (*$p<0.0001$)

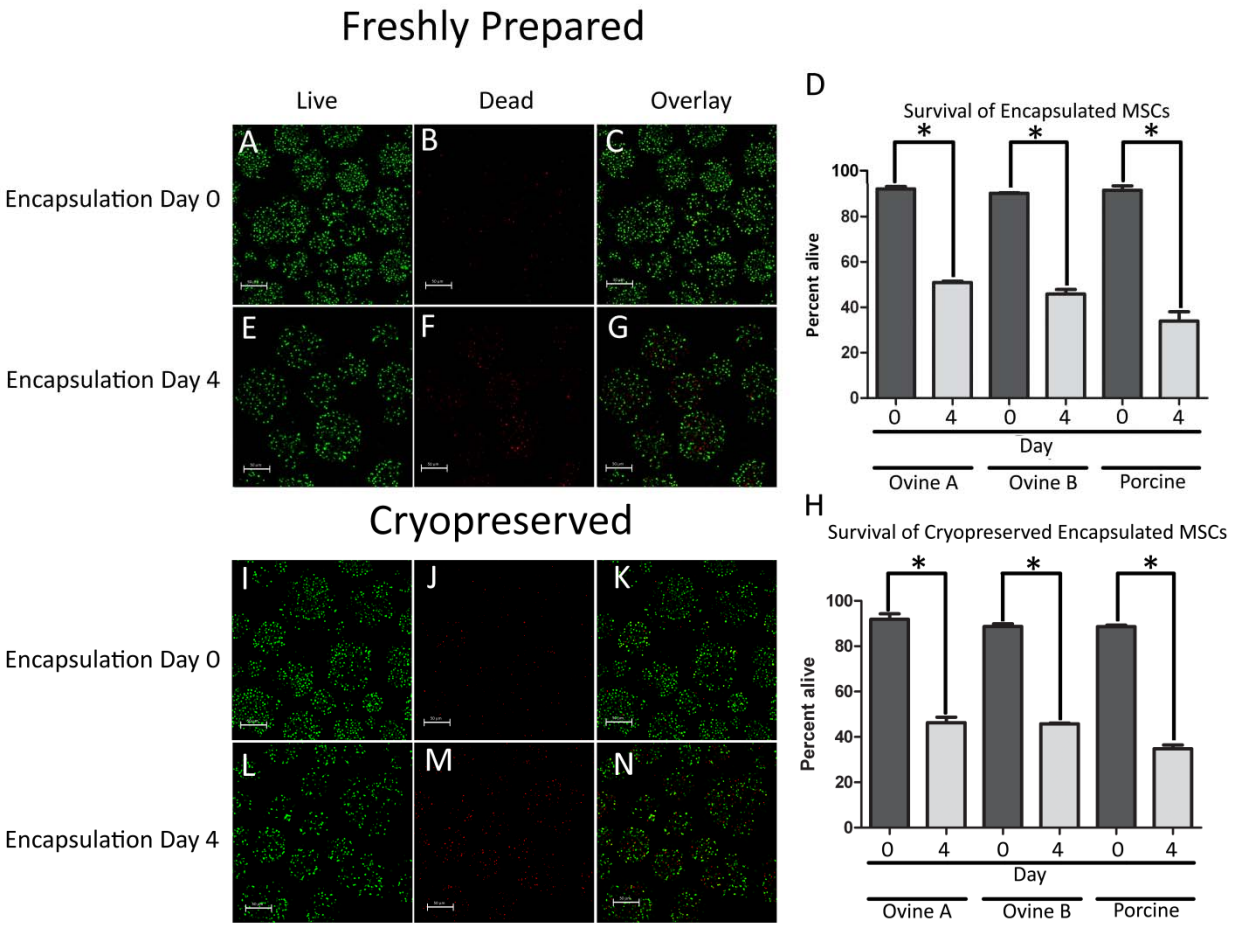

Freshly Prepared
Live
Dead
Overlay
Encapsulation Day 0
Encapsulation Day 4
A
B
C
E
F
G
Cryopreserved
I
J
K
L
M
N
D
Survival of Encapsulated MSCs
Percent alive
100
80
60
40
20
0
0
4
0
4
0
4
Day
Ovine A
Ovine B
Porcine
H
Survival of Cryopreserved Encapsulated MSCs
Percent alive
Day
Ovine A
Ovine B
Porcine

