THE EFFECTS OF THE MICROENVIRONMENT ON THE OSTEOGENESIS OF HUMAN EMBRYONIC STEM CELLS

by

Shelley Elaine Brown

A dissertation submitted in partial fulfillment
of the requirements for the degree of
Doctor of Philosophy
(Biomedical Engineering)
in the University of Michigan
2011

Doctoral Committee:

Professor Paul H. Krebsbach Co-Chair
Professor Scott J. Hollister Co-Chair
Professor K. Sue O'Shea
Assistant Professor Kenichi Kuroda

To my Lord and Savior Jesus for giving me the beautiful gift of life and the gift of pursuing my heart's desires. You have been here every step of the way, whether I realized it or not. I dedicate this thesis and my life's work to you for your glory. Amen. To my mom, dad, brother, grandma, and Yang – thank you for pouring so much love and support into me and for always allowing me to chase my dreams. Life with you has been a privilege and joy, and I love you more than words could ever express.

ACKNOWLEDGEMENTS

I cannot believe that it has been seven years since I first stepped foot on campus and since I arrived in Ann Arbor. This has been the best years of my life to date, and if this is just the prelude to what is to come, I am most excited! The list of people to thank is vast but I have to first acknowledge two very important people. I would like to sincerely thank my advisor, Dr. Paul H. Krebsbach, for grooming me, training me, and instructing me on how to be a critical thinker, a productive scientist, and a motivated biomedical engineer. He is the epitome of what an advisor should be – truly like a father figure. He is always available to talk, give great advice about science, the proper controls, what the data really is stating, etc. But not only about science, but also about personal matters, how to balance everything, music, sports, you name it! Thank you for being the absolute best advisor any graduate student can ask for, I hope to be the type of mentor that you are someday. I would also like to sincerely thank my pastor, Rev. Mark J. Lyons for grooming me, training me, and developing me into a thoughtful, caring, compassionate, and dedicate minister. He is the epitome of what a father in the ministry should be. You have been listened and offered counsel on all life matters, you have seen the peaks and valleys, victory and defeat, joy and pain, and have witnessed and coached me through some of the most important decisions that I have ever made in life. It is because of these two individuals that I am the biomedical engineer and the minister that I am today. They have been so helpful and patient throughout the entire process as I learned how to balance both aspects of my journey here – and for that I am eternally grateful. You all have invested so much into me, and I hope that as I prepare to embark upon my next journey in life, that I will make you proud.

I would sincerely like to thank my dissertation committee: Dr. Scott J. Hollister, who served also as a co-advisor, Dr. Sue O'Shea, and Dr. Kenichi Kuroda. Dr. Hollister was very kind and helpful with my biomaterial design training. The knowledge that I acquired

about biomaterials under his advising is invaluable, and I appreciate that I was able to freely use all of the equipment and materials as needed in order to get the scaffold portion of my project up and running. Thank you for your great advice, and on a more personal note, also for inviting us to your home for lab get togethers where the food (specifically the grilled steak) was delicious! Dr. O'Shea, you have been such an asset to my development as a biomedical engineer who wanted to take the plunge into the world of hESCs. Your guidance and understanding about how hESC culture is more than a notion was so very helpful and I cannot thank you enough. I am grateful that I had the AWESOME opportunity to work with such a powerful scientific tool at such a young stage of my career, and your training at the Stem Cell Core was the start of it all. Dr. Kuroda, thank you for all of the great conversations about science, grant writing, starting a lab and…how to balance family life! It was a pleasure being neighbors and having the opportunity to meet your family and children. Thank you for always being very approachable, very helpful, and an all around fantastic committee member.

PREFACE

This dissertation contains the following published articles and manuscripts:

1. W Tong & SE Brown, PH Krebsbach, "Human Embryonic Stem Cells Undergo Osteogenic Differentiation in Human Bone Marrow Stromal Cell Microenvironments". *Journal of Stem Cells*. 2007; 2(3):139-47.

2. SE Brown, W Tong, PH Krebsbach, "The Derivation of Mesenchymal Stem Cells from Human Embryonic Stem Cells". *Cells Tissues Organs*. 2009;189(1-4):256-60.

3. P Arpornmaeklong, SE Brown, Z Wang, PH Krebsbach, "Phenotypic characterization, osteoblastic differentiation, and bone regeneration capacity of human embryonic stem cell-derived mesenchymal stem cells". *Stem Cells and Development.* 2009 Sep; 18(7):955-68.

4. BB Ward, SE Brown, PH Krebsbach, "Bioengineering Strategies for Craniofacial Bone Repair: A Review of Emerging Technologies". *Oral Diseases.* 2010 Nov; 16(8):709-16.

5. SE Brown and PH Krebsbach, The Derivation of Mesenchymal Stem Cells from Human Embryonic Stem Cells. In *Embryonic Stem Cells – The Hormonal Regulation of Pluripotency and Embryogenesis*. New Delhi, 2011:649-660.

6. LG Villa-Diaz, SE Brown, Y Liu, J Lahann, JM Parent, PH Krebsbach, "Derivation of functional mesenchymal stem cells from human induced pluripotent stem cells cultured in xeno-free and defined conditions". *Manuscript in preparation*

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ABSTRACT

Human embryonic stem cells (hESCs) present a potentially unlimited supply of cells that may be directed to differentiate into all cell types within the body and used in regenerative medicine for tissue and cell replacement therapies. An area of particular interest is stem cell transplantation for bone tissue regeneration. Current techniques used for bone tissue repair employ the use of auto- and allografting methods, however, these methods have inherent limitations that restrict their universal application. The limitations of these reparative strategies suggest that an alternative approach is required, and hESCs may provide a repository of cells for such an approach. One of the major gaps in the knowledge regarding hESCs is the lack of understanding the biological cues from the microenvironment that control and direct differentiation. Previous work has demonstrated that hESCs can be differentiated into osteoblasts, however, how to achieve directed differentiation still remains a pivotal question that remains unanswered. Therefore, we tested the hypothesis that controlling the local *in vitro* and *in vivo* microenvironments can direct osteoblastic differentiation of hESCs**.**

Overall, we demonstrated the importance of cell culture conditions in the *in vitro* microenvironment, and the importance of implantation site and scaffold design in the *in vivo* microenvironment. First, we adapted a transwell co-culture system consisting of hESCs with human bone marrow stromal cells (hBMSCs), which demonstrated that pro-osteogenic soluble signaling factors secreted by hBMSCs into the *in vitro* cellular

microenvironment directed differentiation of hESCs into osteoblasts. Secondly, we reproducibly derived mesenchymal progenitors from hESCs (hES-MSCs) that possess the characteristic hBMSC surface marker expression profile and are capable of undergoing differentiation along the mesenchymal lineage. Subsequently, via FACS analysis, we isolated multiple subpopulations of osteoprogenitors from within the hES-MSC population in order to identify candidate cells for biomaterial studies. Distinct osteoprogenitor cells were identified and when implanted *in vivo* in an orthotopic calvarial defect microenvironment, participated in the bone regeneration process. Lastly, we delivered osteoprogenitor hES-MSCs subcutaneously within designed hydroxyapatite/tri-calcium phosphate (HA/TCP) scaffolds to investigate the effects that porosity has on cell differentiation and overall bone tissue formation. We demonstrated that osteoprogenitors derived from hESCs survive and play a role in *in vivo* bone tissue formation within designed HA/TCP scaffolds with high porosity.

The long-term goal of this research is to further understand the biology of human embryonic stem cell development and more specifically, provide information about the effects the microenvironment has on the osteogenesis of hESCs. Utilizing the knowledge we have acquired on effects of the *in vitro* and *in vivo* microenvironments, we hope to have provided a platform for future studies aimed at developing hESC-based bone tissue engineering strategies.

CHAPTER 1

INTRODUCTION

"With men this is impossible, but with God all things are possible".
-Matthew 19:26 KJV

1.1 Problem Statement

Bone is the principle calcified tissue within vertebrates, which comprises the skeletal system. Bone development occurs through specialized processes called ossification and osteogenesis, which involves integrated and coordinated events such as cellular invasion, cellular cross-talk, matrix deposition, and tissue remodeling in order to form and maintain mechanically stable bone tissue capable of withstanding everyday movement. Over the past few decades, understanding bone repair subsequent to trauma, congenital defects, and osteodegenerative diseases has led to the development of the bone tissue engineering field. Current bone tissue engineering strategies combine the use of bioactive materials, gene therapy, and adult bone marrow stromal cell (hBMSCs) sources. Significant advances in this area have been made. However clinically, bone grafts have remained the standard treatment. The U.S. market is forecasted to reach $2.2 billion in 5 years driven by higher adoption of bone graft substitutes over other orthopedic repair procedures (Global Data Inc., 2011). In order to make tissue engineering more of a clinical reality, one particular area of improvement is the cell source. Although hBMSCs have been used in the clinic (Caplan 2005), there are many limitations to using these cells such as

invasive and painful harvesting procedures, reduced cellular availability, and diminished differentiation capacity. Human embryonic stem cells might provide a repository of cells for an alternative source.

This dissertation aims to understand the influence the microenvironment has on the osteogenesis of human embryonic stem cells (hESCs) in order to develop hESC-based bone tissue engineering strategies for regenerative medicine. This work has two approaches in an effort to investigate the interface of stem cell biology and materials science – it is on one hand a biological study on *in vitro* and *in vivo* hESC survival, progenitor derivation, and participation in the bone forming process. And on the other, a biomaterials study on the influence that scaffold macroscopic architecture has on *in vivo* hESC differentiation.

1.2 Specific Aims

The specific aims for this thesis are as follows:

SPECIFIC AIM 1 - Determine the osteoblastic lineage progression of hESCs in the presence of soluble signaling factors secreted by differentiated human bone marrow stromal cells into the local microenvironment using an indirect transwell co-culture system.

Hypothesis 1: Human bone marrow stromal cells secrete pro-osteogenic growth factors that can contribute to the lineage progression of human embryonic stem cells to functional osteoblasts.

SPECIFIC AIM 2 - Derive, isolate, and characterize mesenchymal progenitors from hESCs (hES-MSCs).

Hypothesis 2a: Human ES-MSC progenitors can be derived from differentiating hESCs and cultured *in vitro* to differentiate along multiple mesenchymal lineages.

Hypothesis 2b: Within the hES-MSC population there exists sub-populations of osteoprogenitor cells that can be isolated via FACS analysis. These populations can be selected, according to their *in vitro* osteogenic potential, and used for subsequent implantation in an orthotopic defect model and in designed biomaterial scaffolds.

SPECIFIC AIM 3 – Direct osteoblast differentiation of hES-MSCs *in vivo* by controlling the microenvironment of HA/TCP scaffolds with varying porosity design.

Hypothesis 3: The local *in vivo* microenvironment of designed biomaterials will regulate and direct the differentiation of hES-MSCs. Varying porosity will change the

overall permeability of the scaffold, thus affecting nutrient diffusion and metabolic byproduct transport within the scaffold. These HA/TCP scaffolds will enhance hES-MSC survival, proliferation, differentiation, and overall bone tissue formation *in vivo.*

1.3 Research Significance

Human embryonic stem cells (hESCs) are derived from the inner cell mass, a group of approximately thirty cells, of four or five day old embryos that have developed into blastocysts that were collected for *in vitro* fertilization (IVF) purposes. They are derived by the selection and expansion of individual colonies rather than clonal expansion of a single cell. Human ES cells are pluripotent cells are capable of unlimited and undifferentiated proliferation in vitro, maintain normal karyotypic characteristics, sustain high levels of telomerase activity, and retain uniform undifferentiated morphology in prolonged culture (Thomson et al. 1998) In addition, the hESCs have the ability to differentiate along the three embryonic germ layers *in vivo* as evidenced by teratoma formation after injection into severe combined immunodeficient (SCID) mice. The teratomas contained gut epithelium (endoderm); cartilage, bone, smooth muscle, and striated muscle (mesoderm); and neural epithelium, embryonic ganglia, and stratified squamous epithelium (ectoderm). They have also been shown to express certain cell surface markers that are widely use to confirm pluripotency, such as stage-specific embryonic antigen SSEA-4, TRA-1-60, TRA-1-81, and alkaline phosphatase. Oct-4, a transcription factor, has been identified as another key indicator of undifferentiation. In order to maintain pluripotentcy, hESCs are typically cultured on mouse embryonic fibroblast (MEF) feeder layers. In addition, the cells are grown under serum-free

conditions using serum replacement (SR) with supplements of basic fibroblast growth factor (bFGF). Under these culture conditions, continuous passaging of hESCs is permitted while maintaining pluripotency and normal karyotypic characteristics. In 2007 there were only 22 available hESC lines for research, however, now there are 128 different lines approved on the NIH Stem Cell Registry (NIH Stem Cell Registry, 2011). This dissertation discusses work using the Bresagen BG01 line and the WiCell H9 stem cell line.

Human embryonic stem cells have the potential to be used in clinical applications for the treatment of illnesses, and offer great promise for research. It has been hypothesized that hESCs may become the basis of therapeutic strategies to treat diseases such as diabetes, heart disease, Alzheimer's, and Parkinson's disease. Another major goal for embryonic stem cell research is the controlled differentiation into specific kinds of cells for the purpose of replacing or regenerating damaged tissue caused by disease or injury. However, this requires that the hESCs are able to differentiate into the desired cell type, survive post-transplantation, and proliferate and differentiate to generate sufficient amounts of tissue.

The osteogenic cells derived from hESCs have tremendous potential, and serve as a tool through which one can characterize early bone development and cellular behavior on bone-related biomaterials. This may increase their usefulness as a source for osteoprogenitor cells as compared to hBMSCs. The generation of osteoblasts from hESCs has been shown to be successful, as evidenced by osteogenic gene expression of runx2,

bone-specific alkaline phosphatase (ALP), osteocalcin (OCN), mineralization confirmed by von Kossa and Alizarin Red staining, and bone nodule formation (Duplomb et al. 2008). One of the first studies cultured hESCs in the presence of defined osteogenic supplements for 21 days and was able to demonstrate mineralization and induction of osteoblastic marker expression (Sotille et al. 2003). To achieve osteoblastic differentiation, hESCs have been co-cultured with primary bone derived cells (PBDs) to induce osteoblast differentiation without the addition of exogenous factors, cultured *in vitro* in the presence of known osteogenic factors without the embryoid body formation step, and shown to give rise to mesenchymal progenitor cells (Ahn et al. 2006; Arpornmaeklong et al. 2009; Barberi et al. 2005; Brown et al. 2009; Cao et al. 2005; de Peppo et al. 2010a; de Peppo et al. 2010b; Evseenko et al. 2010; Karlsson et al. 2009; Karp et al. 2006; Kopher et al. 2010; Kuznetsov et al.; Lian et al. 2007; Olivier et al. 2006; Smith et al. 2009; Tong et al. 2007; Tremoleda et al. 2008; Trivedi et al. 2007; Xu et al. 2004)). However, due to spontaneous differentiation, many of demonstrated that a heterogeneous population of cells still remained. Therefore, we proposed that it was necessary to define the appropriate *in vitro* and *in vivo* microenvironment to address this issue. Therefore, the following dissertation discusses our efforts to investigate the cellular, implantation site, and scaffold design microenvironment effects on the osteogenesis of human embryonic stem cells.

1.4 Dissertation Outline

The thesis is arranged as follows:

Chapter two discusses the literature and background information on human embryonic stem cells, human bone marrow stromal cell biology, the use of scaffolds in tissue engineering, the cellular microenvironment, and clinical applications of mesenchymal progenitors. A large portion of chapter two was written as a chapter (*The Derivation of Mesenchymal Stem Cells from Human Embryonic Stem Cells)* for a book titled, "*Embryonic Stem Cells – The Hormonal Regulation of Pluripotency and Embryo-genesis".*

Chapter three describes an experimental approach to direct and control hESC differentiation towards osteoblasts in a human bone marrow stromal cell microenvironment. This work was published as an original research article in *Journal of Stem Cells.*

Chapters four and five demonstrates our ability to generate mesenchymal progenitors from hESCs that can undergo multilineage differentiation and express a surface marker profile similar to that of hBMCs. Further selection of candidate osteoprogenitor population capable of undergoing *in vitro* mineralization and *in vivo* bone formation when implanted *in vivo* in an orthotopic calvarial defect microenvironment. The work discussed in chapter four was published as an original research article in *Cells Tissues Organs*.

Chapter six describes the use of computer aided design methods and solid free form fabrication techniques for HA/TCP scaffold porosity design. Additionally, this chapter

deals with the subcutaneous implantation of osteoprogenitors isolated from derived from hES-MSCs into high and low porosity scaffolds. Lastly, the effects of macroporosity on cellular differentiation and bone tissue formation are summarized.

Chapter seven discusses conclusions of this dissertation and future challenges and directions.

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CHAPTER 2

BACKGROUND AND LITERATURE REVIEW

2.1 Introduction

Bone tissue engineering is a field of biological research that is based on the principle of restoring function and replacing diseased or damaged tissue through the application of biological and engineering principles. Currently, cancellous bone grafting is the gold standard treatment for healing and managing large bone defects. However, autogenous bone grafting is associated with numerous limitations including difficulties in shaping the graft to fill the defect, the requirement for numerous procedures, infection, postoperative pain, and donor site morbidity (Banwart et. al., 1995; Goulet et. al., 1997). As a result, medical researchers have turned toward tissue engineering as an alternative treatment strategy. Bone regeneration by tissue engineering is dependent on chemical and mechanical influences on osteogenesis and the bone remodeling process. There are three main elements of tissue engineering, known as the tissue engineering triad, that need to be used in a combinatorial approach in order to achieve directed bone tissue formation: 1) The presence of osteoprogenitor cells capable of forming bone, 2) Incorporation of factors with pro-osteogenic effects, and 3) The use of a naturally derived or synthetic osteoconductive scaffold capable of supporting adequate bone formation. Although the tissue engineering triad is an important principle to which biomedical engineers,

biomedical researchers, and clinicians should hold on to, it is vitally important that the overall theme of understanding the microenvironment and how it majorly contributes to cell phenotype, proliferation, behavior, survival, and differentiation. As the field of regenerative medicine continues to progress and if it will in fact progress, then much attention is required to address the overall question: What are the macroscopic factors present within the microenvironment that will not only directly influence a cell, but inherently influence microscopic behavior within the cell? Therefore, from an engineering and, in general research standpoint, it is prudent to develop cell-directed biomaterials that cells respond to on a macroscopic and more importantly, microscopic level. Furthermore, it should be noted that careful attention is now being paid to the cell, which is vital, because heretofore, the fields of stem cell biology and materials science engineering have not intersected paths as much as they perhaps should have. In an effort to develop the most viable and effective scaffolds, biomaterials or substrates intended for cell-based therapeutics, one must first address important developmental biological questions pertaining to the appropriate progenitor cell populations with the characteristics that will produce a useful and clinically relevant constructs for bone repair strategies. This chapter and overall dissertation, in combination with the current body of knowledge, will aim to at least in part address the very important question of understanding the microenvironment effects on hESC-derived osteoprogenitor cell phenotype, proliferation, behavior, survival, and differentiation such that we can gain better insight and understanding of embryonic bone development and bone repair.

2.2 Osteoprogenitor Cell Source

In the early to mid 1970's, hallmark studies were published demonstrating the ability to isolate and derive adult fibroblast cell colonies from the bone marrow stroma and the spleen (Friedenstein et al. 1970); (Friedenstein et al. 1971). These fibroblast-like cells, later termed bone marrow stromal cells (BMSCs) or bone marrow-derived mesenchymal stem cells (MSCs), were shown to proliferate in culture, to continually grow upon passaging while maintaining stable karyotypic characteristics, and were comprised of cells that had multipotent potential to differentiate along multiple mesenchymal cell lineages such as bone, cartilage, fat and could support hematopoietic stem cell (HSC) differentiation (Bab et al. 1984); (Bab et al. 1986); Friedenstein *et al.*, 1970; (Friedenstein et al. 1974b). Numerous studies spurred from these findings, which also led researchers in this area to explore the functions of these cells in vitro and in their normal microenvironment. Bone marrow stromal cells were transplanted *in vivo* to determine if they had the ability to re-establish the marrow microenvironment, and it was reported that the *ex vivo* expanded stromal cells did indeed restore the hematopoietic niche within the bone marrow (Friedenstein et al. 1974a). These experiments further developed the hypothesis that within the bone marrow stroma resided a heterogeneous mixture of cells that function as a repository of progenitors, known as MSCs, that may migrate out of their stem cell niche in response to disease, injury, and aging. Therefore, extensive investigation into the identification of MSCs and their utility for cell-replacement therapies were the basis for a new emerging field known as tissue engineering (Ashton et

al. 1980; Bab *et al.*, 1986; Owen et al., 1988; Beresford 1989; Jaiswal et al. 1997; Krebsbach et al. 1999; Kuznetsov et al., 2001).

In the 1980's and 1990's, many groups further demonstrated that culture-adherent MSCs present in the marrow stroma were capable of differentiation into bone, cartilage, and fat for multiple species such as canine, chicken, rabbit, rat, and mouse (Jaiswal et al. 1997). Using the expertise gained from these culture systems, MSCs were then isolated and propagated from human adult bone marrow (hMSCs) (Bab et al. 1988); (Krebsbach et al. 1997). Human MSCs were then used with site-specific delivery vehicles to repair bone, cartilage, and other connective tissues (Haynesworth et al. 1992a; Haynesworth et al. 1992b). Additionally, a series of monoclonal antibodies were developed to identify characteristic surface markers on hMSCs, which would prove to be beneficial to researchers interested in not only identifying MSCs, but also subpopulations of osteoprogenitor cells (Haynesworth et al. 1992a; Haynesworth et al. 1992b); (Gronthos et al. 1999). Simultaneously, Caplan et al. used the embryonic chick limb bud mesechymal cell culture system as an assay for the purification of inductive factors in bone to further develop the technology for isolating, expanding, and preserving the stem cell capacity of adult human bone marrow-derived mesenchymal stem cells (Caplan 2005). With this newly acquired knowledge and the emerging technologies in biomedical engineering, hMSCs became the principle cell source for cell-based pre-clinical bone tissue engineering studies.

Currently, substantial advances have been made to address clinical needs for regeneration of damaged or diseased tissues. The three main approaches of cell-based clinical therapies that employ the use of hMSCs are: 1) from a tissue engineering standpoint where cells are incorporated into 3D biomaterial scaffolds for the replacement of tissue *in vivo*, 2) from a cell replacement therapy standpoint where allogeneic donor cells are used to replace ablated tumors and diseased cells; and 3) from a inductive standpoint where cells provide cytokine and growth factor cues that stimulate host reparative events and inhibit degenerative events (Caplan 2005). Thus, clinical protocols were developed to establish that autologous hMSCs could be safely implanted back in order to reconstitute the marrow microenvironment for breast cancer and osteogenesis imperfecta (OI) patients following chemotherapy treatment (Koc et al. 2000); (Horwitz et al. 2002). Additionally, hMSCs have been shown to have immunomodulatory effects and could induce immune suppression in patients (Le Blanc et al. 2005); (Aggarwal et al. 2005). Although the use of hMSCs has been successfully used in some cases, there are challenges that scientists and clinicians must overcome before the transplantation of these cells is incorporated into routine clinical practice. Specifically, the classic method to isolate MSCs from bone marrow relies on their capacity to adhere to plastic and proliferation in growth medium containing serum (Olivier et al. 2006). However, cell availability is greatly limited with this method because MSCs are present at low concentrations in the marrow, occurring at less than 1 in 100,000-500,000 nucleated cells (Caplan 2005). Also, the availability of tissues for their isolation remains limiting and requires invasive procedures that may cause severe donor site morbidity.

Therefore, an alternative source for generating MSCs can be found in human embryonic stem cells (hESCs) (Thomson et al. 1998). Human ESCs are an alternative source for generating MSCs due to the fact that they can theoretically be expanded infinitely and also because using these cells would eliminate the need for invasive cell harvesting techniques. Host immune rejection could also be circumvented by the use of autologous hESCs generated from nuclear transfer or from immune compatible allogeneic hESCs. Derivation of mesenchymal stem cells from human ES cells will further the understanding of the differentiation pathways and important cellular events that occur during early human development and could also have useful clinical applications. Because of the therapeutic potential, particularly in the areas of cell therapy and regenerative medicine, derivation of MSCs from hESCs (hESC-MSCs) has specific advantages over the current "gold standard" use of autologous and allogeneic adult hMSCs for bone tissue engineering. (Olivier et al. 2006).

2.2.1 Human Embryonic Stem Cells

The major advancements in the area of stem cell culture, derivation, propagation, and differentiation paved the way for a pivotal discovery that was reported in a 1998 study from the University of Wisconsin. Thomson et. al. described the first successful isolation and long term sustained culture of a small cluster cells from the inner cell mass of four-day old embryos (Thomson et al. 1998). These cells, known as human embryonic stem cells (hESCs), represent a robust biologic tool and model system through which the scientific and medical communities will better understand human development, disease pathophysiology, organogenesis, and mechanisms for cellular differentiation; all of which

will help develop and improve the field of regenerative medicine. These embryonic stem cells are derived by the selection and expansion of individual colonies rather than clonal expansion of a single cell. Human ESCs are pluripotent cells that are presumed to have virtually unlimited proliferation capacity in vitro, maintain normal karyotypic characteristics, sustain high levels of telomerase activity, and retain uniform undifferentiated morphology in prolonged culture (Thomson et al. 1998). In addition, hESCs have the ability to differentiate along the three embryonic germ layers *in vivo* as evidenced by teratoma formation after injection into severe combined immunodeficient (SCID) mice. The teratomas can contain gut epithelium (endoderm); cartilage, bone, smooth muscle, and striated muscle (mesoderm); and neural epithelium, embryonic ganglia, and stratified squamous epithelium (ectoderm). They have also been shown to express certain cell surface markers that are widely used to confirm pluripotency, such as stage-specific embryonic antigen SSEA-4, TRA-1-60, TRA-1-81, and alkaline phosphatase. Oct-4, a transcription factor, has been identified as another key indicator of the undifferentiated state.

To maintain their self renewal capacity, hESCs were originally cultured on mouse embryonic fibroblast (MEF) feeder layers and grown under serum-free conditions using serum replacement (SR) with supplements of basic fibroblast growth factor (bFGF). Under these culture conditions, hESCs have been passaged continuously and maintained pluripotency as well as a normal karyotype. However, it has been reported that hESCs have been successfully cultured with feeder cells of human origin, such as human bone marrow stromal cells (hBMSCs), human placental fibroblasts, human foreskin fibrobalsts

(hFFs), feeders derived from hESCs, and on polymeric substrates in feeder free conditions (Cheng et al. 2003); (Genbacev et al. 2005); (Wang et al. 2005); (Stojkovic et al. 2005); (Hovatta et al. 2003); (Villa-Diaz et al. 2010)). In order to safely use hESCs in a clinical setting, it is imperative that that feeder-free and animal product-free culture conditions are explored further to overcome the risks of cross-transfer of pathogens from xenogeneic sources.

The ability of hESCs to maintain an undifferentiated state indefinitely in culture and to differentiate into all cell types and tissues within the human body has created a high demand for research. Although the cells are of great scientific interest, progression of this type of research has been met with great controversy and resistance due to the ethical concern of destroying early human embryos for derivation of hESC lines (Knowles 2004); (Baschetti 2005); (Gruen et al. 2006)). Nevertheless, once the ethical concerns are abated through placement of the appropriate guidelines and policies on research, the hESC field will not only evolve, but will continue to rapidly progress toward monumental medical and scientific breakthroughs.

2.2.2 Human Embryonic Stem Cell Derived Mesenchymal Stem Cells

The current major goal for hESC research in regenerative medicine is the controlled differentiation into specific progenitor cells for the purpose of replacing or regenerating damaged tissue. Therefore, the ability to obtain large quantities of multipotent cells from hESCs represents a challenge for cell based therapy and tissue engineering strategies that currently rely on human bone marrow stromal cells (hMSCs). Within the diverse

population of hMSCs, there exist early progenitor mesenchymal stem cells capable of self-renewal and multi-lineage differentiation into cell types such as osteoblasts, chondrocytes, and adipocytes (Bianco et al. 2003; Wagers et al. 2004). While hMSCs make a useful source of osteoprogenitor cells for tissue engineering strategies, they have limited proliferation and differentiation capacity. In contrast, hESCs which are able to proliferate indefinitely in vitro, represent a potentially unlimited source of mesenchymal stem cells.

Recent studies demonstrate that the derivation of hESC-MSCS, mesenchymal precursors derived from hESCs, has been achieved via various isolation methods, and the generation of osteoblasts has been achieved in co-culture with primary bone derived cells (PBDs), in the presence of known osteogenic supplements, and in transwell co-culture with hBMSCs (Ahn et al. 2006; Cao et al. 2005; Duplomb 2007; Hwang et al., 2006; Karner et al. 2007; Karp et al. 2006; Sotille et al. 2003; Tong et al. 2007). Although the identification and characterization of hESC-MSCs has been reported, the data are quite vast and varied in terms of the derivation method, cell culture conditions, the mechanism of differentiation (epithelial-mesenchymal transition vs neural crest stem cell-mesenchymal differentiation), multilineage differentiation potential, and surface markers used to select for a pure mesenchymal stem cell subpopulation. As the field continues to evolve, careful attention should be placed on standardizing these parameters along clinical-grade good manufacturing practice (GMP guidelines). Through the isolation and identification of hESC-MSCs and the ability to produce a large supply of progenitor cells that can be

genetically modified, the field hESC-MSC based tissue engineering and regenerative medicine strategies holds great promise.

2.2.3 Derivation Methods

Thorough and extensive investigation into the definition, differentiation, and identification of mesenchymal stem cells has occurred over the last three decades. However, there are fundamental mechanistic and developmental concepts that remain poorly understood. The foundation laid by pioneers in the MSC field has provided current researchers with a breadth of knowledge to draw upon because the same fundamental questions are being investigated to identify the true ''MSC" from differentiating hESCs. Many investigators state that although MSCs isolated from the adult bone marrow have been shown to differentiate *in vitro* and *in vivo*, as well as have been successfully used in a clinical setting to repopulate the marrow environment in cancer patients, harvesting and utilizing adult hMSCs has disadvantages such as tissue availability, donor site morbidity, and host immune rejection (Caplan 2005; Horwitz et al. 2002; Karp et al. 2006). Therefore, hESCs have been the topic of great discussion and interest as a potential repository of cells that can provide an unlimited number of specialized mesenchymal stem cells known as hESC-MSCs.

Numerous isolation protocols have been reported describing successful derivation and differentiation of hESC-MSCs (Arpornmaeklong et al. 2009; Barberi et al. 2005; Brown et al. 2009; de Peppo et al. 2010a; de Peppo et al. 2010b; Evseenko et al. 2010; Hwang et

al., 2006; Karlsson et al. 2009; Karp et al. 2006; Kopher et al. 2010; Kuznetsov et al.; Lian et al. 2007; Olivier et al. 2006; Smith et al. 2009; Trivedi et al. 2007; Xu et al. 2004). One of the first reports of the derivation of a MSC-like progenitor population was in 2004, where fibroblast-like hESC derivatives were infected with a human telomerase reverse transcriptase (hTERT) retrovirus, as a result showed extended proliferative capacity, supported undifferentiated growth of hESCs as a feeder layer, and differentiated into osteoblasts (Xu et al. 2004). Following that study, another group reported the successful production of hESC-MSCs when cultured on murine OP9 stromal cells in the presence of heat-inactivated FBS, and indicated that the hESC-MSCs had a similar immunophenotype to hMSCs after flow cytometry was performed to purify the hESC-MSC population from the stromal cell feeder (Barberi et al. 2005). Another method for hESC-MSC production involved the use of spontaneously differentiated hESC colonies. The cells obtained became morphologically fibroblastic and homogenous after multiple passages, possessed a characteristic MSC immunophenotype, and supported hESC and hematopoietic progenitor cell growth (Olivier et al. 2006). Of particular importance, two reports showed the ability to reproducibly derive clinically compliant hESC-MSCs in a xeno-free environment where all contaminating animal-derived components were replaced with human-derived or recombinant components. Thus, they cultivated a hESC-MSC line suitable for clinical use ((Karlsson et al. 2009; Lian et al. 2007). Other groups described similar findings, demonstrating that hESCs had the ability to reproducibly proliferate, differentiate, and commit to the mesodermal lineage in various cell culture conditions (both in monolayer and 3D) while retaining their multilineage differentiation potential and self renewal capacity, further demonstrating their high potential for tissue

engineering applications (Arpornmaeklong et al. 2009; Brown et al. 2009; de Peppo et al. 2010a; de Peppo et al. 2010b; Evseenko et al. 2010; Kopher et al. 2010; Lian et al. 2007; Smith et al. 2009; Trivedi & Hematti 2007).

In summary, multiple approaches have attempted to achieve the most direct and efficient derivation of hESC-MSCs. A variety of studies have compared using the embryoid body (EB) step versus omitting this step, using multiple media formulations with and without serum, and using feeder-free cultures versus co-culture. These reports greatly contributed to the field, however, a concensus on the most appropriate method of isolation and culture is absolutely necessary to make hESC-MSC based therapies in a clinical setting a reality.

2.2.4 Osteoprogenitor Cell Differentiation from hESCs

Currently, there are major gaps in the knowledge about the growth factors and three-dimensional milieu that influence and direct osteoblast differentiation. The generation of osteoprogenitors from hESC-MSCs has been shown to be successful as evidenced by osteogenic gene expression of runt-related transcription factor 2 (Runx2), collagen type 1A (Col1A1), bone-specific alkaline phosphatase (ALP), and osteocalcin (OCN); mineralized matrix confirmed by von Kossa and Alizarin Red staining; bone nodule formation *in vitro;* and bone formation *in vivo* in diffusion chambers and transplants to orthotopic sites (Arpornmaeklong et al., 2010; Duplomb 2007). One of the first differentiation studies used cultured hESCs in the presence of defined osteogenic supplements for 21 days, and was able to demonstrate mineralization and induction of

osteoblastic marker expression (Sotille et al. 2003). Human ESCs have been co-cultured with primary bone derived cells (PBDs) to induce osteoblast differentiation without the addition of exogenous factors, and cultured *in vitro* in the presence of known osteogenic factors without the embryoid body (EB) formation step – both studies confirming that hESCs have the capacity to differentiate into osteoblasts (Ahn et al. 2006; Karp et al. 2006). Whereas, other findings suggest that 12 day EB-derived hESC-MSCs are equally capable of undergoing multilineage differentiation *in vitro* (Cao *et al*., *2*005). It has also been shown that hESC-MSCs can not only differentiate into functional osteoblasts and adipocytes and express markers characteristic of hMSCs, but they can also be successfullly transduced with an osteogenic lineage specific Col2.3-GFP lentivirus in order to track and isolate cells as they underwent differentiation. The transgene construct used has been shown to be a useful tool for studying hBMSC differentiation (Brown *et al*., 2009). When the hESC-MSCs began as pre-osteoblasts there was low GFP expression, however, increased GFP expression was detected after 28 days culture in osteogenic medium, suggesting that hES-MSCs differentiated into mature osteoblasts. The ability to track differentiation allowed the isolation of osteoprogenitor cells from the derived hESC-MSC population. These studies suggest that in particular, the osteoprogenitor populations derived from hESCs have tremendous potential, and can serve as a tool through which we can characterize early bone development and cellular behavior on bone-related biomaterials.

2.2.5 Gene Transcription and Proteomic Array Analyses

The therapeutic capacity of hESC-MSCs to treat a variety of diseases lies within their capability to different into numerous cell phenotypes to repair or regenerate tissues and organs. However, it remains to be determined if transplanted MSCs, whether of hESC or adult stem cell origin, contribute to and integrate within the majority of newly formed tissue, or perhaps via paracrine action mediate and stimulate host repair and regeneration. To that end, investigation into the therapeutic potential of the hESC-MSC paracrine proteome has been conducted. Within the study, defined serum-free culture medium was conditioned by hESC-MSCs and subsequently analyzed via multidimensional protein identification and cytokine antibody array analysis (Sze et al. 2007). The array data revealed over 200 unique gene products that play a role in biological processes such as metabolism, defense, response, and tissue differentiation including vascularization, hematopoiesis, and skeletal development. These processes and pathways are associated with numerous cellular processes that are activated to participate in injury, repair, and regeneration, as well as to facilitate immune cell migration to the site of injury, ECM remodeling, and increases in cellular metabolism (Sze et al. 2007). The identification of a large number of MSC secretory products that can act as paracrine modulators provides insight into the potential mechanism of action by which hESC-MSCs may participate in tissue repair and disease treatment.

Another study investigated the gene expression profile of differentiating hESC-MSCs and reported that during derivation major transcriptional changes occurred, resulting in an expression profile very similar to that of hMSCs (de Peppo et al. 2010b). The major

questions addressed were how the transcriptome may be affected by the hESC-MSC derivation process and whether hESCs and their MSC derivatives were distinct or equivalent to one another. The findings in the hESC-MSC population revealed a down-regulation in pluripotency genes such as the *OCT* family of genes, *NANOG, TDGF1, LIN28, GDF3,* and *ZIC3,* down regulation in tumor development *p53*-associated genes *LTBP2* and *TFAP2A,* up-regulation of mesodermal lineage commitment genes such as *RUNX2, TGBR2, BMPR2,* and *TFAP2A,* and up-regulation of genes supportive of craniofacial development and osteogenesis such as *DLX1, DLX2,* and *MSX1*. Lastly, and importantly, the immunological profile of hESC-MSCs displayed lower expression than hMSCs of HLA-ABC and HLA-DR, two markers characteristic of the inflammatory immune response. These findings suggest that the hESC-MSCs may be more immuno-privileged than hMSCs, thus another piece of evidence supporting the notion that hESC-MSCs represent a suitable alternative for cell transplantation therapies (Romieu-Mourez et al. 2007); (de Peppo et al. 2010a)).

2.2.6 Epithelial-Mesenchymal Transition

Cells within the body are derived from a single cell, with variations of cell phenotypes resulting from expression of a specific and defined transcriptome, thus further imparting diversity in cellular signaling and function. Epithelia are considered to be highly plastic during embryogenesis and have the ability to shuttle back and forth between mesenchyme and epithelia through the process know as epithelial-mesenchymal transition (EMT). It is one mechanism that gives rise to mesenchymal-like behavior to cells in numerous different settings (Kalluri 2009). Historically, it has been proposed that epithelial cells

have to be terminally differentiated to in order to perform defined functions involved in organ development. However, experimental evidence has suggested that epithelial cells can alter their phenotype based on the influence of microenvironment (Boyer et al. 2000). Therefore, EMT has been accepted as a mechanism by which fibroblasts and mesenchymal cells are formed in injured tissues. In the adult, the process of EMT occurs during tissue regeneration and wound healing by facilitating mesenchymal cell migration to invade surrounding tissues. This was described as one of the three EMT subtypes that occurs, and is also suggested to be an underlying mechanism for derivation of hESC-MSCs (Zeisberg et al. 2009); (Ullmann et al. 2007).

It has been reported that hESCs grown in monolayer in feeder-free conditions, without MEFs or other supporting cells, form uniform sheets of epithelial cells after removal from standard feeder culture systems (Boyd et al. 2009; Ullmann et al. 2007). The uniform epithelial sheets exhibit characteristic mesodermal gene expression patterns that appear to undergo EMT that results in a highly proliferative population of cells that over time become uniformly homogenous with a mesenchymal stem cell morphology. It is in fact these homogenous cells that many researchers identify as hESC-MSCs, which have the ability to differentiate along multiple mesenchymal cell lineages *in vitro.* More specifically, these studies find that the hESCs that underwent mesenchymal differentation in monolayer culture were over 80% positive for E-cadherin, a characteristic epithelium marker, and maintained expression while cell morphology changed. Additionally, the cells that were undergoing apparent EMT were positive for the characteristic markers such as CD73, CD90, CD 105 and CD166, and negative for CD31, CD34, CD45, CD133

and CD146, further confirming the formation of a mesenchymal progenitor cell population (Boyd et al. 2009). The key significance of these studies is the finding that hESCs are behaving in culture in a manner similar to that of normal embryogenesis, thus underscoring the importance of using hESCs as a tool for better understanding overall human development.

2.3 Tissue Engineering Strategies for Human Clinical Applications

A major challenge for using stem cells in a clinical setting is the need to identify an ideal stem cell candidate that is multipotent while retaining its self-renewal capacity. Although hMSCs make a useful source of progenitor cells for tissue engineering strategies, as evidenced by their multipotent potential and immunosuppressive characteristics, their limited proliferative and differentiation capacity represent an obstacle for therapeutic application. In contrast, hESCs with their ability to proliferate indefinitely *in vitro* and multi lineage differentiation capacity represent an unlimited source of progenitor cells, specifically, mesenchymal progenitor cells. Therefore, it is necessary to establish clinical-grade GMP protocols for the derivation, identification, and isolation of hES-MSCs, to produce large quantities of genotypically homogenous progenitor cells that can be modified, and to fully characterize these cells for tissue regeneration strategies. Tissue engineering is an emerging field of research aimed at regenerating functional tissues by combining cells with a supporting substrate or biomaterial that possesses design characteristics that deliver progenitor cells and important signalling molecules in a spatially and temporally controlled manner, while promoting vascularization and tissue

invastion into the interior of the scaffold. Ideally, biomimetic scaffolds designed for hESC-MSC based tissue engineering strategies would contain inductive signaling cues for proliferation and differentiation, possess composite material properties that conferred the ability to generate multi-layered hybrid tissues, and have tunable three-dimensional geometrical architecture that appropriately restores form and function to anatomical defects or diseased tissues.