Figure 5.3 BMP-2 production in microencapsulated MSCs. A) Cells were transduced transduced with 15,000 vp/cell adenoviral BMP-2 prior to encapsulation and plated out freshly or cryopreserved. (+,# $p<0.01$), B) Ovine A and ovine B cryopreservation and BMP-2 transduction effect on MSC viability on day of encapsulation (day of thaw for cryopreserved samples). And C) 4 days after encapsulation ($p<0.05$).

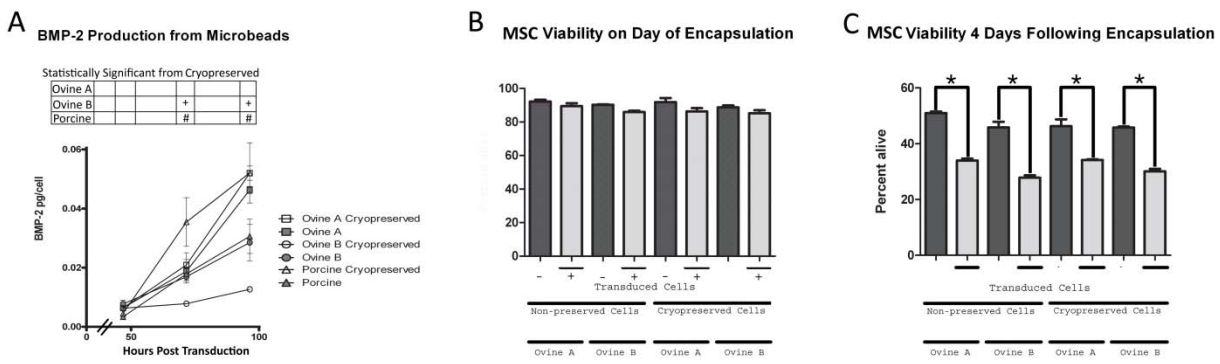

A
BMP-2 Production from Microbeads
Statistically Significant from Cryopreserved
Ovine A
Ovine B
Porcine
+
#
BMP-2 pg/cell
0.06
0.04
0.02
0.00
0
50
100
Hours Post Transduction
Ovine A Cryopreserved
Ovine A
Ovine B Cryopreserved
Ovine B
Porcine Cryopreserved
Porcine
B
MSC Viability on Day of Encapsulation
100
80
60
40
20
0
Transduced Cells
Non-preserved Cells
Cryopreserved Cells
Ovine A
Ovine B
Ovine A
Ovine B
C
MSC Viability 4 Days Following Encapsulation
Percent alive
60
40
20
0
Transduced Cells
Non-preserved Cells
Cryopreserved Cells
Ovine A
Ovine B
Ovine A
Ovine B

Figure 5.4 Structural analysis of freshly prepared and cryopreserved encapsulated MSCs: A) Phase contrast of encapsulated ovine A MSCs showed clear borders on microbeads. B) SEM of MSC microbeads showed a uniform surface. C) SEM of MSC microbeads of all sizes showed uniform structure. D) Phase contrast of cryopreserved encapsulated MSCs did not show appreciable damage E) SEM of cryopreserved MSC microbeads showed a uniform surface. F) SEM of cryopreserved encapsulated MSCs showed no damage to beads of various sizes.