Within the hESC field, the use of 3D scaffolds has been employed in only a few reports (Arpornmaeklong *et al.,* 2009; Ferreira *et al.,* 2007; Kaufman *et al.,* 2010; Levenberg *et al.,* 2003; Kim *et al.,* 2007; Kuznetsov *et al.,* 2010; Smith *et al.,* 2009). Investigators have used collagen scaffolds for hepatocye differentiation, and porous polylactic/polyglycolic biomaterial sponges to direct neural, chondrogenic, or hepatocytic lineages (Levenberg et al., 2003). While other studies have shown that 3D porous alginate scaffolds to provide a conducive environment for generation of well-vascularized embryoid body derived hESCs (Ferreira et al., 2007). Within the bone tissue engineering field, the use of architecturally designed scaffolds with hESC-MSCs is seen even less frequently. It has been reported that hESC-MSCs were capable of forming bone tissue *in vivo* when implanted subcutaneously after 8 weeks in the presence of BMP-2 (Kim *et al.,* 2007). In 2009, Arpornmaeklong et al. reported the influence of composite collagen scaffolds on the osteogenic differentiation of hESC-MSCs *in vitro* as indicated by osteogenic gene induction, increased ALP activity, and the presence of mature bone ECM proteins; all of which are characteristic of the osteoblast phenotype. From an *in vivo* standpoint, enriched osteoprogenitor cells were encaspulated in fibrin

gels mixed with ceramic particles and implanted in a rat calvarial defect model. After six weeks, the identification of transplanted hESC-MSCs in newly formed bone verified the role that MSCs derived from hESCs played in the bone regeneration process (Arpornmaeklong *et al*., 2009). Another study demonstrated that hESC-MSCs can form mineralized tissue *in vitro* when cultured on 3D nanofibrous polylactic acid (PLLA) in the presence of BMP-7, illustrating the capability of hESC-MSCs to differentiate in 3D culture for bone regeneration purposes (Smith *et al.,* 2009). Most recently, a comprehensive study investigated multiple media formulations and cell culture conditions for efficient derivation of a homogenous hESC-MSC population. To determine their *in vivo* osteogenic potential, cells were implanted up to 16 wks with biphasic ceramic particles and histology revealed cells of human origin were embedded with the bone, including broad areas of multiple intertwining trabeculae (Kuznetsov *et al.,* 2010).

It is hypothesized that the hESC-MSCs not only require a 3D biomaterial, but also inductive cues. This suggests that for tissue formation, hESCs may require additional biological cues such as pro-osteogenic factors for attachment, proliferation, and directed differentiation on biomaterials. For bone formation specifically, hESCs may require an osteoconductive biomaterial with not only the appropriate scaffold architecture, but one that also can associate cellular and molecular elements to increase cellular response to the biomaterial. To that end, within this body of work we have also aimed to address the cellular response of hESC-MSC progenitors to designed osteoconductive hydroxyapatite/tri-calcium phosphate (HA/TCP) biomaterials.

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CHAPTER 3

HUMAN BONE MARROW STROMAL CELL MICROENVIRONMENT EFFECTS ON HUMAN EMBRYONIC STEM CELLS

3.1 Introduction

Human embryonic stem cells (hESCs) may offer an unlimited supply of cells that can be directed to differentiate into all cell types within the body and used in regenerative medicine for tissue and cell replacement therapies. Previous work has shown that exposing hESCs to exogenous factors such as dexamethasone, ascorbic acid and β-glycerophosphate can induce osteogenesis. The specific factors that induce osteogenic differentiation of hESCs have not been identified yet, however, it is possible that differentiated human bone marrow stromal cells (hMBSCs) may secrete factors within the local microenvironment that promote osteogenesis. In this chapter we report that the lineage progression of hESCs to osteoblasts is achieved in the presence of soluble signaling factors derived from differentiated hBMSCs. For 28 days, hESCs were grown in a transwell co-culture system with hBMSCs that had been previously differentiated in growth medium containing defined osteogenic supplements for 7-24 days. As a control, hESCs were co-cultured with undifferentiated hBMSCs and alone. Von Kossa and Alizarin Red staining as well as immunohistochemistry confirmed that the hESCs co-cultured with differentiated hBMSCs formed mineralized bone nodules and secreted

extracellular matrix protein osteocalcin (OCN). Quantitative Alizarin Red assays showed increased mineralization as compared to the control with undifferentiated hBMSCs. RT-PCR revealed the loss of pluripotent hESC markers with the concomitant gain of osteoblastic markers such as collagen type I, runx2, and osterix. We demonstrate that osteogenic growth factors derived from differentiated hBMSCs within the local microenvironment may help to promote hESC osteogenic differentiation.

Human embryonic stem cells (hESCs) present a potentially unlimited supply of cells that may be directed to differentiate into all cell types within the body and used in regenerative medicine for tissue and cell replacement therapies. An area of particular interest is stem cell transplantation for bone tissue regeneration where hESCs may be used to repair skeletal defects. One of the major gaps in the knowledge regarding hESCs is the lack of understanding of the growth factors and three-dimensional signals that control differentiation. Current techniques used for bone tissue repair employ the use of auto- and allografting methods, however, these methods have inherent limitations that restrict their universal application. The limitations of these reparative strategies suggest that an alternative approach is required, and hESCs may provide a repository of cells for such an approach. Previous work has shown that exposing hESCs to exogenous factors such as dexamethasone, ascorbic acid and β-glycerophosphate can induce osteogenesis *in vitro* (Cao et al. 2005; Karp et al. 2006; Sotille et al. 2003). However, the specific factors that regulate and influence the commitment of hESCs along the osteoblast lineage have not yet been identified. It is possible that soluble factors secreted by human bone marrow

stromal cells (hBMSCs) may provide the necessary signaling molecules to direct osteogenic differentiation of hESCs.

When bone marrow is cultured *in vitro*, adherent non-hematopoietic cells proliferate and exhibit characteristics of bone marrow stroma *in vivo*. Within this diverse population of hBMSCs there exist early progenitor mesenchymal stem cells that are capable of self-renewal and have multi-lineage differentiation potential into cell types such as osteoblasts, chondrocytes, and adipocytes. In the presence of dexamethasone, ascorbic acid and β-glycerophosphate, it has been demonstrated that hBMSCs can be differentiated readily into mineralizing osteoblasts both *in vitro* and *in vivo* (Bianco et al. 2003; Jaiswal et al. 1997; Kuo et al. 2003; Wagers et al. 2004). The *in vitro* equivalent of bone formation is characterized by the formation of mineralized nodules, increased alkaline phosphatase activity, and up regulation of osteoblastic genes such as runx2, osteocalcin, bone sialoprotein, and collagen type I (Beresford et al. 1993; Jaiswal et al. 1997; Kalajzic et al. 2005; Krebsbach et al. 1997). The use of this well-defined *in vitro* model allows the control of the differentiation state of hBMSCs at varying time points within their lineage progression towards functional osteoblasts. Subsequently, soluble factors derived from hBMSCs may be controlled, thus enabling the establishment of a co-culture system that stimulates hESC differentiation.

Therefore, the goal of this study was to determine if the lineage progression of hESCs toward osteoblasts could be directed by soluble signaling factors derived from differentiated hBMSCs. Because the differentiation of hBMSCs *in vitro* is so well

characterized, the ability to manipulate and control hBMSCs along with the osteogenic factors derived from them will be integral to controlling the osteoblastic differentiation of hESCs. In this chapter, we demonstrate that osteogenic growth factors secreted by hBMSCs into the local microenvironment can promote osteoblastic differentiation of hESCs, and the secretion of these factors was dependent on the state of cell differentiation.

3.2 Experimental Materials and Methods

3.2.1 Human ESC Culture

The BG01 cell line was obtained from Bresagen, Inc. (Atlanta, GA) and cultured on irradiated mouse embryonic fibroblast (MEF) feeder layers at a density of approximately 19,000 cells/cm^2 onto 60 mm dishes (0.1% gelatin-coated). The hESC culture medium consisted of 80% (v/v) DMEM/F12, 20% (v/v) knockout serum replacement (KOSR), 200mM L-glutamine, 10mM nonessential amino acids (all obtained from Invitrogen), 14.3M β-mercaptoethanol (Sigma, St. Louis, MO), and 8 ng/ml bFGF (Invitrogen, Carlsbad, CA). Cell cultures were incubated at 37°C in 5% CO_2 in air and 95% humidity with medium changes everyday and manually passaged once per week. To induce osteogenic differentiation, the hESCs were made into embryoid bodies (EBs) and then seeded onto 0.1% gelatin-coated 6-well plates and cultured in hBMSC osteogenic medium (OS) consisting of 90% α-MEM (v/v), 10% heat-inactivated fetal bovine serum (FBS), 200 mM L-glutamine, and 10 mM nonessential amino acids (all obtained from Invitrogen) with 100 nM dexamethasone, 10 mM β-glycerophosphate, and 50 μM ascorbic acid for 4 weeks with medium changes every 48 hours (Sotille et al. 2003).

3.2.2 Human ESC/hBMSC Transwell Co-Culture

The hBMSCs were isolated from patients at the University of Michigan using IRB approved protocols. The hBMSCs were plated at 2,000/cm^2 onto transwell inserts (0.4 μm pore, Corning, Corning, NY) and allowed to differentiate for 7-24 days in OS with complete medium changes every 48 hours. Two days prior to adding the hBMSC transwell inserts, the hESCs were either made into EBs or manually passaged directly from MEFs (omitting EB formation) and then seeded onto gelatin-coated 6-well plates. After 7, 14 or 24 days of differentiation, hBMSCs on transwell inserts were added to the 6-well plates and cultured in hBMSC growth medium (GM) without osteogenic supplements for an additional 28 days with medium changes every 48 hours. This method allows for the passage of soluble molecules while preventing direct cell-cell contact.

3.2.3 Reverse Transcriptase PCR

Total RNA was obtained using Trizol (Invitrogen) and purified using the Qiagen RNEasy kit with DNase I treatment (Qiagen, Valencia, CA). Reverse transcription of 1 μg of RNA was performed using the Superscript III kit (Invitrogen). Taq DNA Polymerase (Invitrogen) was used to amplify the cDNA. The PCR conditions were as follows: 2 minutes at 94ºC; followed by cycles of 45" denaturation at 94ºC, 45" annealing at 56ºC, and 60" extension at 72ºC. Primer sequences were as follows (forward, reverse): *oct4* (GAA GGT ATT CAG CCA AAC, CTT AAT CCA AAA ACC CTG G); *nanog* (GAC TGA GCT GGT TGC CTC AT, TTT CTT CAG GCC CAC AAA TC); *runx2* (CAT GGT GGA GAT CAT CGC, ACT CTT GCC TCG TCC ACT C); *osterix* (GCA GCT

AGA AGG GCG TGG TG, GCA GGC AGG TGA ACT TCT TC); *collagen1-Col-1* (GGA CAC AAT GGA TTG CAA GG, TAA CCA CTG CTC CAC TCT GG) [14]; *osteocalcin-OCN* (ATG AGA GCC CTC ACA CTC CTC, GCC GTA GAA GCG CCG ATA GGC); *GAPDH* (TGA AGG TCG GAG TCA ACG GAT TTG GT, CAT GRG GGC CAT GAG GTC CAC CAC) (Bielby et al. 2004; Brimble et al. 2004; Miura et al. 2003; Noth et al. 2002). PCR products were analyzed on a 1.5% agarose gel with ethidium bromide staining. Imaging was obtained using a Fluor-S system (Biorad).

3.2.4 Mineralization Assays by Alizarin Red S and von Kossa Staining

Cell cultures were fixed in 4% paraformaldehyde for 30 minutes, washed twice with PBS and then stained. Alizarin Red S (Sigma) staining was used to determine the presence of mineralized nodules. Fixed cells were incubated for 1 hour in 1% Alizarin Red S solution and then washed twice with water. Von Kossa staining was used to determine the presence of phosphate. Fixed cells were incubated for 1 hour in 5% $AgNO_3$ solution and exposed to bright light for at least 30 minutes. For mineralization quantification, Alizarin Red S precipitate was extracted using a 10% acetic acid/20% methanol solution for 45 minutes. Spectrophotometric measurements of the extracted stain were made at 450 nm.

3.2.5 Immunofluorescence

Cell cultures were fixed for 30 minutes at room temperature with 4% paraformaldehyde. After washing with PBS, cells were permeabilized for 10 minutes with 10% (v/v) Triton X-100 in PBS, then washed twice with a serum wash containing 1% (v/v) sodium azide, followed by a blocking step containing 0.5% (v/v) Triton X-100 and 1% (v/v) sodium

azide for 30 minutes at room temperature. Dilution buffer containing 2 μg/ml polyclonal rabbit anti-human osteocalcin (Santa Cruz Biotechnology, Santa Cruz, CA) was added and incubated at 4°C overnight. Following incubation, the cells were rinsed twice with serum wash and incubated in the dark with 8 μg/ml FITC-labeled secondary antibodies for 30 minutes and counterstained with DAPI. The cells were then washed with PBS and fluorescence was observed using a Nikon Eclipse TE3000.

3.2.6 Statistical Analysis

Results were evaluated using the student *t* test. Statistical significance was set at the 95% confidence level with a p-value < 0.05.

3.3 Results

3.3.1 Human ESCs Undergo Osteogenic Differentiation and Give Rise to Condensed Mineralized Nodules in the Presence of hBMSCs

Human bone marrow stromal cells are able to differentiate into osteoblasts both *in vitro* and *in vivo*. Therefore, we postulated that exposing hESCs to hBMSCs undergoing osteoblastic differentiation would stimulate differentiation of hESCs along the osteogenic lineage (Bianco et al. 2003; Jaiswal et al. 1997; Kuo et al. 2003; Wagers et al. 2004). For the transwell co-culture experiments, we used EB-derived cells. Human ESCs were plated onto gelatin-coated 6-well plates two days prior to co-culture with hBMSCs. Prior to plating the hESCs, hBMSCs were seeded onto gelatin-coated 0.4 μm pore transwell inserts and differentiated in growth medium plus defined osteogenic supplements (OS) for 7 days, 14 days, or 24 days. As controls, hESCs were co-cultured with

undifferentiated hBMSCs or cultured alone in growth medium without osteogenic supplements (GM). At each specific time point (7, 14 or 24 days), the differentiated hBMSCs were added to the hESCs that had been plated onto gelatin-coated 6-well plates. The two cell types were then co-cultured in GM for an additional 28 days. Since the two cell types were not in direct physical contact, the hESCs were exposed to soluble signaling factors derived only from differentiated hBMSCs. In addition, hESCs were grown in osteogenic medium as previously described (Cao et al. 2005; Sotille et al. 2003).

Mineralized nodule formation is the hallmark of *in vitro* osteogenic differentiation. Therefore, after 28 days the cells and extracellular matrix (ECM) produced by co-cultures were stained by von Kossa and Alizarin Red to detect the presence of phosphate and calcium, respectively (Bonewald et al. 2003; Wergedal et al. 1969). The hESCs in co-culture with 14 day differentiated hBMSCs formed bone nodules that stained positively for von Kossa and Alizarin Red (Fig. 3.1C, D). In contrast, cells grown with undifferentiated hBMSCs only showed minimal levels of mineral deposition and weak staining (Fig. 3.1A, B). As expected, the hESCs grown in the presence of osteogenic supplements for 28 days stained positive for a mineralized matrix (data not shown).

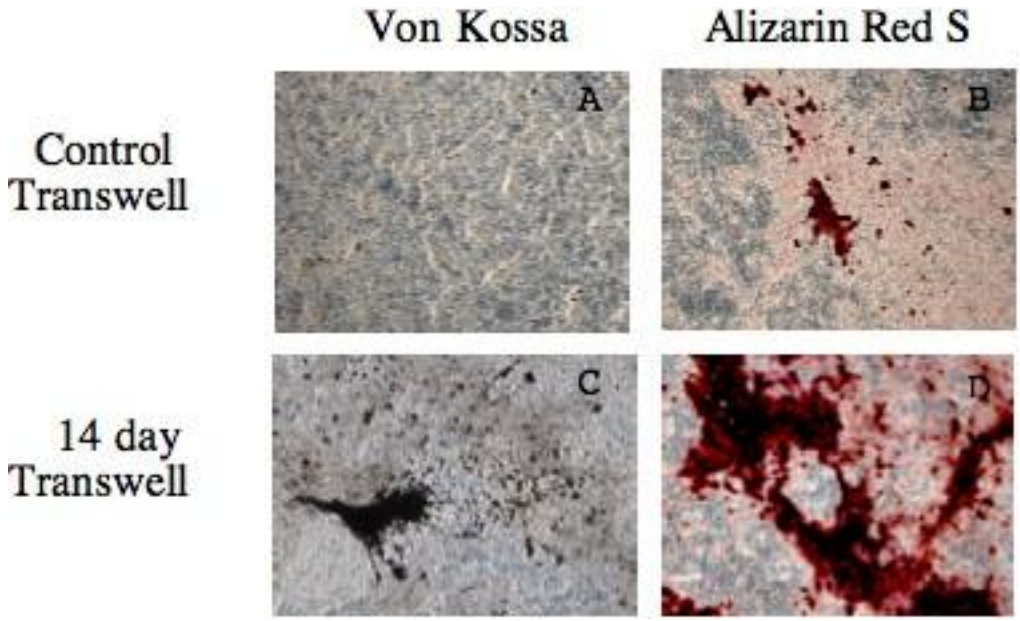

Figure 3.1. **Human embryonic stem cells mineralize when co-cultured with differentiated hBMSCs.** hBMSCs were grown on transwell inserts in osteogenic induction medium for 14 days. Differentiated hBMSCs were co-cultured with hESCs for an additional 28 days. Von Kossa (A, C) and Alizarin Red S (B, D) staining of hESCs co-cultured with differentiated or undifferentiated hBMSCs. Magnification = 10x.

Calcium deposition can also be quantified by extracting the Alizarin Red stain and subsequent spectrophotometric readings of Alizarin Red uptake. Therefore, we also performed quantitative Alizarin Red assays to assess the extent of bone nodule mineralization. There was a marked increase in mineralization within the transwell co-cultures with differentiated hBMSCs as compared to the controls. The hESCs exposed to hBMSCs differentiated for 7 days showed a 1.7-fold increase in Alizarin Red

concentration (µg/ml) above control, a 2.5-fold increase for 14 day differentiated hBMSCs, and a 1.8-fold increase for cells exposed to hBMSCs differentiated for 24 days (Fig. 3.2). Taken together, the upward trend of Alizarin Red content and greater von Kossa staining indicates that higher levels of mineral deposition could be found in hESCs exposed to differentiating hBMSCs.

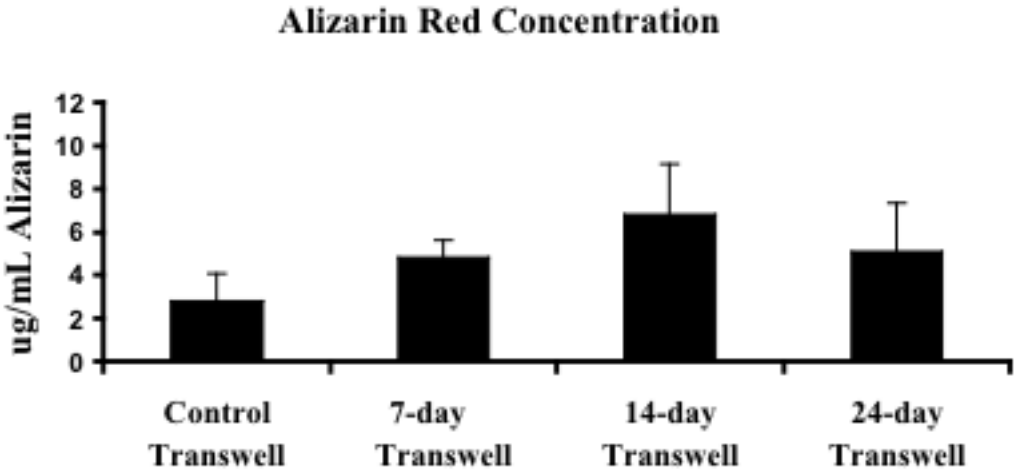

Figure 3.2. Quantitative analysis of Alizarin Red assays. hBMSCs were grown on transwell inserts in osteogenic induction medium for 7, 14 or 24 days. Differentiated hBMSCs were co-cultured with hESCs for an additional 28 days. Comparison of Alizarin Red staining between co-cultures with undifferentiated hBMSCs and experimental groups consisting of 7, 14 and 24 day differentiated hBMSCs.

3.3.2 Human ESCs Respond to the hBMSC Transwell Co-Culture System and Express Osteogenic Specific Markers

Osteoblastic lineage commitment can be observed by the expression of bone specific transcription factors runx2 and osterix, and with collagen type I production. Runx2 is a homolog of the *Drosophila* Runt protein that acts as a transcriptional regulator of osteoblast differentiation, and osterix is a zinc finger containing transcription factor

required for osteoblastic differentiation that acts downstream of runx2 [Ducy et al. 1997; Olsen et al. 1996). Collagen type I is the most abundant protein found in bone ECM (Olsen et al. 1996). Therefore, in order to further confirm osteogenic differentiation, we analyzed the expression of these bone markers after 28 days in co-culture (Fig. 3.3). RT-PCR analysis demonstrated that undifferentiated hESCs exhibit strong expression of the hESC pluripotency markers Oct-4 and Nanog, whereas they were absent in the transwell co-cultures and OS conditions (Fig. 3.3, Lane 1).

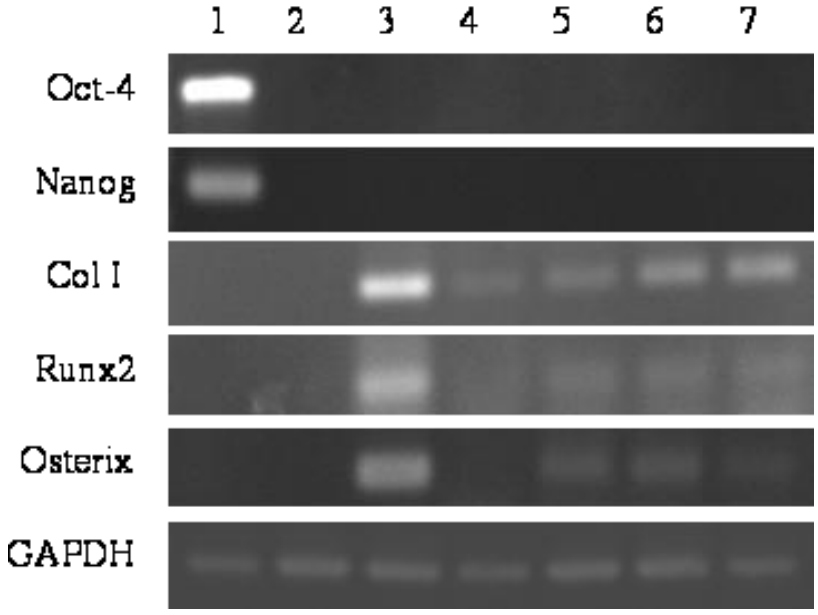

Figure 3.3. Human embryonic stem cells express osteoblastic markers when co-cultured with differentiated hBMSCs. hBMSCs were grown on transwell inserts in osteogenic induction medium for 7, 14 or 24 days. Differentiated hBMSCs were co-cultured with hESCs for an additional 28 days. Lane 1: undifferentiated hESCs; lane 2: hESC in growth medium; lane 3: hESC in osteogenic medium; lane 4: transwell co-culture with undifferentiated hBMSCs (control); lane 5: transwell co-culture with 7 day differentiated hBMSCs; lane 6: transwell co-culture with 14 day differentiated hBMSCs; lane 7: transwell co-culture with 24 day differentiated hBMSCs.

3.3.3 Human ESCs Co-Cultured with Differentiated hBMSCs Secrete the ECM Protein Osteocalcin

Fully differentiated osteoblasts have the ability to form three-dimensional nodules with many of the immunohistochemical markers of bone and also form mineralized matrix. These nodules are thought to represent the end stage of differentation of osteoprogenitor cells *in vitro* (Purpura et al. 2004). It has also been shown that three-dimensional bone nodules derived from hESCs stain positive for osteocalcin (OCN) (Bonewald et al. 2003). Results from our study show that immunostaining with antibody to OCN had strong immunoreactivity localized to clusters of hESCs co-cultured with 7, 14, and 24 day differentiated hBMSCs, whereas there was no evidence of OCN staining for hESCs exposed to undifferentiated hBMSCs (Fig. 3.4). We believe the clusters that formed in the differentiated hBMSC transwell co-cultures are condensed bone nodules comprised of hESC-derived osteoblasts that secrete osteocalcin. The hESCs readily adhered to the culture plates and proliferated extensively in the presence of hBMSCs as evidenced by DAPI staining (Fig. 3.4). As compared to hESCs grown alone or with undifferentiated hBMSCs, the cells in transwell co-culture with differentiated hBMSCs displayed bone nodule formation and mineralized matrix deposition.

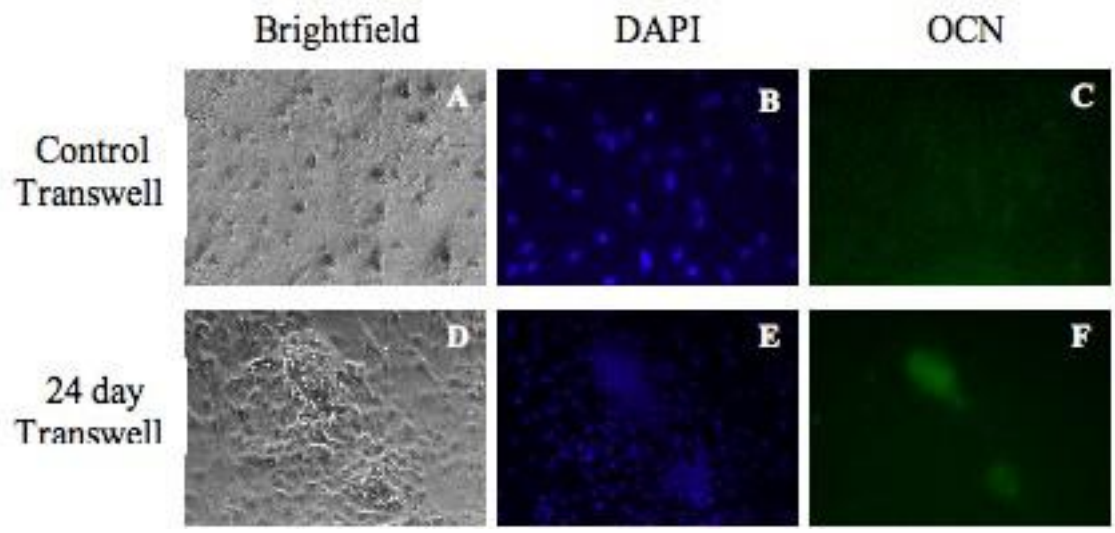

Figure 3.4. Human embryonic stem cells express osteocalcin when co-cultured with differentiated hBMSCs. hBMSCs were grown on transwell inserts in osteogenic induction medium for 24 days. Differentiated hBMSCs were co-cultured with hESCs for an additional 28 days. Expression of osteocalcin with DAPI counterstaining of hESC co-cultures with undifferentiated hBMSCs (A-C) and 24 day differentiated hBMSCs (D-F). Magnification = 10x.

3.4 Discussion

Bone marrow is a complex tissue comprised of hematopoietic precursors, as well as a connective tissue network referred to as bone marrow stroma. It has been demonstrated that culture-adherent cells present in the marrow stroma have the capability to differentiate along multiple mesenchymal lineages [21]. Therefore, within the stroma itself exists a heterogeneous population of cells including osteoprogenitor cells that can proliferate and differentiate into mature osteoblasts. These multipotent cells are referred to as mesenchymal stem cells (MSCs), and have been serially passaged without lineage progression and subsequently shown to form cartilage, bone, fat and mature stromal cell lineages [2, 5, 22-24]. Due to our knowledge about the differentiation potential of human MSCs, we hypothesized that exposing hESCs to hBMSCs at various time points along

the osteogenic differentiation pathway would contribute to directed osteoblastic differentiation of hESCs. The temporal expression of cell growth and osteoblast phenotype-related genes during osteoblast growth and differentiation has been established for primary osteoblasts, where three principle periods of osteoblast phenotype development have been identified – proliferation, ECM development and maturation, and mineralization phases. During the proliferation phase (approximately 0-12 days in culture) collagen type I is predominantly expressed. The ECM development and maturation phase (approximately 12-21 days in culture) is characterized by high alkaline phosphatase expression, and within the mineralization phase (approximately 21-35 days in culture) osteocalcin is highly expressed as cell aggregates, or nodules, become mineralized in the presence of dexamethasone, ascorbic acid and β-glycerophosphate (Stein et al. 1993). Therefore, we chose to differentiate our hBMSCs for 7, 14, and 24 days to determine if soluble factors secreted at different developmental time points would contribute to hESC differentiation.

Mineralization assays, RT-PCR and immunohistochemistry data described in this study show that by exposing the hESCs to soluble factors secreted by differentiated hBMSCs during each of the osteoblast phenotype development periods, we were able to achieve osteogenic differentation without the addition of any other exogenous factors. The osteogenic response observed can be compared to that of hBMSCs in osteogenic medium (Haynesworth et al. 1995). There were substantially higher levels of Alizarin Red staining found in hESC co-cultures with differentiated hBMSCs than co-cultures wth undifferentiated hBMSCs. More specifically, the cells exposed to hBMSCs differentiated

for 14 days gave rise to highly mineralized bone nodules as evidenced by exhibiting the strongest von Kossa and Alizarin Red staining, as well as the highest Alizarin Red concentration as compared to the other transwell co-cultures. Although the increase in Alizarin Red content was not statistically significant between the control transwell and differentiated hBMSC transwell co-cultures, the 1.5-2.5 fold increase in concentration suggests that increased mineralization occurs when hESCs are exposed to soluble factors derived from differentiated hBMSCs. Additionally, the expression of bone-specific transcription factors, runx2 and osterix, as well as collagen type I, further confirmed that we successfully obtained hESC-derived osteoblasts. Gene expression of runx2, osterix, and collagen type I was found to be upregulated by co-culture conditions with differentiated hBMSCs, which is consistent with a mature osteoblast phenotype. There were low levels of bone specific gene expression within the control transwell co-culture with undifferentiated hBMSCS, and this is likely due to the fact that spontaneous differentiation occurs, thus yielding a small population of cells undergoing osteogenic differentiation. When the cells were cultured in osteogenic supplements for 28 days there was significantly stronger induction of bone specific gene expression as compared to the transwell co-cultures. This suggests that the growth factors secreted by the hBMSCs are osteo-inductive, however, further investigation regarding the identification and temporal expression of these secreted factors is needed to optimize this co-culture system. Lastly, immunostaining of condensed bone nodules within the differentiated hBMSC transwell co-cultures verified the presence of osteocalcin, a late marker of osteogenesis that corresponds with induction of mineralization (Karp et al. 2006). Such differentiation was not observed in hESCs co-cultured with undifferentiated hBMSCs (Figure 4C).

Human embryonic stem cells hold promise for future regenerative medicine strategies. They were first derived from the inner cell mass of blastocyst stage embryos and have since been studied to determine their capacity to differentiate into all cell types (Odorico et al. 2001; Thomson et al. 1998). Osteoblastic differentiation of mouse, human, and monkey embryonic stem cells *in vitro* has previously been shown using the traditional osteogenic supplements dexamethasone, ascorbic acid and β-glycerophosphate (Karp et al. 2006; Buttery et al. 2001; Yamashita et al. 2005). Additionally, co-culture systems have been used to direct differentiation of both murine and human ES cells. Murine ES cells were shown to differentiate into osteoblasts using fetal murine osteoblasts in transwell co-culture, and hESCs were shown to differentiate into osteoblasts using direct co-culture with primary bone derived cells (Ahn et al. 2006, Buttery et al. 2001). Also, hESCs have been proven to have the capacity to differentiate into cartilage both *in vitro* and *in vivo* when exposed to primary chondrocytes in a transwell co-culture system (Vats et al. 2006).

This study differs from previous reports on the osteogenic differentiation of hESCs in two important ways: 1) Using an indirect transwell co-culture system; and 2) Controlling the state of differentiation of hBMSCs to induce hESC differentiation. The osteogenic response of the hESCs to the co-culture system may be explained by the fact that differentiated hBMSCs provide necessary osteo-inductive signals. Collectively, these data strongly support the hypothesis that the lineage progression of hESCs to osteoblasts can be directed by soluble signaling factors secreted by differentiated hBMSCs within the local cell environment. This co-culture system indicates the importance of cellular

communication and coordination; therefore, determining the role of pro-osteogenic growth factors derived from hBMSCs and the influence of the microenvironment on osteogenic hESC differentiation is essential. Understanding the regulatory mechanisms that control osteoblastic differentiation will provide segue towards developing a useful cell source for bone tissue engineering repair. Future studies include performing proteomic analyses to identify and define the secreted factors, as well as systematically altering the microenvironment to further investigate the co-culture system we have developed and how it contributes to the osteogenic lineage progression of hESCs.

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CHAPTER 4

THE DERIVATION OF MESENCHYMAL STEM CELL PROGENITORS FROM HUMAN EMBRYONIC STEM CELLS

4.1 Introduction

Human embryonic stem cells (hESCs) hold promise for tissue regeneration therapies by providing a potentially unlimited source of cells capable of undergoing differentiation into specified cell types. Several preclinical studies and a few clinical studies use human bone marrow stromal cells (hBMSCs) to treat skeletal diseases and repair damaged tissue. However, hBMSCs have limited proliferation and differentiation capacity, suggesting that an alternate cell source is desirable, and hESCs may serve this purpose. In this chapter we describe a protocol for the reproducible derivation of mesenchymal stem cells from hESCs (hES-MSCs). The hES-MSCs have a similar immunophenotype to hBMSCs, specifically they are CD73+, STRO-1+ and CD45–, and are karyotypically stable. The derived hES-MSCs are also capable of differentiating into osteoblasts and adipocytes. When the hES-MSCs were genetically modified with the lineage-specific Col2.3-GFP lentivirus and cultured in osteogenic medium, increased GFP expression was detected over time, indicating the hESMSCs have the capacity to differentiate down the osteogenic lineage and had progressed toward a mature osteoblast phenotype.

The current major goal for human embryonic stem cell (hESC) research is the controlled differentiation into specific progenitor cells for the purpose of replacing or regenerating

damaged tissue. Therefore, the ability to obtain large quantities of multipotent cells from hESCs represents a challenge for cell-based therapy and tissue engineering strategies that currently rely on human bone marrow stromal cells (hBMSCs). Within the diverse population of hBMSCs, there exist early progenitor mesenchymal stem cells capable of self-renewal and multilineage differentiation into cell types such as osteoblasts, chondrocytes and adipocytes [Bianco et al., 2003; Wagers and Weissman, 2004]. While hBMSCs make a useful source of osteoprogenitor cells for tissue engineering strategies, they have limited proliferation and differentiation capacity. On the other hand, hESCs, which are able to proliferate indefinitely in vitro, represent a potentially unlimited source of osteoprogenitor cells. Recent studies have shown that mesenchymal precursors have been derived from hESCs via various isolation methods, and the generation of osteoblasts has been achieved in the presence of known osteogenic supplements and coculture with primary bone-derived cells [Sotille et al., 2003; Cao et al., 2005; Ahn et al., 2006; Karp et al., 2006; Olivier et al., 2006; Duplomb, 2007; Lian et al., 2007; Tong et al., 2007]. However, the identification and characterization of a pure osteoprogenitor cell population has yet to be achieved. Osteoprogenitor cells derived from hESCs have tremendous potential, as they can serve as a tool through which one cannot only characterize early bone development and cellular behavior on bone-related biomaterials, but also have application in regenerative medicine. Therefore, the goal of this study was to derive progenitor cells from hESCs that can undergo differentiation along mesenchymal lineages. These cells are known as hESC-derived mesenchymal stem cells (hES-MSCs). We generated hES-MSCs and relied on functional and morphological criteria to identify them. The criteria included growth on tissue culture plastic, expression

of characteristic mesenchymal stem cell surface markers and the ability to undergo multilineage differentiation. Moreover, we were able to transduce the derived hES-MSCs with bone-specific lentivirus Col2.3-GFP in order to track cells undergoing osteoblast differentiation. By isolating hES-MSCs, we have the ability to produce a large supply of osteoprogenitor cells that can be genetically modified and used for tissue engineering strategies.

4.2 Materials and Methods

4.2.1 Human ESC Culture

The BG01 cell line was obtained from Bresagen Inc. (Atlanta, Ga., USA) and cultured on irradiated mouse embryonic fibroblast feeder layers at a density of $4x10^5$ cells/plate (0.1% gelatin coated, 60 mm). The hESC culture medium consisted of 80% (v/v) DMEM/F12, 20% (v/v) knockout serum replacement, 200 mM L -glutamine, 10 mM nonessential amino acids (all obtained from Invitrogen, Carlsbad, Calif., USA), 14.3 M β-mercaptoethanol (Sigma, St. Louis, Mo., USA) and 4 ng/ml bFGF (Invitrogen). Cell cultures were incubated at 37° C in 5% CO_2 in air and 95% humidity with medium changes everyday and manually passaged once per week.

4.2.2 Derivation of hES-MSCs

To induce mesenchymal differentiation, embryoid bodies were formed and cultured in suspension for 7 days with hESC growth medium in low-attachment culture dishes. Then, approximately 70 embryoid bodies/well were plated onto 0.1% gelatin-coated 6-well plates in the presence of hBMSC growth medium (α-MEM, 10% FBS, 200 mM L-glutamine and 10 m M nonessential) and 4 ng/ml bFGF. After EB attachment, cells were

cultured for up to 2 weeks to reach confluence, then trypsinized and passaged in a ratio of 1:3. Cells were continually passaged into T-75 flasks until a homogeneous fibroblastic morphology appeared. Differentiation into osteoblasts and adipocytes was performed as previously described, and von Kossa, alizarin red and oil red O staining was performed according to standard protocols [Barberi et al., 2005].

4.2.3 Col2.3-GFP Lentiviral Infection

The Col2.3-GFP lentivirus was a kind gift from Dr. Peng Liu (Aastrom Bioscience Inc., Ann Arbor, Mich., USA). Initially, cells were seeded onto 6-well plates at 10,000 cells/cm^2. The following day, cells were transduced with 0.5 ml of Col2.3-GFP lentivirus along with 5 μg/ml polybrene for 4h with shaking. Then, complete growth medium was added and cells were further cultured overnight. Approximately 18–24h later, the medium was aspirated and either control growth medium or osteogenic medium was added. The cells were then cultured for 28 days.

4.2.4 Immunohistochemistry

Immunofluorescent staining for mesenchymal stem cell surface markers was performed on the hES-MSCs. After cells were fixed for 30 min with 4% paraformaldehyde, they were permeabilized for 10 min with 10% (v/v) Triton X-100 in PBS, then washed twice with a serum wash containing 1% (v/v) sodium azide, followed by a blocking step containing 0.5% (v/v) Triton X-100 and 1% (v/v) sodium azide for 30 min at room temperature. Dilution buffer containing 2 μg/ml polyclonal rabbit anti-human CD45 and CD73, and monoclonal mouse anti-human STRO-1 (Santa Cruz Biotechnology, Santa Cruz, Calif., USA) were added and incubated at 4°C overnight. Following incubation, the cells were rinsed twice with serum wash and incubated in the dark with 8

μg/ml FITC-labeled and Texas red-labeled secondary antibodies for 30 min and counterstained with DAPI. Cells were then washed with PBS and fluorescence was observed using a Nikon Eclipse TE3000.

4.3 Results

To confirm that our differentiation protocol into mesenchymal progenitor cells from hESCs was efficient, the expression of surface antigens similar to adult mesenchymal stem cells was evaluated. Immunofluorescent staining on hES-MSCs demonstrated that cells were positive for CD73 and STRO-1, and negative for the hematopoietic marker CD45 (Fig. 4.1).

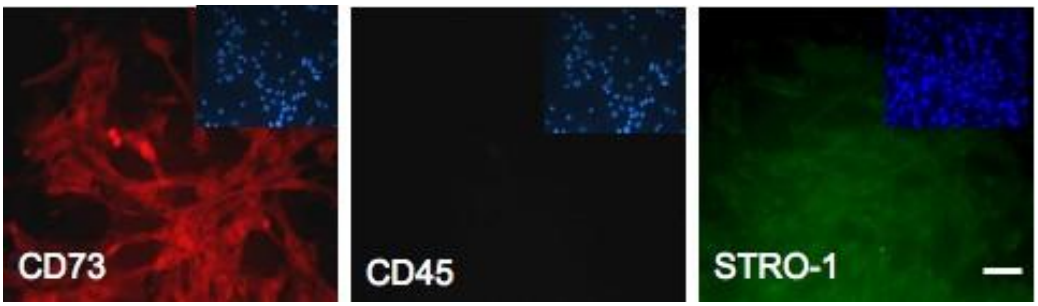

Figure 4.1 Characteristic mesenchymal stem cell markers are expressed by derived hES-MSCs. The hES-MSCs are positive for CD73 and STRO-1, but negative for CD45. Insets show DAPI nuclear counterstaining. Scale bar = 100 μm.

To verify that the derived hES-MSCs can undergo differentiation along mesenchymal lineages, osteogenic and adipogenic differentiation were performed according to standard differentiation protocols previously described [Barberi et al., 2005]. Differentiation of hES-MSCs towards functional osteoblasts was achieved after being cultured in osteogenic medium for 4 weeks (Fig. 4.2). Tissue mineralization was demonstrated by

von Kossa and alizarin red assays. Moreover, RT-PCR analysis showed the expression of bone-specific transcription factor Runx2 and the extracellular matrix protein osteocalcin (OCN) mRNA, compared to control cells cultured in growth medium without osteogenic supplements. Both genes are upregulated in mature osteoblasts. Differentiation of hES-MSCs towards functional adipocytes was achieved after being cultured in adipogenic medium for 4 weeks (Fig. 4.2). Positive oil red O staining confirmed the presence of lipid droplets, and PPAR-γ gene expression validated the induction of adipogenesis, compared to control hES-MSCs cultured without supplements.