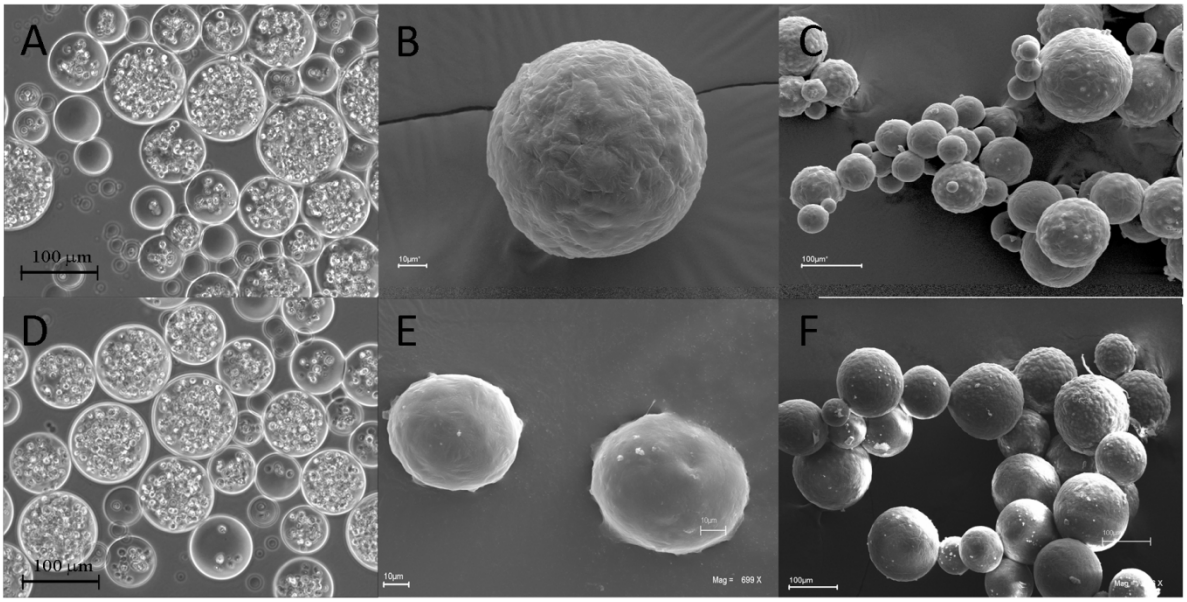
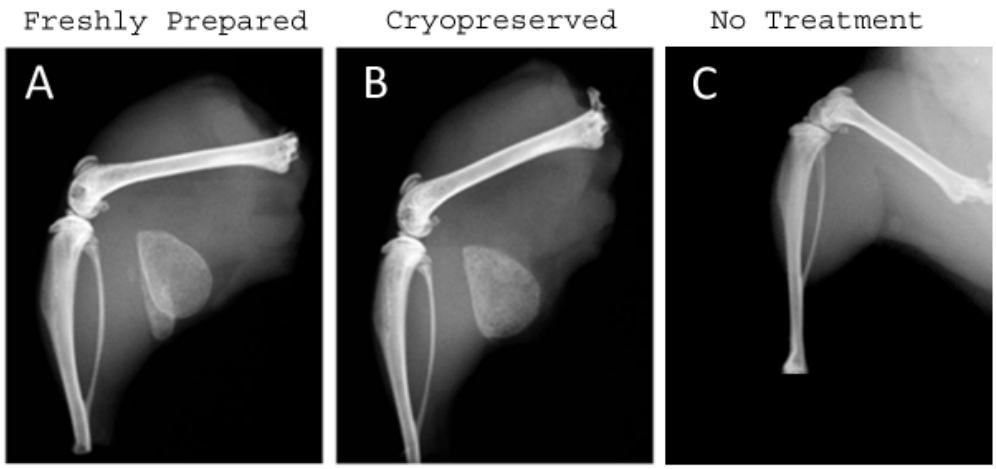