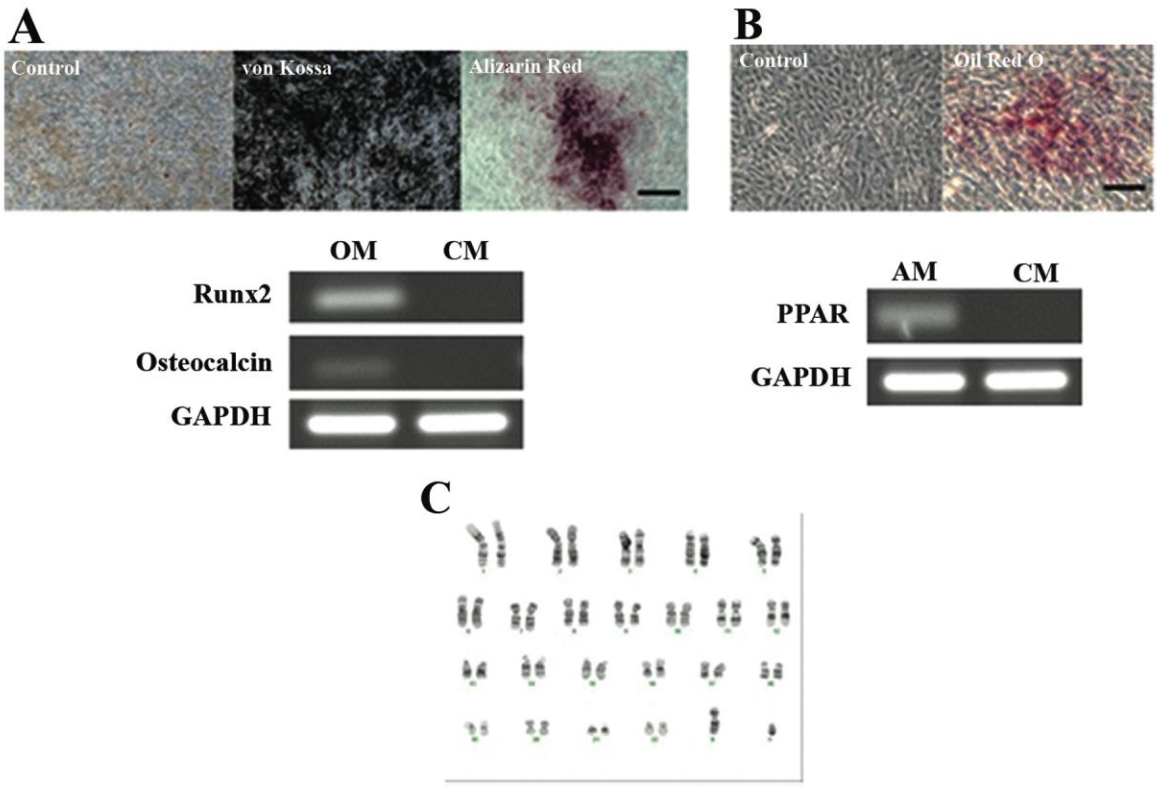

Fig. 4.2. hES-MSCs undergo differentiation along mesenchymal lineages. **A.** Osteoblast differentiation evidenced by positive von Kossa and alizarin red staining, and Runx2 and osteocalcin gene expression compared to control. **B.** Adipogenic differentiation shown by positive oil red O staining and PPAR-γ gene expression compared to control. **C.** Karyotype analysis of hES-MSCs showing no chromosomal

abnormalities. OM = Osteogenic medium; CM = growth medium without osteogenic supplements; AM = adipogenic medium. Scale bars = 100 μm.

The ability to genetically modify hESC-MSCs would be quite useful in future tissue regeneration therapies. hES-MSCs were transduced with the lineage-specific transgene Col2.3-GFP, which is activated as cells differentiate into osteoblasts [Krebsbach et al., 1993]. After viral transfection, hES-MSC were cultured with either normal growth media or osteogenic media over a 28-day time period. In control groups where Col2.3-GFP-transduced hES-MSCs were cultured with normal growth media, no GFP expression was noticed. Meanwhile, a gradual increase in GFP expression was observed during the time course of osteogenic differentiation, as cells progressed towards a mature osteoblast phenotype (Fig. 4.3).

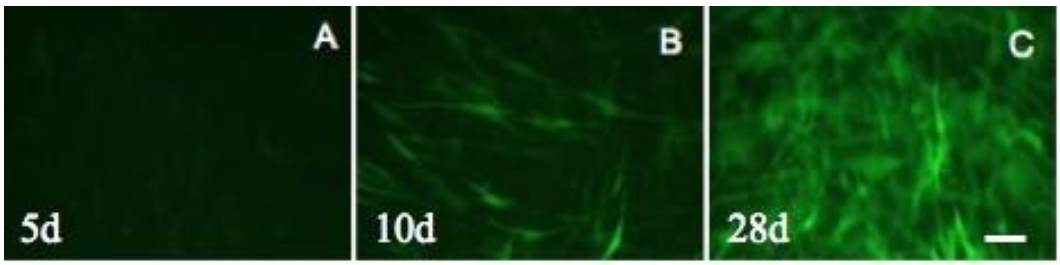

Fig. 4.3. Commitment to the osteogenic lineage of hES-MSCs transduced with Col2.3-GFP lentivirus. GFP expression increases over time as hES-MSCs differentiate along the osteogenic lineage. hES-MSCs cultured for 5 (**a**), 10 (**b**) and 28 days (**c**) in growth medium with osteogenic supplements. Scale bar = 100 μm.

4.4 Discussion

Human ESC research is a rapidly developing field, and has the potential to impact the medical and scientific community immensely. In general, stem cell research advances the knowledge about how an organism develops and how progenitor cells migrate from the stem cell niche to the site of damaged or diseased tissue. It is vitally important that we

begin to explore hESC biology in order to realize the potential of hESCs to cure diseases [Thomson et al., 1998]. Currently, there are major gaps in our knowledge about the growth factors and three-dimensional milieu that influence and direct osteoblast differentiation. The generation of osteoblasts from hESCs has been shown to be successful, as evidenced by osteogenic gene expression of Runx2, bone-specific alkaline phosphatase, and OCN, mineralization confirmed by von Kossa and alizarin red staining, and bone nodule formation [Duplomb, 2007]. One of the first differentiation studies cultured hESCs in the presence of defined osteogenic supplements for 21 days, and was able to demonstrate mineralization and induction of osteoblastic marker expression [Sotille et al., 2003]. hESCs have been cocultured with primary bone-derived cells to induce osteoblast differentiation without the addition of exogenous factors, and cultured in vitro in the presence of known osteogenic factors without the embryoid body formation step – both studies confirming that hESCs have the capacity to differentiate into osteoblasts [Ahn et al., 2006; Karp et al., 2006]. The osteogenic lineages derived from hESCs have tremendous potential and serve as a tool through which we can characterize early bone development and cellular behavior on bone-related biomaterials.

Mineralization assays, RT-PCR analysis and immunofluorescent staining demonstrated that according to our protocol we derived a mesenchymal stem cell population from hESCs. Characterizing the hES-MSCs via immunofluorescent staining demonstrated the expression of cell surface markers that are characteristic of mesenchymal stem cells. The positive expression of CD73 and STRO-1, as well as the lack of expression of CD45 show that the cells possess a similar surface antigen expression profile as hBMSCs. Studies have identified the STRO-1+ fraction of adult hBMSCs as osteogenic precursors,

suggesting that we can further isolate osteoprogenitor cells from the derived hES-MSC population [Gronthos et al., 1994, 1999; Stewart et al., 1999]. Functional differentiation results demonstrated the potential of derived hES-MSCs to become osteoblasts and adipocytes. The osteogenic and adipogenic response of hES-MSCs is similar to that of hBMSCs in osteogenic and adipogenic medium [Pittenger et al., 1996]. Von Kossa and alizarin red staining revealed the hES-MSCs formed mineralized bone nodules in culture, and the ex-pression of bone-specific transcription factor Runx2 and extracellular matrix protein OCN suggests the presence of mature osteoblasts. Oil red O staining revealed the ability of hES-MSCs to differentiate into phenotypically mature adipocyte cells with cytoplasmic lipid droplets. Additionally, the expression of PPAR-γ further confirmed differentiation into adipocytes.

In order to track and isolate cells as they undergo differentiation, gene delivery is a useful tool that can be used. The proposed transgene construct is an osteogenic lineage-specific Col2.3-GFP lentivirus, which has been a useful tool for studying hBMSC differentiation [Kalajzic et al., 2002, 2005]. Therefore, we used the Col2.3-GFP transgene to study hES-MSC differentiation along an osteoblast lineage. When the hES-MSCs began as pre-osteoblasts there was low GFP expression, however, there was an increase in GFP expression after 28 days of culture in osteogenic medium, suggesting that hES-MSCs differentiated into mature osteoblasts. The ability to track differentiation allows us to isolate osteoprogenitor cells from the derived hESMSC population.

In summary, although hBMSCs make a useful source of osteoprogenitor cells for tissue

engineering strategies, their limited proliferation and differentiation capacity represents an obstacle for therapeutic application. On the other hand, hESCs with their ability to proliferate indefinitely in vitro and multilineage differentiation capacity represent an unlimited source of osteoprogenitor cells. Here we present a protocol for the derivation, identification and isolation of hES-MSCs with osteogenic capacity, which gives us the ability to produce large quantities of genotypically homogenous progenitor cells that can be modified for tissue regeneration strategies. Future work in the area of identifying a subset of osteoprogenitors, based on immunophenotype and osteogenic potential, within the hES-MSC progenitor population would also provide a supply of cells through which we can understand cellular differentiation and overall bone development. In the next chapter, we will describe our investigation into identifying sub-populations of osteoprogenitors capable of *in vitro* differentiation and *in vivo* bone formation.

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CHAPTER 5

IN VITRO DERIVATION OF OSTEOPROGENITORS FROM HUMAN EMBRYONIC STEM CELLS AND IN VIVO BONE FORMATION IN AN ORTHOTOPIC CALVARIAL DEFECT MICROENVIRONMENT

5.1 Introduction

Advanced studies that suggest cells derived from hESCs can be used in regenerative medicine have developed significantly. Of particular interest in the field of regenerative medicine is the treatment osteodegenerative diseases, including bone loss caused by trauma, neoplasia, reconstructive surgery, or congenital defects. Advancements in stem cell research have suggested that hESCs can serve as a potential source of mesenchymal stem cells (hES-MSCs), and subsequently functional osteoprogenitors for bone regeneration strategies with clinical applications. Research in the area of directed differentiation into specific progenitor cells for the purpose of replacing or regenerating damaged tissue has continued to be one of the major goals within the hESC field. To that end, being able to reproducibly derive large quantities of multipotent cells from hESCs represents an alternative for cell based therapy and tissue engineering strategies that currently rely on human bone marrow stromal cells (hBMSCs). For nearly the past decade, numerous studies have been published demonstrating that mesenchymal

precursors can be derived from hESCs via various isolation methods, as well as successful differentiation of hESCs into osteoblasts in the presence of known osteogenic supplements and co-culture with primary bone derived cells (Sotille et al, 2003; Bielby et al, 2004; Barberi et al, 2005; Cao et al, 2005; Ahn et al, 2006, Karp et al, 2006; Olivier et al, 2006; Duplomb et al, 2007; Karner et al, 2007; Tong et al, 2007; Arpornmaeklong et al, 2009; Brown et al, 2009). Although these studies provided information about the practical use of MSCs derived from hESCs for bone regeneration, the actual identification and characterization of a pure osteoprogenitor cell population has yet to be achieved. Therefore, to address this question, the main goals of the study discussed in this chapter were: 1. To derive and identify sub-populations of osteoprogenitor hES-MSCs based on surface marker expression via fluorescence-activated cell sorting (FACS) analysis; 2. To characterize and determine the *in vitro* osteogenic potential of these isolated osteoprogenitors; and 3. To investigate their *in vivo* osteogenic potential when implanted into mouse calvaria defects. Amongst the numerous populations that were FAC-sorted, here we report the selection of the top two candidate hES-MSC osteogprogenitor populations: CD105/CD73 and ALP populations. From an *in vitro* standpoint, the different populations demonstrated varying degrees of mineralization and differentiation as evidenced by Alizarin Red staining, mineralization quantification, and gene expression, with the CD73/CD105 population exhibiting the greatest osteogenic potential *in vitro*. Further characterization of osteoprogenitor sub-populations within hES-MSCs revealed that CD73 and CD105 expression increased over time as the cells differentiated, whereas ALP was expressed at lower levels over the entire culture period. This suggested that ALP cells represented a much smaller fraction of the overall

mesenchymal progenitor cell population. To determine *in vivo* osteogenic potential, derived CD73/CD105 and ALP osteoprogenitors were differentiated in medium containing osteogenic supplements for 7 days and then implanted into critical sized calvaria defects of SCID mice for 8 weeks. MicroCT analysis and histology revealed early bone formation in the mouse calvaria, and furthermore, human mitochondrial staining confirmed the participation of the transplanted human cells in the bone reparative process. Taken together, the study discussed in this chapter demonstrates the capability of deriving and isolating distinct hES-MSC osteoprogenitor sub-populations based on their cell surface marker expression, and the ability to produce a large supply of functional osteoprogenitor cells capable of regenerating bone *in vivo* that have great potential for use in tissue engineering strategies.

5.2 Materials and Methods

5.2.1 Human ESC Culture

The H9 cell line was obtained from WiCell (Madison, WI) and cultured on irradiated mouse embryonic fibroblast feeder (MEF) layers at a density of $3x10^5$ cells/plate (0.1% gelatin coated, 60 mm). MEFs were obtained from Global Stem, Inc (Rockville, MD). The hESC culture medium consisted of 80% (v/v) DMEM/F12, 20% (v/v) knockout serum replacement, 200 m M L -glutamine, 10 m M nonessential amino acids (all obtained from Invitrogen, Carlsbad, CA), 14.3M β-mercaptoethanol (Sigma, St. Louis, Mo., USA) and 4 ng/ml FGF-2 (Invitrogen). Cells were incubated at 37° C in 5% CO_2 in air and 95% humidity with medium changes everyday and manually passaged once per week.

5.2.2 Derivation of hES-MSCs

To induce differentiation of hESCs into MSCs, hESC colonies were mechanically dissociated from the MEF feeder layers and embryoid bodies (EBs) were formed via suspension culture for 7 days with human cell conditioned medium (hCCM) in low-attachment culture dishes (Invitrogen). Then, approximately 70 EBs/well were plated onto 0.1% gelatin-coated 6-well plates in the presence of MSC growth medium (α-MEM, 10% FBS, 200 mM L-glutamine and 10 mM nonessential amino acid supplement and 8 ng/ml FGF2. After EB attachment, cells were cultured up to 2 weeks to reach confluence, then trypsinized and passaged at a ratio of 1:3. Cells were continually passaged in T-75 flasks until a homogeneous fibroblast morphology appeared.

The characterization of MSCs included functional differentiation assays *in vitro* into osteoblasts, adipocytes, and chondrocytes according to previously described protocol as well as immunophenotype analysis. Cell surface antigen profiling was performed using fluorescent-activated cell sorting (FACS). MSCs were harvested using 0.25% trypsin, and after neutralization, single cell suspensions were washed in cold 0.5% BSA (Sigma) in DPBS and incubated at a concentration of $1x10^6$ cells/mL in 1 µg/mL unconjugated goat anti-human IgG (Invitrogen) on ice for 15 min to block nonspecific binding. Samples ($2.5x10^5$ cells) were then incubated on ice with the optimal dilution of fluorochrome-conjugated monoclonal antibody (mAb) in 1 µg/mL unconjugated goat anti-human IgG in the dark. All mAbs were of the immunoglobulin G1 (IgG1) isotype. The following conjugated antibodies were used in the analyses: allophycocyanin (APC)-

conjugated antibodies against CD45 and ALP, fluorescein isothiocyanate (FITC)-conjugated mAbs against SSEA-4, and phycoerythrin (PE)-conjugated mAbs against CD73, CD105, CD166, and STRO-1. All antibodies were from BD Pharmingen (TM, San Jose, CA) except for CD105, which was obtained from eBioscience (San Diego, CA). After 30 min incubation, cells were washed twice with ice cold 0.5% BSA in DPBS. Nonspecific fluorescence was determined by incubating cells with respective fluorochrome conjugates raised against antihuman IgG1. At least 10,000 events were acquired for each sample using a FACSCalibur instrument (Becton Dickinson, San Jose, CA) and cell flow cytometry data were analyzed using CELLQUEST software (Becton Dickinson).

5.2.4 *In Vitro* Differentiation and Characterization of FAC-sorted hES-MSC Osteoprogenitor Populations

The hES-MSC osteoprogenitor populations used in this study were: CD73, CD105, CD166, ALP, and CD73/CD105 positive cells, with unsorted hES-MSCs and hBMSCs (Lonza Walkersville Inc., Walkersville, MD) as control. To induce osteogenic differentiation, cells were cultured in osteogenic medium comprised of 90% α-MEM (Gibco), 10% heat inactivated fetal bovine serum (FBS) (Gibco), 50 μM ascorbic acid, 10 mM β-glycerophosphate and 100 nM dexamethasone (all from Sigma) (Sottile et al., 2003). Culture medium was changed every 48 hours.

For adipocyte differentiation, the cells were incubated in DMEM supplemented with 15% FCS, 100 U/ml penicillin, 100 μg/ml streptomycin, 12 mM L-glutamine, 5 μg/ml insulin

(Sigma), 50 μM indomethacin (Sigma), 1 ×10^{-6} M dexamethasone (Sigma), and 0.5 μM 3-isobutyl-1-methylxanthine (IBMX; Sigma). Media was changed 2 times per week for 3 weeks. Cells were fixed with 10% formalin for 20 minutes at RT and stained with Oil Red (Sigma) in ethanol (Sigma) for 20 minutes at RT.

For chondrocyte differentiation, cells were incubated in DMEM that was supplemented with 15% FCS, 100 U/ml penicillin, 100 μg/ml streptomycin, 12 mM L-glutamine, 5 μg/ml insulin (Sigma), 50 μM indomethacin (Sigma), 1 ×10^{-6} M dexamethasone (Sigma), and 0.5 μM 3-isobutyl-1-methylxanthine (IBMX; Sigma). Media was changed 2 times per week for 3 weeks. Cells were fixed with 10% formalin for 20 minutes at RT and stained Safranin O (Sigma) in ethanol (Sigma) for 20 minutes at RT.

For histological analysis, cell cultures were fixed in 4% paraformaldehyde for 30 minutes, washed twice with PBS and then stained. Alizarin Red S (Sigma) staining was used to determine the presence of mineralized nodules. Fixed cells were incubated for 1 hour in 1% Alizarin Red S solution and then washed twice with water. For mineralization quantification, Alizarin Red S precipitate was extracted using a 10% acetic acid solution for 30 minutes. Spectrophotometric measurements of the extracted stain were made at 405 nm with high and low range 2-fold serial dilutions of 2 mM stock Alizarin Red standard. For gene expression analysis, osteoblast-associated genes such as Runx2, Col I, and OCN were performed to assess phenotypic differentiation into osteoblasts.

5.2.5 Quantitative PCR Analysis

Quantitative RT-PCR was performed to determine expression levels of osteoblast-associated genes (*Runx2, Collagen Type I,* and *Osteocalcin)* and adipocyte-associated gene (*PPAR-γ).* Gene expression levels were determined in undifferentiated hESCs, unsorted hES-MSCs, FAC-sorted hES-MSC osteoprogenitor populations at the second passage (P2) post-sorting, and hBMSCs. Total RNA was extracted using Trizol (Invitrogen) and 1 µg of RNA was reverse transcribed into cDNA using Superscript III Reverse Transcriptase (Invitrogen). Two µl of the diluted reverse transcribed cDNA (RT reaction, 1:5 in RNase free-water) was amplified in a 30 µl PCR assay volume, using the TaqMan Gene Expression Master Mix (Applied Biosystems, Foster City, CA) and 20x Target primers and Probe (unlabled PCR primers and a FAM^{TM} dye-labled TaqMan® minor groove binder (MGB) probe) (Applied Biosystems). The expression of the genes was measured by qRT-PCR on an ABI Prism 7700 Sequence Detection System (Applied Biosystems). The levels of the target genes were correlated to standard concentrations and normalized to the levels of beta-actin (ACTB) as an endogenous reference. Subsequently, the expression levels of investigated genes were normalized to the expression levels of undifferentiated hESCs (H9 passage 25) and reported as fold changes.

5.2.6 Preparation of hES-MSCs for implantation

Unsorted, CD73/CD105, and ALP hES-MSCs were harvested using 0.25% trypsin-0.53 mM EDTA (Gibco), and cell pellets were re-suspended in 5 mg/ml human plasma fibrinogen (Sigma) at a concentration of $2x10^6$ cells per 40 µl in 1.5 ml tubes. The fibrinogen cell suspension were then pipetted directly into 5 x 3 mm cubic pieces of

Gelfoam. Following absorption by the Gelfoam sponges, five µl of human thrombin, 200 unit/ml (Sigma, MO) were added and placed immediately on ice until implantation. Gelation of the fibrin gel was observed within 10 minutes. In each of the groups, 300 ng of rhBMP-2 (Sigma) was added to half of the implants (n=3).

5.2.7 Surgical procedure and cell transplantation in a craniofacial defect model

All procedures were approved by the University of Michigan Committee on the Use and Care of Animals. Four 5 week-old female immunocompromised mice (N:NIH-bg-nu-xid; Harlan Sprague Dawley, Inc., NC) were anesthetized by intraperitoneal injections of ketamine, (Ketaset, 75 mg/kg, Fort Dodge Animal Health, IA) and xylazine, (Ansed, 10 mg/kg, Lloyd laboratories, IA). A semilunar scalp incision was made from right to left of the post-auricular areas, and a full-thickness flap was elevated. The periosteum overlying the calvarial bone was completely resected. A trephine was used to create a 3-mm craniotomy defect proximal to the sagittal sinus and the calvarial disk was removed. The incisions were closed with 4-0 Chromic Gut suture (Ethicon/Johonson&Jhonson, NJ). All mice were sacrificed 8 weeks after the implantation.