A
100 µm
B
10µm
C
100µm
D
100 µm
E
10µm
Mag = 699 X
F
100µm

Figure 5.5 BMP-2 transduced MSCs bone formation in a mouse model for heterotopic ossification. 3 X 10^6 Ovine A and Ovine B MSCs transduced with 15,000 vp/cell were injected in to the hind limb of a NOD/SCID mouse. The resulting heterotopic ossification was observed by X-RAY for A) freshly prepared BMP-2 encapsulated MSCs B) Cryopreserved BMP-2 encapsulated MSCs and C) no treatment control

X-Ray



CHAPTER 6

CONCLUSIONS

Embryonic stem cell derived treatments entered the first clinical trials for spinal cord injury and macular degeneration in 2010, and there is much hope for these promising cell based treatments in regenerative medicine. Since embryonic and induced pluripotent stem cells can be differentiated into any one of the 220 cell types in the human body, their potential to regenerate damaged and diseased tissue is limited mainly by their ability to be developed *in vitro*. Understanding the effects of growth factors and morphogens will allow for cultures to more closely resemble what is found *in vivo.* As these cells move into therapeutics, finding ways to make the treatments compatible with the patient's immune system and will prevent the need for lifelong immunosuppressive therapy. Furthermore, to create a conducive environment and successful integration, the addition of anti-inflammatory factors and growth factors will likely be required. Thus despite relative differences between these three chapters that can all be used for achieving the most successful therapeutics possible. The conclusions and critical analysis of the finding presented in this work will help to further validate these findings and move therapeutics closer to the clinic.

In chapter 2 we established that ESCs, ESC derived neural cells, and differentiating neural cells exhibited similar structural changes as seen in the ICM and developing nervous system. Additionally we examined the effect of complex culture conditions on the structure of these cells which further increased the similarities to *in vivo* development. ESCs like cells of the

ICM had a high level of protein synthesis with abundant polyribosomces. ESCs also resembled the ICM with immature golgi, endoplasmic reticulum and mitochondria formation. The ESCs also expressed microvilli like the ICM which helps to mediate signaling. As these cells developed into neural cells, the progression of structure changes was representative of neural development *in* vivo with neural cells having developed defined somas and long processes ending in specialized structures, lamellipodia and filopodia, that resembled neural growth cones. The ESC derived neurons when cultured with BDNF and serum also showed overall structural organization similar to what is seen in the brain with cells of differing phenotypes being found in a layered formation. These results showed that ESC and derived neural cells resembled *in vitro* ICM and developing nervous system through both intracellular and structural organization indicating that they are a viable model for human neural structural development.

In Chapter 3 we attempted to reprogram peripheral blood mononuclear cells (PBMCs) to induced pluripotent stem cells (iPSCs) using the transcription factors POU5F1/OCT4, SOX2, KLF4, LIN28, and NANOG. These factors had previously been shown to reprogram cells into pluripotent stem cells in differing combinations. The PBMCs in this study failed to integrate all vectors, but proliferative colonies were formed. The two derived lines showed expression of POU5F1/OCT4 with and without KLF4. Despite generating proliferative cell lines, this integration was unsuccessful in generating cells expressing the markers of ESCs/iPSCs: TRA-1-81, TRA-1-60, SSEA4, and E-cadherin; however, the produced lines, termed partially reprogrammed cells (PT-RPs), were capable of differentiation into all three germ lineages through both undirected differentiation and neural enhancing differentiation. The differentiated cultures resulted in the formation of all three germ layers (endoderm, mesoderm and ectoderm) as observed through marker expression of Desmin, Alpha fetoprotein and Nestin/Tuj.

Furthermore, with defined culture conditions these cells formed neural cells as seen through Nestin and Tuj expression and expressed markers of a differentiated phenotype as seen with the RT-PCR of NeruoD, calbindin, HuB, HuC, HuD, and MAP-2. These findings are important because no other group has shown the formation of partially reprogrammed cells with only POU5F1/OCT4 when grown in ESC culture medium can be proliferated and give rise to all three germ lineages and differentiated neural cells. These results show that lack of full reprogramming to an embryonic stem cell like state did not prohibit lineage conversion. This allows for cellular reprogramming using only one factor, minimizing the need to integrate additional reprogramming factors known to be proto-oncogenes.