5.2.8 Radiology, histology and micro-CT analyses

Radiographic analysis was performed immediately after the calvariae were harvested with the use of a microradiographic apparatus (Faxitron X-ray Corporation, IL). Then the calvaria were immediately fixed in aqueous buffered zinc formalin, Z fix (Anatech, MI) and then scanned for the micro-computed tomography (µCT) analysis. Calvaria were subsequently decalcified with a 10% EDTA solution for 2-3 weeks, dehydrated with gradient alcohols and embedded in paraffin. Coronal sections 5 µm in thickness were cut and stained with hematoxylin and eosin. Specimens were embedded in 1% agarose and

placed in a 34 mm diameter tube and scanned over the entire length of the skulls using a microCT system (μCT100 Scanco Medical, Bassersdorf, Switzerland). Scan settings were: voxel size 12 μm, medium resolution, 70 kVp, 114 μA, 0.5 mm AL filter, and integration time 500 ms. The center of the defect was identified and a cylindrical volume of interest (3 mm diameter) was drawn centered around the defect. Analysis was performed using the manufacturer's evaluation software and a fixed global threshold of 20% (200 on a grayscale of 0–1000) was used to segment bone from non-bone. Total bone volume (BV, mm3) was computed and calibrated to the manufacturer's hydroxyapatite (HA) phantom.

5.2.9 Statistical analyses

Data are expressed as the mean value ± the standard error of the mean (mean ± SEM) and analyzed by one-way analysis of variance. Statistical significance was set at $p<0.05$.

5.3 Results

5.3.1 Derivation of hES-MSCs

Human ESCs were cultured on MEFs as distinct colonies prior to being harvested as EBs (or cell aggregates) for 7 days prior to being plated onto gelatin coated dishes to begin mesenchymal stem cell (MSC) derivation (Fig 5.1 A and B). At passage 0 (P0), EBs (or cell aggregates) were plated at high density and attached overnight. Following attachment, cells with heterogeneous morphologies began to grow from the loosely attached EBs until confluence. Then cells were trypsinized and re-plated over multiple passages, which supported growth of different supporting cell types at P1 until a

homogenous fibroblast morphology appeared at P5 (Fig. 5.1 C and D).

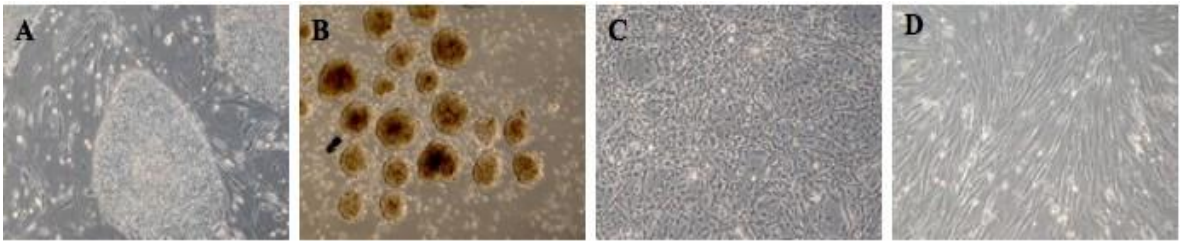

Figure 5.1 hES-MSCs have a homogenous fibroblastic morphology after five passages. A. Brightfield image of an undifferentiated hESC colony. B. Embryoid Bodies (EBs) grown in suspension for 7 days. C. Passage 0 cells after initial plating onto gelatin coated dishes showing heterogeneous morphology. D. P5 hES-MSCs showing their homogeneous fibroblastic morphology.

To determine if the similarity between surface markers in the hES-MSCs and hBMSCs, FACS analysis was performed at passages 1, 4, and 9 (P1, P4 and P9, respectively). The MSCs derived from hESCs exhibited a panel of markers characteristic of hBMSCs such as CD73, CD105, STRO-1, ALP, and CD166, and were negative for hematopoeitic stem cell markers such as CD45 (Figure 5.2). By P4, hES-MSCs were 76% for SSEA-4, 95% positive for CD73, 90% positive for CD105, and 0.4% positive for CD45, which was similiar to P4 hBMSCs that were 76% ±3 for SSEA-4, 81% ±5 for CD73, 76% ±4 for CD105, and 0.15% ±2 positive for CD45. The immunophenotype for the hES-MSCs was consistent over multiple passages, with a decrease in MSC marker expression only at the ninth passage.

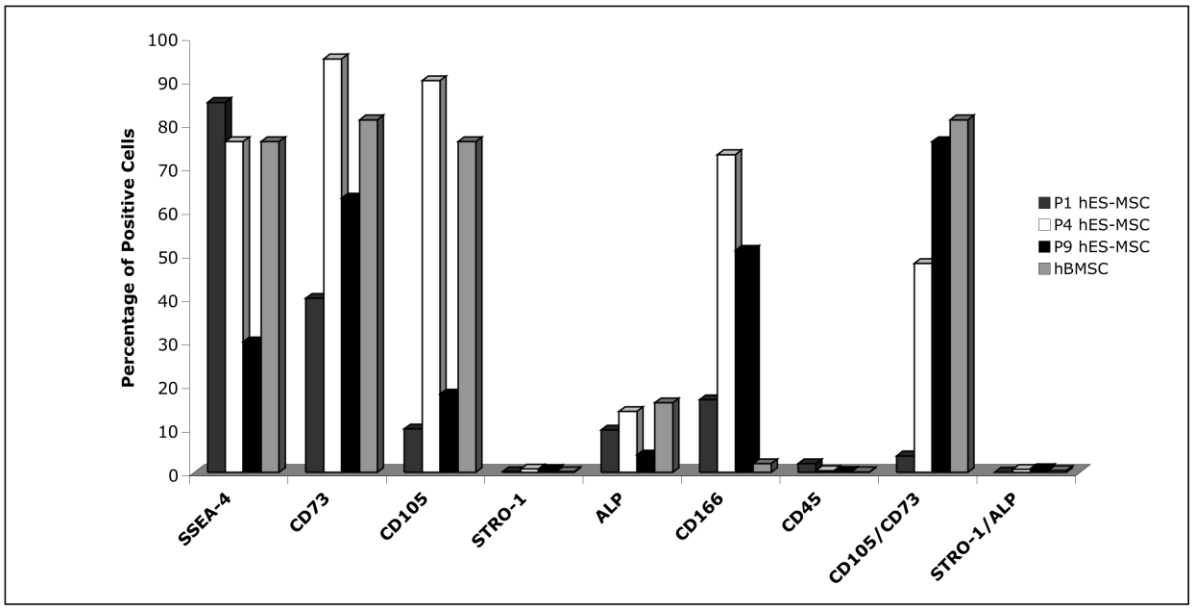

Figure 5.2 hES-MSCs express characteristic adult hBMSC surface markers. The expression of characteristic MSC cell-surface markers was analyzed by flow cytometry at different passages (1, 4 and 9). As a control, human bone marrow cells (P2) were used.

To verify phenotypic differentiation into osteoblasts, adipocytes, and chondrocytes, functional assays were performed to determine the ability of the hES-MSCs to undergo multilineage differentiation along the mesenchymal lineage. Although robust chondrogenic differentiation was not observed, adipogenic and osteoblastic differentiation were achieved as evidenced by histochemical staining and gene expression analysis. Osteogenic differentiation was demonstrated after culture for 28 days in medium containing osteogenic supplements as evidenced by positive Alizarin Red S staining of calcium deposition and mineralization within differentiated hES-MSC cultures (Fig. 5.3A). Gene analysis by qRT-PCR revealed increased mRNA expression of osteoblast related genes such as *runt-related transcription factor 2 (Runx2)*, and bone matrix protein *osteocalcin (OCN)*, as compared to control hES-MSCs grown in growth medium with osteogenic supplements (Fig. 5.3B). Adipogenic differentiation was demonstrated by positive Oil Red O staining of lipid droplets present within the cells

further confirmed differentiation into functional adipocytes (Fig 5.3C) Quantitative PCR analysis revealed a 6-fold increase in *peroxisome proliferator activated receptor gamma* (PPAR-γ) mRNA expression in hES-MSCs cultured for 28 days in medium containing adipogenic supplements as compared to control hES-MSCs cultured in only growth medium without supplements (Fig. 5.3D).

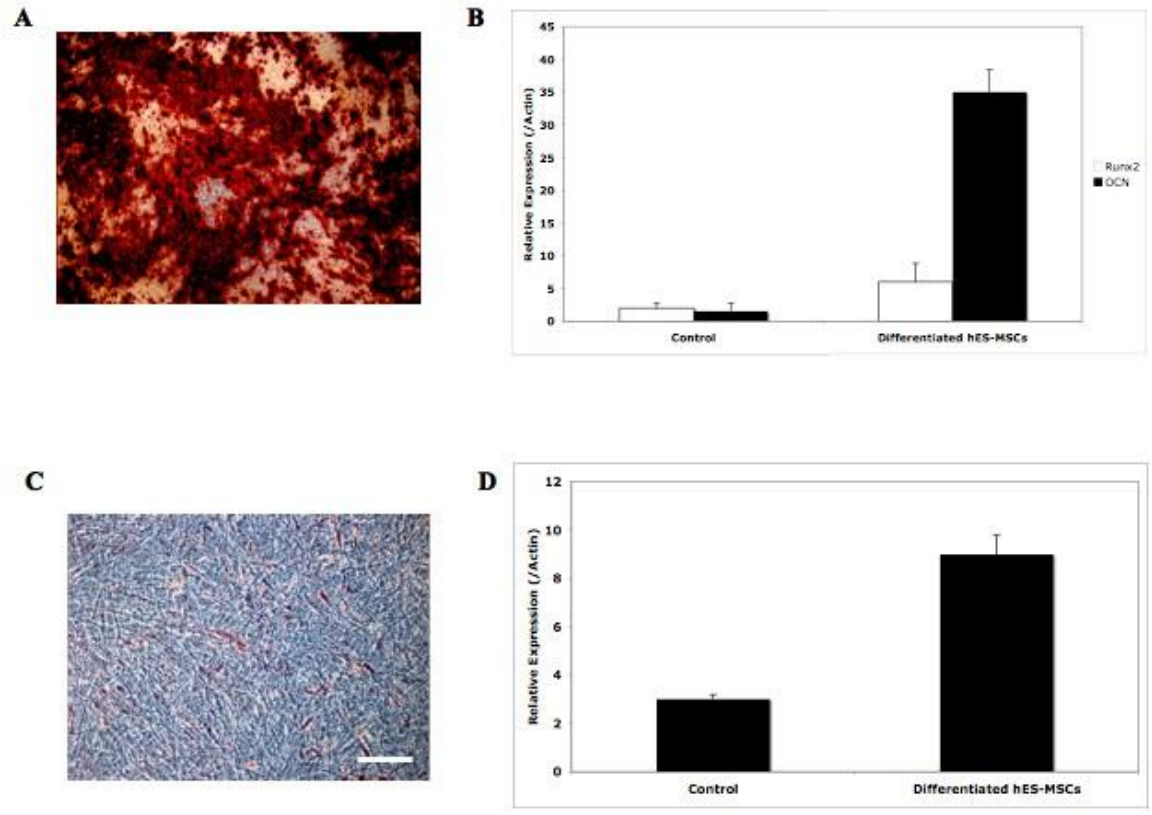

Figure 5.3 hES-MSCs differentiate into functional osteoblasts and adipocytes. A. Alizarin Red S staining indicating calcium deposition in mineralized cultures. Up-regulation of osteogenic-related genes: *runx2* and osteocalcin (OCN). C. Oil Red O staining of lipid indicating the presence of lipid droplets in adipocytes. D. Up-regulation of adiogenic-related gene *peroxisome proliferator activated receptor gamma* (PPAR-γ). Samples were analyzed by qRT-PCR, data normalized to human β-*Actin* expression and results shown are the mean and SEM of three experiments.

5.3.2 Characterization of the *in vitro* osteogenic potential of FAC-sorted osteoprogenitor subpopulations of hES-MSCs

Following hES-MSC derivation, multilineage differentiation and FACS analysis of marker expression, distinct subpopulations of cells were isolated using markers most highly expressed by the hES-MSCs. Therefore, following FACS analysis, unsorted hES-MSCs were singly and doubly stained with APC- and PE-conjugated monoclonal antibodies (mAbs) for CD73, CD105, CD166, and ALP. To ensure that distinct singly positive populations were isolated, cells were double stained in two marker combinations. For instance, CD73+ cells were also stained for CD105, CD166 and ALP to ensure that cells positive for only CD73 were sorted and collected for further culture (data not shown). This was done for each subpopulation analyzed in this study. Only in the case of CD73/CD105 cells were double positive cells collected.

Characterization of CD73, CD105, CD166, ALP, and CD73/CD105+ populations showed that the cells had different morphologies, with the CD73/CD105 and ALP populations possessing fibroblastic morphology most similar to hBMSCs as compared to the unsorted hES-MSCs and the other FAC-sorted subpopulations (Fig. 5.4). When cultured in osteogenic medium (OM) for 28 days, Alizarin Red staining showed that CD73/CD105 subpopulation, ALP subpopulation, and hBMSCs mineralized most robustly as compared to the unsorted hES-MSCs, CD73, CD105, and CD166 FAC-sorted subpopulations.

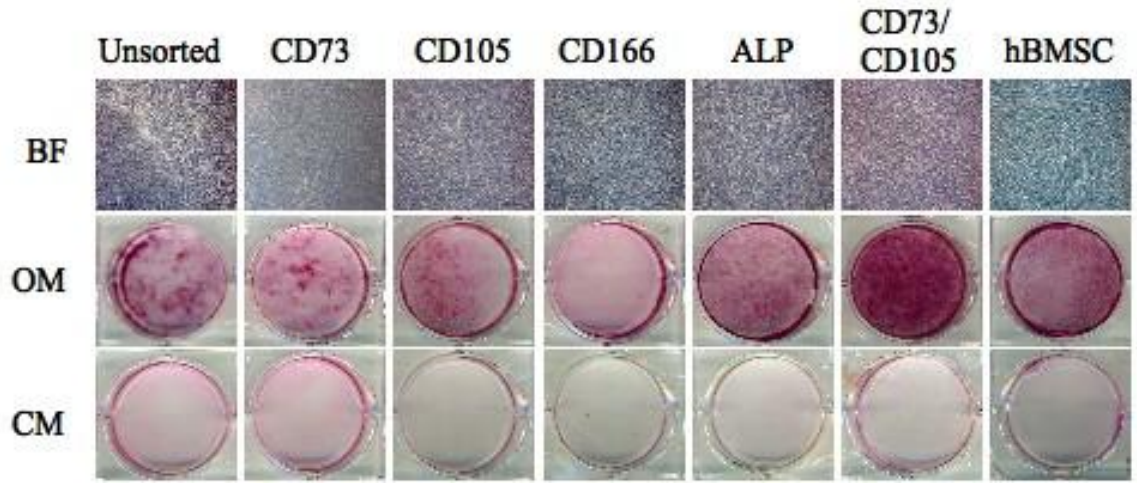

Figure 5.4 CD73/CD105+ and ALP+ cells display enhanced in vitro mineralization. Top panel: Brightfield (BF) images of unsorted, CD73+, CD105+, CD166+, ALP+, CD73/CD105+, and hBMSCs illustrating differences in morphology at P4. Middle panel (OM): Comparison of the extent of *in vitro* mineralization by Alizarin Red staining when cells were cultured in osteogenic medium for 28 days. Bottom panel (CM): Alizarin Red staining of each population grown in control medium without osteogenic supplements for 28 days.

To quantify the extent of mineralization, Alizarin Red S staining was extracted and spectrophotometric readings at 405 nm were taken. The negative control for this experiment was unsorted hES-MSCs cultured in growth medium without osteogenic supplements for 28 days. The concentrations of Alizarin Red S staining for each population were as follows: Negative Control (10 µM); Unsorted hES-MSCs (375 µM); CD73 (250 µM); CD105 (200 µM); CD166 (125 µM); ALP (750 µM); CD73/CD105 (1,750 µM); and hBMSCs (675 µM) (Fig. 5.5). Out of the eight experimental groups, the highest levels of Alizarin Red S staining were seen in hBMSCs, the ALP subpopulation, and the CD73/CD105 subpopulation, with the CD73/CD105 cells being the most highly differentiated.

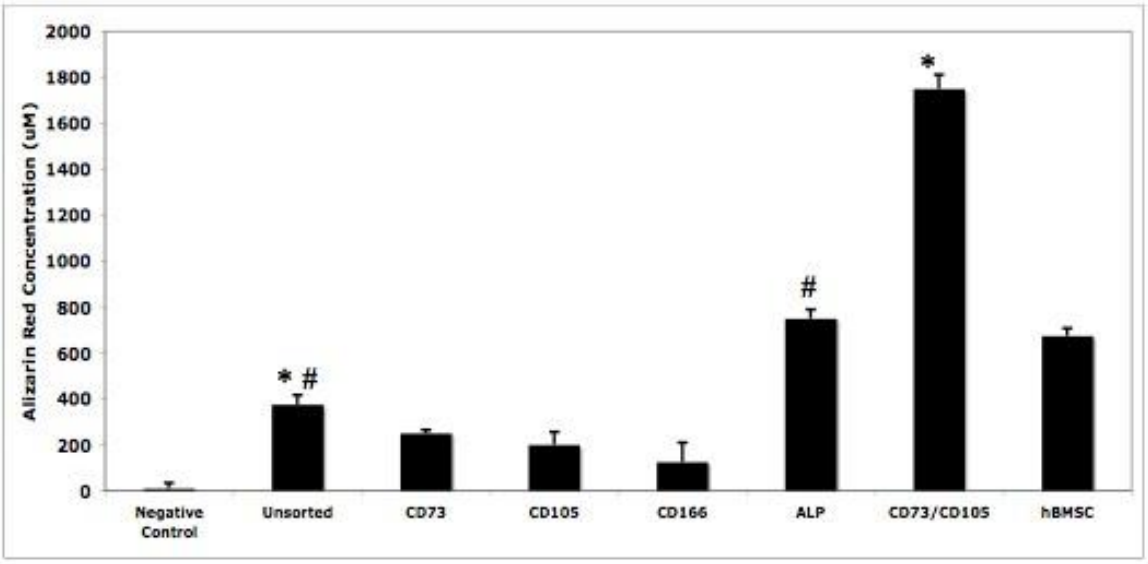

Figure 5.5 FAC-sorted CD73/CD105+ cells show increased mineralization. The extent of mineralization was quantified by Alizarin Red staining, showing significantly increased mineralization of the CD73/CD105+ and ALP+ populations as compard to the negative control (grown in CM) and unsorted populations.

Quantitative PCR (qPCR) analysis was performed to further characterize the *in vitro* osteogenic differentiation potential of the FAC-sorted subpopulations. Each group was cultured in OM for 28 days and gene expression for *runt-related transcription factor 2 (Runx2), collagen type I (ColI)* and *osteocalcin (OCN)* analyzed at 1-4 week time points. At week 3, the CD73/CD105 and ALP subpopulations expressed the highest levels of Runx2, with a decrease in expression over all populations at week 4. This was expected given that Runx2 is expressed early in the osteoblast differentiation cascade (Fig. 5.6). At week 4, the CD73/CD105 and ALP subpopulations expressed the highest level of ColI and OCN, which are both late markers of osteoblastic differentiation associated with matrix maturation and mineralization, respectively (Figs. 5.7 and 5.8). In contrast, the single positive CD73, CD105, and CD166 cells, as well as the controls, all had lower expression of ColI and OCN over all time points observed.

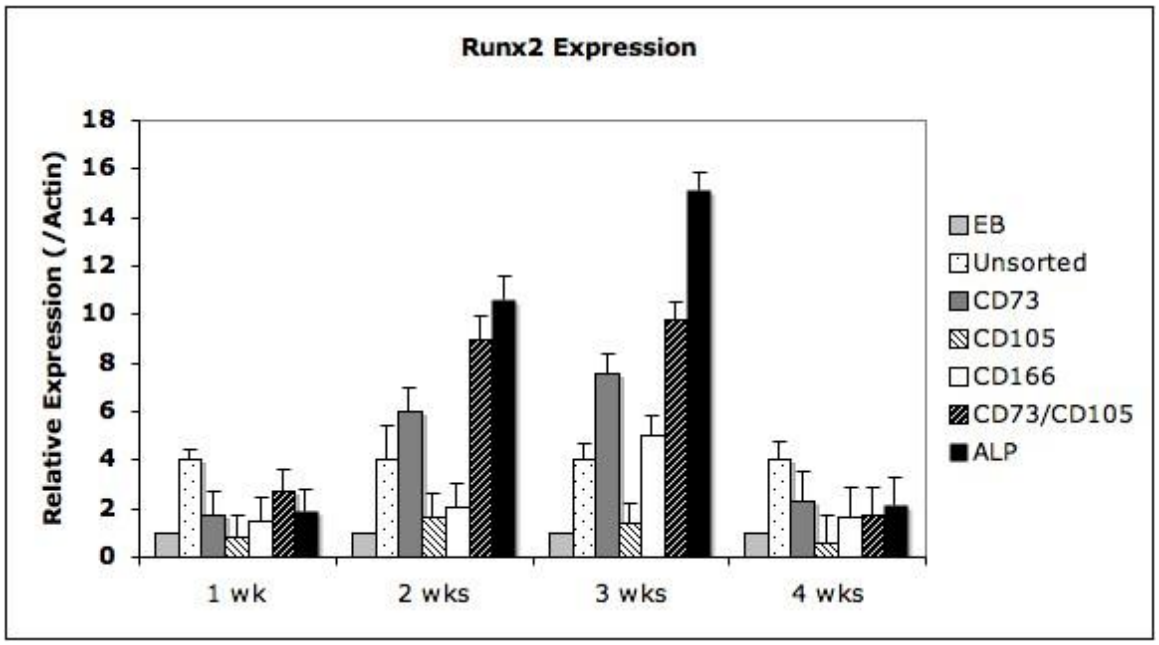

Figure 5.6 FAC-sorted CD73/CD105 and ALP+ cells express highest levels of Runx2. Upregulation of the early transcription factor Runx2 at 2 and 3 wks in CD73/CD105 and ALP populations as compared to unsorted populations, indicating osteoblastic differentiation**.** Samples were analyzed by qRT-PCR, data normalized to human β-*Actin* expression and results shown are the mean and SEM of three experiments

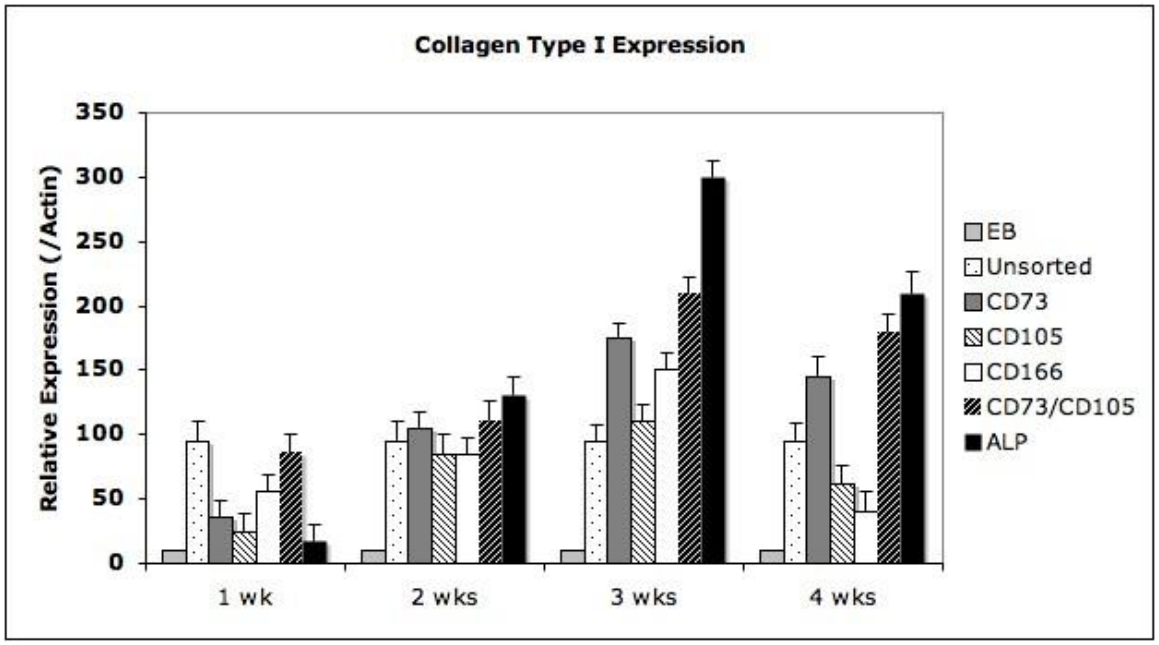

Figure 5.7 FAC-sorted CD73/CD105 and ALP+ cells express the highest levels of Collagen Type I. Upregulation of the bone matrix protein ColI at 3 wks in the

CD73/CD105 and ALP populations as compared to unsorted populations, indicating osteoblastic differentiation and matrix maturation**.** Samples were analyzed by qRT-PCR, data normalized to human β-*Actin* expression and results shown are the mean and SEM of three experiments.

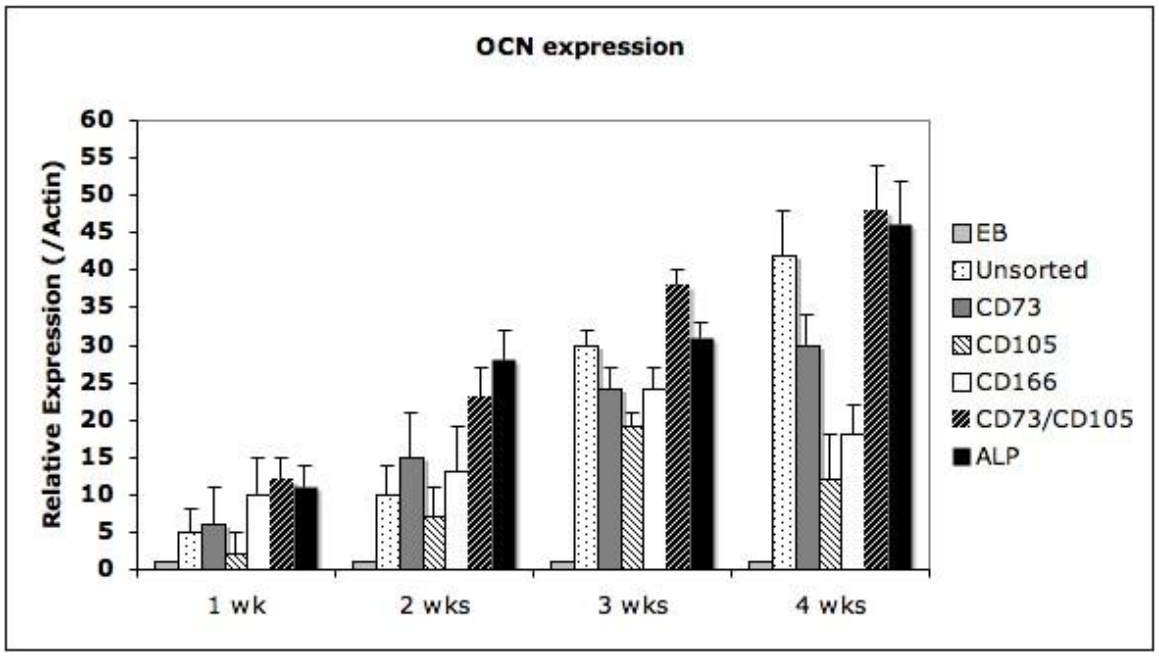

Figure 5.8 FAC-sorted CD73/CD105 and ALP+ cells express highest levels of OCN. Upregulation of the bone matrix protein OCN 3 and 4 wks for CD73/CD105 and ALP populations as compared to unsorted populations, indicating osteoblastic differentiation and matrix mineralization**.** Samples were analyzed by qRT-PCR, data normalized to human β-*Actin* expression and results shown are the mean and SEM of three experiments.

5.3.3 Characterization of the *in vivo* osteogenic potential of hES-MSCs in an orthotopic microenvironment

To determine the capability of unsorted hES-MSCs, and the two candidate osteoprogenitor populations - sorted CD73/CD105 and sorted ALP cells - to regenerate bone *in vivo*, cells treated with osteogenic factors for 7 days were transplanted into calvaria defects of immuncompromised mice. After 8 weeks, animals were euthanized and specimens were analyzed by microCT and histology. MicroCT scans of the calvaria

from the unsorted, CD73/CD105, and ALP populations show the production of mineralized tissue within and surrounding the defect. Unexpectedly, the gel foam carrier used to deliver the cells was not entirely degraded; however, microCT scans reveal that the implant surrounding the defect also contains mineralized tissue (Fig. 5.9).

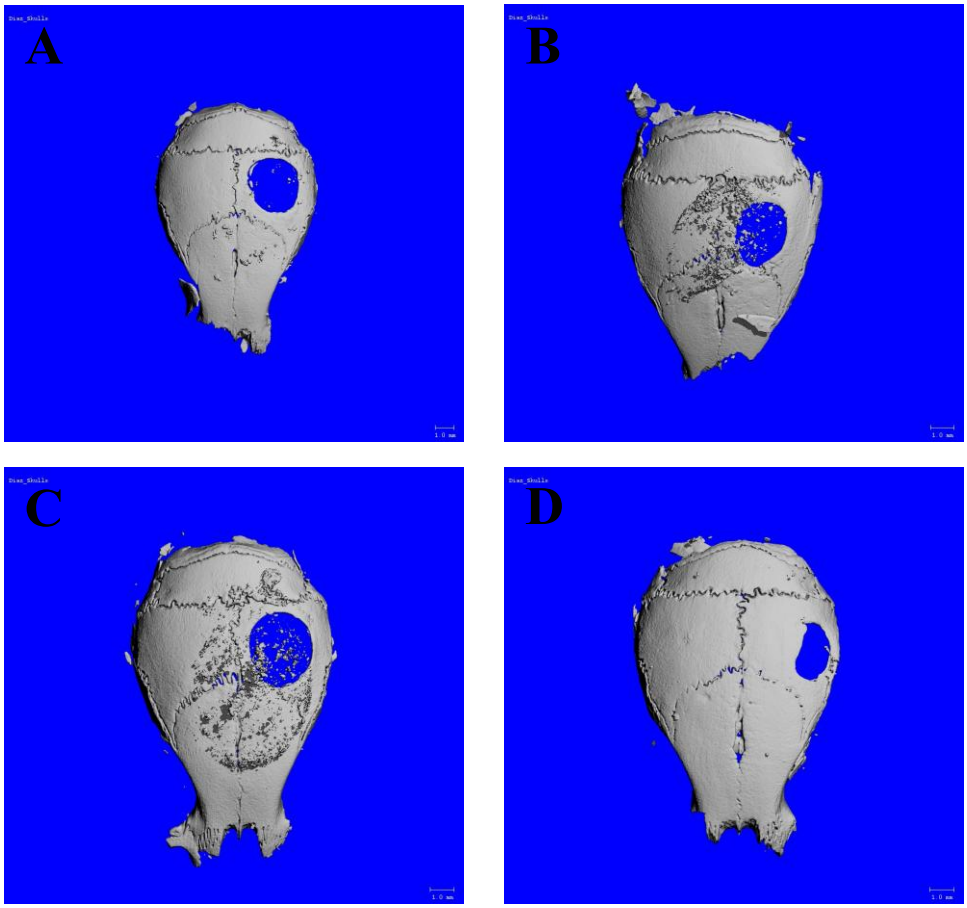

Figure 5.9 MicroCT scans show the presence of mineralized tissue surrounding the defect. 3D reconstruction of microcomputerized tomography (micro-CT) scans of calvaria after 8 weeks of implantion for empty (fibrin gel only) (A.), Unsorted hES-MSCs (B), CD73/CD105+ osteoprogenitors (C), and ALP+ (D). Calvaria shown here are representative images, with n=6 for each group.

Quantitative analysis demonstrated a 1.5-fold increase in volume of new bone formed in calvaria transplanted with CD73/CD105 cells, and a 2.1-fold increase in volume in calvaria transplanted with ALP cells, compared to the empty control calvaria (Fig. 5.10).

The bone volume was not significantly different across the unsorted, CD73/CD105, and ALP populations.

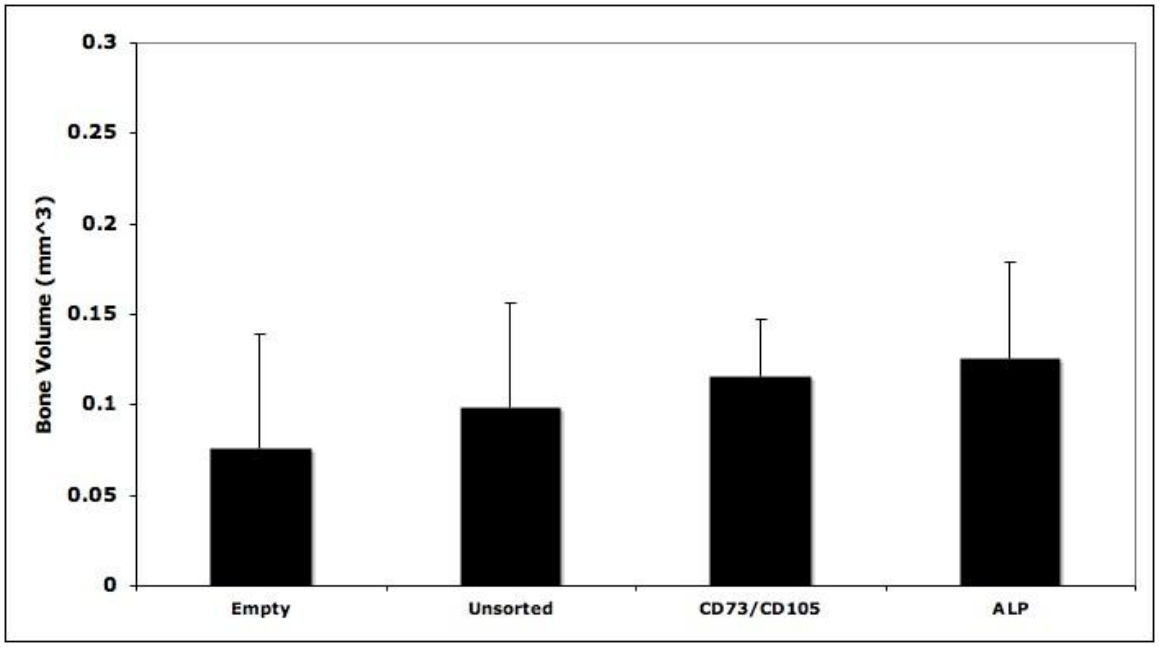

Figure 5.10 Osteoprogenitor populations show increased bone volume. Calvaria were embedded in 1% agarose and scanned over the entire length of the skulls using a microCT system, with a cylindrical region of interest (ROI) through the center of the 3 mm defect to determine bone volume (BV, mm^3). Quantitative analysis demonstrated a 1.5-fold increase in volume of new bone formed in calvaria transplanted with CD73/CD105 cells, and a 2.1-fold increase in volume in calvaria transplanted with ALP cells, as compared to the empty control calvaria. Samples were not statistically different, however, n=4 for empty and n=6 for the calvaria with transplanted cells.

Histological evaluation showed new bone formation was observed within the central region and on the margin of the defect. For calvaria in which CD73/CD105 and ALP cells were implanted, osteocytes were surrounded by woven bone, proliferating osteoblasts lining the exterior of the bone spicules, as well as small bone marrow cavities within the newly formed bone (Figs. 5.11 and 5.12) were observed. In contrast, there was

no obvious formation of mature bone in the unsorted hES-MSC group, although mineralized matrix formation appears to have occurred. Fibrous connective tissue filled the rest of the defect. To determine the contribution of the transplanted CD73/CD105 and ALP subpopulations in new bone formation, specific anti-human monoclonal antibodies that do not cross-react with murine cells were used. Positive staining for human mitochondria were observed in proliferating osteoblasts surrounding the bony spicules formed within the defect, as well as in osteocytes embedded in the newly-formed bone for both the CD73/CD105 and ALP populations (Figs. 5.12 and 5.13). Staining was not observed in the surgical margins of the native mouse bone and also was not observed in the negative control sections that were only treated with secondary antibody (Figs. 5.12D and 5.13D).

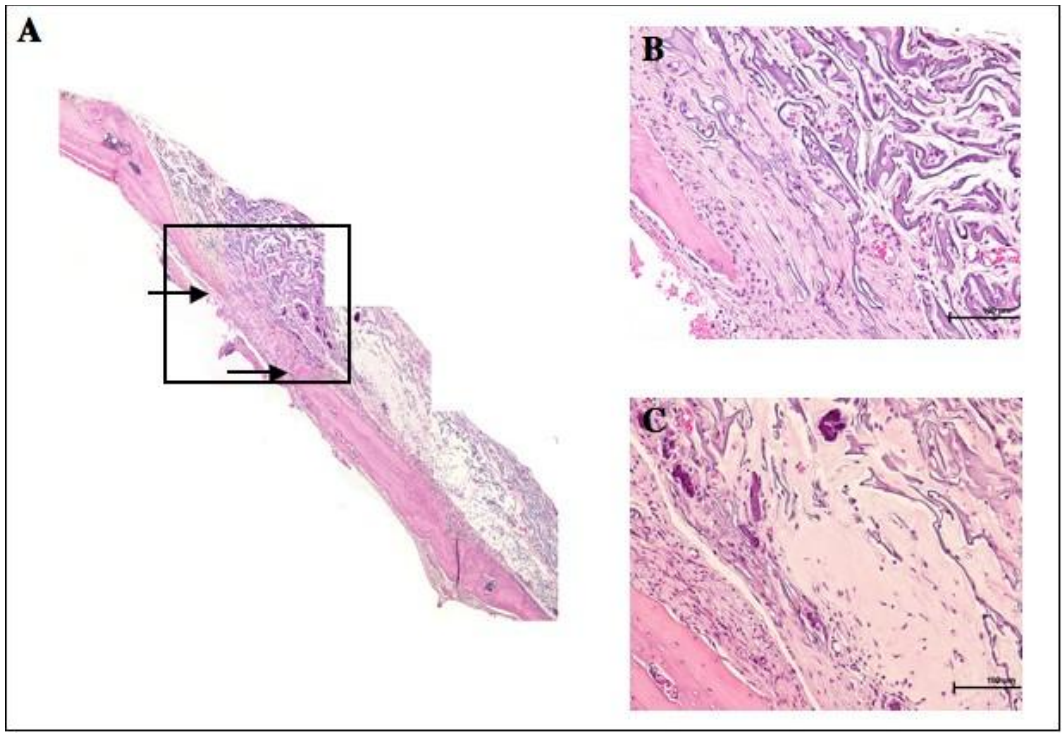

Figure 5.11 Implanted unsorted hES-MSCs exhibit early mineralized matrix formation *in vivo*. A. Reconstructed image stained with H&E showing the area of the defect. Arrows indicate defect margins, and black box indicating area of focus. B and C. Higher magnification images of H&E staining within the defect of mineralized tissue. Scale bar in B-C= 50 μm.

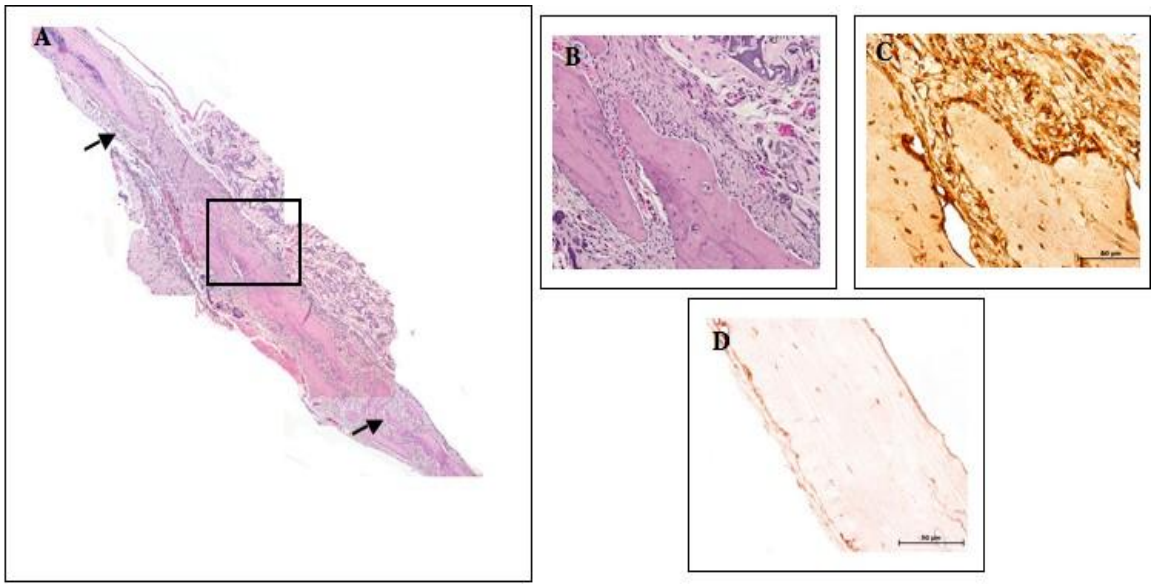

Figure 5.12 Implanted CD73/CD105+ cells play a role in *in vivo* bone formation
A. Reconstructed image stained with H&E showing area of new bone formation. Arrows indicate defect margins, and black box indicating area of focus. B. Higher magnification image of H&E staining within the defect. C. Immunostaining with human mitochondrial antibody confirming that the origin of the regenerated bone was from implanted CD73/CD105+ cells. D. Immunostaining of control mouse calvarial bone. Scale bar in B-D= 50 μm.