In the third study, chapter 4, we used mesenchymal stem cells (MSCs) for genetic engineering in a model for bone formation. With the potential complications seen in the current gold standard for bone regeneration, bone grafting, new methods for effectively treating complicated fractures and nonunions are needed. To fill this need we examined MSCs transduced with a potent osteoinductive initiator, BMP-2, in an adenoviral vector and implanted cell containing microbeads in a mouse model for bone formation. In addition to being a vector for gene delivery, MSCs are capable of immune modulation and can help to reduce inflammation. The MSCs encapsulated biocompatible polymer, poly (ethylene glycol) (PEG), was capable of initiating bone formation in a mouse model for heterotopic ossification. While this finding proved the potential of this therapeutic, as this technology progresses to the clinic there is a need for developing a process to make the engineered encapsulated MSCs available for immediate use. We were able to show that cryopreservation did not adversely affect cell viability in both encapsulated MSCs and encapsulated genetically modified MSCs when compared to the freshly prepared samples. Additionally, there was no observable defects in the structure of the

microbeads unlike previous reports in Alginate microbeads. The cryopreserved encapsulated MSCs were capable of producing bone in the mouse model for heterotopic ossification as seen with the freshly prepared samples. These results indicate that cryopreservation is a valid method for preserving the viability and function of this valuable therapeutic.

Future Studies:

The results from chapter 2 indicated that there are similarities between the structural development of the ICM and hESCs as well as the developing nervous system and a hESC derived neural line. However these studies failed to show synapse formation. While this study demonstrated that cells did develop in *vitro* as expected this shortcoming of synaptic formation is of monumental importance. As neural cells function through synapses, finding ways to increase synaptic formation are vital to have these cells truly resemble what is found *in vivo*. Present literature suggests that these studies were preformed too early during *in vitro* differentiation to capture the formation of synapses. Glial cells have been shown to be vital for the development of functional synapses and the lack of glial cells within this study produce findings that may not be true at later time points. These studies looked at the differentiation of the cells at 5, 6 and 7 weeks and it has been shown by other researchers that glial cells do not develop before 7-9 weeks. In future studies, examining the cultures for glial formation should be a prerequisite before ultrastructural analysis. Additionally, for correlation to function the cells should be examined for synaptic transmission through patch clamp or microelectrode array recordings and

areas of activity should be selectively examined and characterized for synaptic formation. This will allow for correlation between the analyzed synapses and their observed functionality.

In chapter 3 many findings require further examination for validation. To allow for clear analysis the findings are separately addressed below.

Finding: PBMCs can be reprogrammed with only one factor, POU5F1/OCT4. This finding is not irrefutable because the experiments were not done in a clean system. The presence of additional factors (SOX2, c-MYC, KLF4, Lin28, and NANOG) involved in the transduction of the PBMCs does not allow for definitive analysis of the effect of POU5F1/OCT4. To rectify this, future studies should be done using only POU5F1 to verify that no other populations containing additional exogenous reprogramming factors could exist within the culture.

Finding: SOX-2 was not required for neural differentiation. While it was not expressed through genetic modifications, SOX2 is regulated by POU5F1/OCT4. All future experiments should verify lack of SOX2 through flow cytometery.

Finding: POU5F1/OCT4 transduction of PBMC resulted in lineage differentiation capabilities of derived cells. While the study established that PBMCs could not generate colonies in hESC medium without genetic manipulation, looking at the potential of the PBMCs to differentiate in permissive medium should also be verified. This will allow for the comparison that POU5F1/OCT4 is having an effect on the switching lineage capability of these cells, and not that it is just allowing for continuous proliferation.

Finding: Continued POU5F1/OCT4 expression inhibits the differentiation capacity of PT-RPs. POU5F1/OCT4 expression was seen both before and after differentiation in the cultures. The fluorescent images of neural cells indicate that the differentiation process did not create a homogenous population as seen in the hESCs. It is possible that the expression of

POU5F1/OCT4 resulted from an undifferentiated population not from continued expression from the differentiated cells. Co-staining of POU5F1/OCT4 with differentiated markers can address this issue.

Additionally using an inducible promoter driving POU5F1/OCT4 in this system can examine the effect of exogenous POU5F1/OCT4 during differentiation. This system would allow for POU5F1/OCT4 to be expressed or reduced with and without differentiation to examine if maintenance of the partially reprogrammed state is dependent on exogenous POU5F1/OCT4 expression. Being able to turn off the exogenous gene will allow for dissecting the difference between the potential of partially reprogrammed cells to differentiate and the effect of POU5F1/OCT4 to alter differentiation potential.

Finding: PBMCs may have an advantage over other cell types in generating partially reprogrammed cells. Comparisons between labs often are too variable to make direct conclusions. Thus to validate this finding the cell types mentioned from other research groups in the chapter, MSCs, karatinocytes and fibroblasts should all be transduced under the same conditions as PBMCs. This theory can be further validated through use of the inducible POU5F1/OCT4.

To further validate this claim once colonies are generated and POU5F1/OCT4 turned off, the cells can be differentiated into various germ lineages. The lineages can then be purified to create homogenous populations. In these populations the inducible gene can be reactivated to examine the propensity of the derived cells to return to a reprogrammed state to further examine the belief that original cell phenotype plays a role in the partial reprogramming process. While this system would be cleaner in a mouse model from tissues of a tetraploid complement mouse, it

is likely from previous reports that the partially reprogrammed cells will fail to generate chimeras and prevent the formation of *in vivo* derived tissues.

In Chapter 4 the cryopreservation of the encapsulated cells resulted in excellent viability and preserved BMP-2 production. The large difference between the BMP-2 produced from the monolayer and the BMP-2 produced from the microbeads is of concern. This was a huge loss of therapeutic potential and for therapeutic purposes there is a need for more encapsulated cells to equal the production of the cells in a monolayer. The PEG microbeads may have provided an unsuitable environment for the MSCs. The viability of the cells, even without genetic manipulation, was greatly reduced by day 4 with roughly 40-50% showing survival. Other research groups have found that by incorporating extracellular matrix proteins that MSC survival in PEG encapsulated beads was significantly increased. To create a more biologically permissive environment, incorporation of extracelluar matrix proteins should be done and validated for cell viability and BMP-2 production from PEG encapsulated cells. Additionally the use of platelet rich plasma can be done during the encapsulation process to create a natural source of extracellular matrix proteins and growth factors to promote a more permissive environment for the encapsulated cells while still maintaining biological compatibility for future therapeutic uses.

The studies used in the preceding chapters are connected through their progression towards making the most viable therapeutics from stem cells. As human ESCs provide a model for human development, induced pluripotent stem cells and partially reprogrammed cells can be compared to human ESCs. These comparisons allow researchers to understand differences that may have arisen during the reprogramming process. Two of the major roadblocks of ESCs being translated into the clinic are embryo destruction and incompatibility with the patient's immune

system. Reprogrammed cells are capable of overcoming both of these barriers. In every injury and disease, inflammation will be a complication whether it is induced by surgical disruption or a characteristic of the disease. This inflammation will likely reduce the integration and survival of therapeutically transferred cells. Using cells in conjunction with microencapsulation technology can help to provide a conductive environment for cellular regeneration. With MSCs being capable of dampening the immune response, encapsulated MSCs can be introduced alone or prepared with growth factors that would promote the survival and integration of transplanted pluripotent derived cells. With the capabilities of the encapsulated cells to be prepared in advance and cryopreserved this can provide a more regulated production of combinations of these therapeutics.