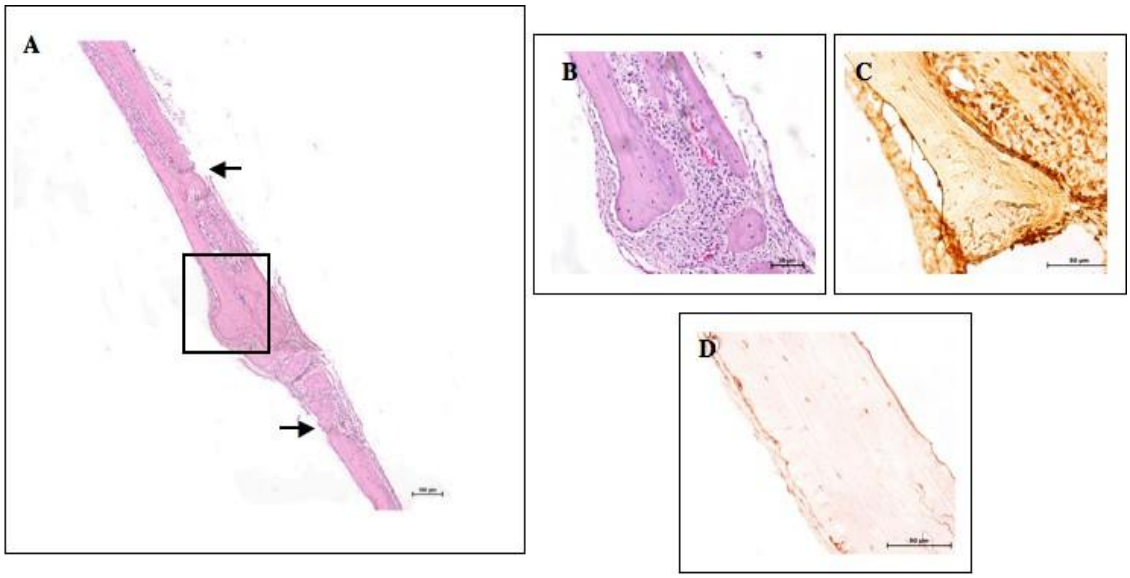

Figure 5.13 Implanted ALP+ cells play a role in *in vivo* bone formation.
A. Reconstructed image stained with H&E showing area of new bone formation. Arrows indicate defect margins, and black box indicates area of focus. B. Higher magnification image of H&E staining within the defect. C. Immunostaining with human mitochondrial antibody confirming that the origin of the regenerated bone was from implanted ALP+ cells. D. Immunostaining of control mouse calvarial bone. Scale bar in A= 100 μm, while in B-D= 50 μm.

5.4 Discussion

Given the complex nature of craniofacial bone, the use of human embryonic stem cells (hESCs) has been suggested as a repository of cells that can be isolated, manipulated, and utilized for future cell-based engineering strategies (Thomson et al, 1998). Osteoprogenitor cells derived from hESCs have tremendous potential, as they can serve as a tool through which one can not only characterize early bone development and cellular behavior on bone-related biomaterials but also have application specifically in craniofacial bone repair. To enhance the understanding of the differentiation pattern and bone formation capacity of hESCs in a skeletal defect, investigators have studied the complete temporal pattern of osteoblastic differentiation of hESMSCs in a long-term culture, as well as the influence of a three-dimensional matrix on the osteogenic differentiation and bone formation capacity of hES-MSCs in the calvarial defect. It was found that incubation of hES-MSCs in osteogenic medium induced osteoblastic differentiation in a similar chronological pattern to previously reported human bone marrow stromal cells (hBMSCs) and primary osteoblasts. Furthermore, it was also demonstrated that differentiation was enhanced by culture in three-dimensional collagen scaffolds (Arpornmaeklong et al, 2009). The fate of transplanted cells in the bone formation process was verified by identifying the presence of human cells in the matrix of the newly formed bone, suggesting that hES-MSCs represent an osteoprogenitor population that can be sorted, enriched and manipulated for use in craniofacial engineering strategies. Of particular importance is the fact that the lineage progression through both mesodermal and neural crest lineages can be controlled for hESCs, addressing one of the major challenges unique to healing the craniofacial skeletal given

its dual embryonic origins (Jiang et al, 2002; Mao et al, 2006; Lian et al, 2007; Brown et al, 2009; Goldstein et al, 2010). Given the characteristics of craniofacial bone, we believed this was a good model to test the hypothesis that we could isolate subpopulations of osteoprogenitors from derived hES-MSC based on surface markers, identify the best candidates through *in vitro* mineralization studies, and demonstrate their ability to participate in *in vivo* bone formation.

These results add to the current body of knowledge regarding osteoblastic differentiation of hESCs, as only a few reports have shown hESC survival, differentiation, and participation in bone formation within either a defect model or when transplanted subcutaneously (Arpornmaeklong et al., 2010; Kuznetsov et al., 2011). Moreover, it is not yet possible to identify an osteoprogenitor population within the overall MSC population has not yet been achieved. Here, we show that there are subpopulations of progenitor cells, based on their surface marker expression profile, that have varying degrees of *in vitro* and *in vivo* osteogenic potential. The *in vitro* differentiation comparison between all the FAC-sorted groups (CD73+, CD105+, CD166+, ALP+, and CD73/CD105+) shows that there are distinct populations that possess different growth characteristics, percentages of positive cells within the unsorted hES-MSCs, and abilities to mineralize in culture in the presence of osteogenic supplements. Based on the extent of *in vitro* mineralization as demonstrated by positive Alizarin Red S staining, quantification of mineralization and gene expression analysis, we conclude that the CD73/CD105 and ALP populations have the greatest *in vitro* osteogenic potential. This could be due to the fact that CD73, also known as ecto-5-nucleotidase, has been shown to play a role in MSC

migration in bone fracture repair (Ode et al., 2011). CD105, also known as endoglin, is a type I membrane glycoprotein that is part of the TGF-β1 receptor complex, thus it may be involved in the binding of other TGF-βs and BMPs. And ALP, also known as alkaline phosphatase, which is essential for the proper formation of mineral and calcium deposits within the bone matrix. Hence, we have potentially selected for cells that are expressing important genes needed for osteoblastic development, and in the case of the CD73/CD105 population, have also included supporting cells that further directed and supported enhanced differentiation along the osteogenic lineage.

To examine the potential use of derived hES-MSCs in cell therapies and regenerative medicine, cell-transplantation assays were performed in immunocompromised mice with the goal of developing bone *in vivo*. Histological and microCT image reconstruction confirmed the formation of new bone within the calvaria defects of mice treated with transplanted hES-MSCs after 8 weeks. Randomly distributed and unorganized bone fragments were found within the calvarial defects. Furthermore, positive immunostaining of proliferating osteoblasts and osteocytes for monoclonal antibodies to human mitochondria confirmed the participation of transplanted hES-MSCs and FAC-sorted osteoprogenitors in the regenerated bone. The overall goal of this study was not to heal the defect from a tissue engineering standpoint, but rather to address the question as to whether hESC progenitors can survive *in vivo*, maintain their differentiated state when implanted, not form teratomas, and play a role in tissue regeneration. The fact that the transplanted cells, specifically the CD73/CD105+ and ALP+ populations, participated in bone regeneration *in vivo* in a calvarial defect mouse model suggests these cells became

functional MSCs and osteoprogenitors that can be used as a tool for future cell transplantation studies of bone repair, including craniofacial bone, in response to disease and/or trauma.

In summary, mesenchymal progenitors can be derived from hESCs and osteogprogenitor cells within the hES-MSC population can be isolated and identified based on surface marker characteristics. Specifically, based on *in vitro* and *in vivo* results, as well as the large quantity of cells that can be isolated, we concluded the CD73/CD105+ osteoprogenitor population is the best candidate for biomaterial studies. Future work investigating factors such as the ideal number of transplanted cells, survival and distribution, *in vitro* osteogenic induction prior to transplantation, and even more importantly, the optimal cell carrier with osteoconductive properties, is necessary to the fully characterize the usefulness of these cells in regenerating bone. Additionally, although not a particular goal of this work, it is important to identify a clinically compliant culture system under xeno-free conditions that can support hESC culture and subsequent differentiation to targeted cell types and tissues. Reports have shown that a synthetic polymer coating known as PMEDSAH, along with human cell conditioned medium (hCCM) supports hESC growth, maintains pluripotency over multiple passages, and retains the differentiation capacity of the hESCs (Villa-Diaz et al., 2010). Utilitizing this type of fully defined microenvironment for the derivation and culture of osteoprogenitor cells would be one step closer to identifying a system that would make using hESCs as a cell source a clinical reality. Taken together, efficient derivation of MSCs and successful isolation of osteoprogenitor subpopulations that have unique

osteogenic potential in *in vitro* and *in vivo* microenvironments provides information for the future design of hESC-based bone tissue engineering strategies. The information garnered from this study was used as the platform for the study in the following chapter, which will discuss our investigation of scaffold design effects on hES-MSC differentiation and *in vivo* bone tissue formation.

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CHAPTER 6

THE MICROENVIRONMENT EFFECTS OF HYDROXYAPATITE/TRI-CALCIUM PHOSPHATE (HA/TCP) SCAFFOLD POROSITY DESIGN ON OSTEOPROGENITORS DERIVED FROM HUMAN EMBRYONIC STEM CELLS

6.1 Introduction

6.1.1 Overview

Bone tissue engineering is a field in which major advancements have been made to treat bone defects due to extensive injury, diseases, or congenital malformations (Marot et al., 2010). Due to current limitations in tissue grafting, alternatives for bone repair such as biologically active biomaterial constructs are in great need. In order for these strategies to become a clinical reality, we must continue to characterize scaffold properties, structural and material properties, and determine how these properties affect cellular behavior. The role of the tissue engineering scaffold is to provide a synthetic ECM to which cell can adhere, proliferate, differentiate, and regenerate tissue. From a design perspective, scaffolds are required to have mechanical strength, bioactivity, and biocompatibility. Specifically for bone tissue engineering, scaffolds are commonly used to support vascular invasion, cellular retention, facilitate nutrient diffusion, and undergo resorption as new bone tissue forms (Hollinger et al., 2005). Therefore, when deciding on the scaffold to use for bone repair, the most appropriate design parameters for the target tissue of interest need to be carefully considered. Important parameters that influence bone deposition rate,

depth of infiltration, and the overall extent of bone formation are scaffold size, pore size, pore geometry, pore architecture and interconnectivity, macroporosity, and permeability (Adachi et al., 2006; Hollister et al., 2005). In terms of macroporosity, there is no definitive conclusion as to how this important parameter affects bone regeneration, thus it is important to investigate this parameter from a biomaterial scaffold design perspective.

6.1.2 Porosity Design

Given the fact that hESCs have high metabolic demand, we hypothesized that high scaffold porosity (or permeability) will promote osteogenic differentiation of hES-MSC osteoprogenitord. To test this hypothesis, scaffold macroporosity was manipulated by changing the diameter of the scaffold strut (or wall). Using Interactive Data Language™ programs (IDL), strut diameters of two different sizes were used to create the "High" and "Low" porosity scaffolds for this study. For instance, a scaffold with smaller strut diameters will have a larger amount of void space, and in turn have a greater number of pores; thus giving us the ability to design a scaffold with higher porosity (and overall higher permeability). Reports have shown and discussed the effects that porosity (and permeability) can have on cell survival and overall tissue formation, suggesting this is an important parameter to investigate (Hollister et al., 2005; Liu et al., 2007; Mankani et al., 2011; Weir et al., 2010, Mitsak et al., 2011). For clarification, porosity and permeability are considered simultaneously here because they are two highly interrelated parameters. When one alters scaffold porosity, scaffold permeability is also affected, and vice versa. Porosity is considered to a function of the amount of void space within the scaffold, whereas permeability is considered to be a function of pore interconnectivity. Definition

wise, these two structural parameters are different, and it is difficult to tease out the exact difference between the two. Although permeability was measured for this study and shown to be significantly different between the two scaffolds, to be consistent, the overall effects that porosity has on osteoprogenitor differentiation will be discussed.

6.1.3 Ceramics

As previously stated, scaffolds used for bone tissue engineering methods must have mechanical strength, bioactivity, and biocompatibility in order to support vascular invasion, cellular retention, facilitate nutrient diffusion, and undergo resorption as new bone tissue forms. Specifically for bone tissue engineering, scaffolds are commonly used to support vascular invasion, cellular retention, facilitate nutrient diffusion, and undergo resorption as new bone tissue forms. It is important to select a suitable material that has the appropriate mechanical properties to match elastic properties of bone, and similar chemical compositon to support osteoconduction. Given these requirements, we have chose hydroxapatite-tri-calcium phosphate (HA/TCP), an osteoconductive biphasic ceramic biomaterial, as the delivery vehicle for hESC osteoprogenitors *in vivo*.

HA/TCP is an osteoconductive biomaterial that has displayed excellent biocompatibility, osteoconductivity, and osseointegration (Hollinger et al., 2005; Ruan et al., 2006; Schopper et al., 2005). In general, calcium phosphate biomaterials are widely used as substitutes for autogenous bone grafts when bone reconstruction is considered. Hydroxyapatite (HA), ($Ca_{10}(PO_4)_6(OH)_2$), is essentially a porous calcium phosphate found in hard tissues such as bones and teeth, and is crystallographically similar to bone mineral. Tricalcium phophate is a slow resorbing compound derived from marine coral,

and TCP is a faster resorbing material that encourages new bone growth. HA and TCP can be combined into biphasic calcium-phosphate materials that have been shown to be highly osteoinductive and can achieve better bone formation (Habibovic et al., 2006a, Habibovic et al., 2006b; Schopper et al., 2005).

6.1.4 Experiment

Given what has been reported about the importance of porosity and the biocompatibility of an osteconductive material such as HA/TCP, this chapter will discuss our efforts to study the local microenvironment effects of HA/TCP scaffold porosity design (and in turn differences in permeability) on osteoprogenitors isolated and derived from human embryonic stem cells.

In addition to scaffold design considerations, another major component of the tissue engineering paradigm is the cell source. Therefore, it is vital that further investigation on the interface between the cell and the bfiomaterial is made. In order to engineer a microenvironment that supports tissue formation, a combinatorial approach to tissue engineering is necessary in that multiple factors such as biochemical signals and cell survival contribute to whether one achieves their targeted functional tissue equivalent. That is why we chose to address how scaffold properties, in this case the porosity design parameter, affects hESC differentiation and the role that hES-MSC osteoprogenitors play in overall bone tissue formation. We hypothesized that porosity is a parameter that can be manipulated to study the effects on osteogenesis. According to previous reports, we believed that changes in porosity (and overall permeability) affect oxygen and blood diffusion, nutrient exchange, cellular communication, and metabolic byproduct waste

transport, in turn affecting osteoprogenitor cell differentiation and bone tissue formation within the scaffold. In order to test this hypothesis, we utilized Interactive Data Language™ programs, solid free form fabrication (SFF) techniques, and 3D printing machines to build HA/TCP scaffolds with varying porosities (and significantly different permeabilities). We altered the diameter of the scaffold strut, which created two different scaffold porosities (and permeabilities), while keeping other design parameters such as pore size and pore shape constant. The resulting high (50% porosity) and low (30% porosity) scaffolds were seeded with hBMSCs, unsorted hES-MSCs, and sorted CD73/CD105 osteoprogenitors derived from hESCs, and implanted subcutaneously for 4 weeks to investigate the effects of porosity on cellular differentiation and bone tissue formation.

6.2 Materials and Methods

6.2.1 Human ESC Culture

The H9 cell line was obtained from WiCell Inc. (Madison, WI) and cultured on irradiated mouse embryonic fibroblast feeder layers at a density of $4x10^5$ cells/plate (0.1% gelatin coated, 60 mm). The hESC culture medium consisted of 80% (v/v) DMEM/F12, 20% (v/v) knockout serum replacement, 200 mM L-glutamine, 10 mM nonessential amino acids (all obtained from Invitrogen, Carlsbad, CA), 14.3 M β-mercaptoethanol (Sigma, St. Louis, Mo., USA) and 4 ng/ml FGF-2 (Invitrogen). Cell cultures were incubated at 37° C in 5% CO_2 in air and 95% humidity with medium changes everyday and were manually passaged once per week.

6.2.2 Derivation of hES-MSCs

To induce mesenchymal differentiation, embryoid bodies were formed and cultured in suspension for 7 days with hESC growth medium in low-attachment culture dishes. Then, approximately 70 embryoid bodies/well were plated onto 0.1% gelatin-coated 6-well plates in the presence of hBMSC growth medium (α-MEM, 10% FBS, 200 mM L-glutamine and 10 mM nonessential amino acids) and 4 ng/ml bFGF. After EB attachment, cells were cultured for up to 2 weeks to reach confluence, then trypsinized and passaged in a ratio of 1:3. Cells were continually passaged into T-75 flasks until a homogeneous fibroblastic morphology appeared. Prior to implantation, the unsorted hES-MSCs, CD73/CD105+ hES-MSCs, iPS-MSCs, and hBMSCs were cultured in osteogenic medium for 7 days. Differentiation into osteoblasts was performed according to previously described a protocol (Barberi et al., 2005).

6.2.3 Scaffold Design and Fabrication

Using Interactive Data Language™ programs (IDL; Research Systems, Inc., Boulder, CO), three-dimensional (3D) scaffolds were designed to have varying strut sizes, creating "high" (S50) and "low" (S30) macroporosity designs. Based on the amount of void space available as a result of the difference in strut size, which was the only structural difference, these scaffolds were designed to have 50% and 30% porosity, respectively. Inverse wax molds of each design were built on a Solidscape MM2 3D printer (SolidScape Inc., Merrimack, NH). Once molds were obtained, HA/TCP scaffolds were casted and sintered according to well established protocols in the STEG Hollister Laboratory.

6.2.5 Scaffold Permeability Characterization

Experimental permeability was measured with a custom permeability chamber designed by Dr. Jessica Kemppainen (2007) in the Hollister lab. Water flow through the chamber exerted a constant hydraulic pressure on the scaffolds, allowing permeability to be measured. Within LabView (National Instruments, Austin, TX), an equation derived from Bernoulli's equation (with a frictional loss correctional term) and Darcy's Law was used to compute permeability (Li and Mak, 2005).

6.2.6 Preparation of Cell Populations and Scaffolds for Implantation

Unsorted, CD73/CD105+, iPS-MSCs and hBMSCs were harvested using 0.25% trypsin-0.53 mM EDTA (Gibco), and cell pellets were re-suspended in 5 mg/ml human plasma fibrinogen (Sigma) at a concentration of $2x10^6$ cells per 40 µl in 1.5 ml tubes. In a 96-well plate, the fibrinogen cell suspensions were then pipetted directly into 6 x 3 mm scaffolds with high and low porosity (n=20 for each scaffold design, n=6 for each cell population). Total number of scaffolds used was 60. Five µl of human thrombin, 200 unit/ml (Sigma, MO) was added to each scaffold and placed immediately on ice until implantation. Gelation of the fibrin gel was observed within 10 minutes. For scaffold preparation, 24hrs prior to implantation scaffolds were sterilized in 100% Ethanol, then rinsed in sterile water for 12hrs, and finally soaked in Dulbecco's Modified Eagle Medium (DMEM) for an additional 4hrs (Invitrogen) before cell seeding.

6.2.7 Subcutaneous Implantation

All procedures were approved by the University of Michigan Committee on the Use and Care of Animals. Surgical instruments were sterilized prior to surgery using a steam autoclave and were alternated between animals. Ten 5 week-old female immunocompromised mice (N:NIH-bg-nu-xid; Harlan Sprague Dawley, Inc., NC) were anesthetized with intraperitoneal injections of ketamine, (Ketaset, 75 mg/kg, Fort Dodge Animal Health, IA) and xylazine, (Ansed, 10 mg/kg, Lloyd laboratories, IA). One mid-longitudinal skin incision of about 1 cm in length was made on the dorsal surface of each mouse and 4 subcutaneous pockets were formed in each quadrant of the animal by blunt dissection. A single implant (6mm Diameter x 3mm Height) was placed into each pocket with up to 6 implants per animal. Incisions were closed with 4-0 Chromic Gut sutures (Ethicon/Johnson & Johnson, NJ). All mice were sacrificed 4 weeks after the implantation.

6.2.8 Quantitative PCR Analysis

Quantitative RT-PCR was performed to determine expression levels of osteoblast-associated genes [*Alkaline Phosphatase (ALP)* and *Bone Sialoprotein (BSP)]*. Gene expression levels were determined for undifferentiated hESCs, unsorted hES-MSCs, FAC-sorted hES-MSC osteoprogenitor population CD73/CD105 at four passages after sorting (P4 post-sorting), P4 iPS-MSCs and P4 hBMSCs. Total RNA was extracted from dissected implants using Trizol (Invitrogen) and 1 µg of RNA was reverse transcribed into cDNA using Superscript III Reverse Transcriptase (Invitrogen). Two µl of the diluted

reverse transcribed cDNA (RT reaction, 1:5 in RNase free-water) was amplified in a 30 µl PCR assay volume, using the TaqMan Gene Expression Master Mix (Applied Biosystems, Foster City, CA) and 20x Target primers and Probe (unlabled PCR primers and a FAM^{TM} dye-labled TaqMan® minor groove binder (MGB) probe) (Applied Biosystems). The expression of the genes was measured by qRT-PCR on an ABI Prism 7700 Sequence Detection System (Applied Biosystems). The levels of the target genes were correlated to standard concentrations and normalized to the levels of beta-actin (ACTB) as an endogenous reference. Subsequently, the expression levels were normalized to gene expression in undifferentiated hESCs (H9 passage 65) and reported as relative expression normalized to beta actin.

6.2.9 Micro-Computed Tomography and Histological Analyses

Scaffolds were immediately fixed in aqueous buffered zinc formalin, Z fix (Anatech, MI) and then analyzed via micro-computed tomography (µCT) analysis. Specimens (n=4) were scanned in 70% ethanol in a 1.5 ml centrifuge tube placed inside a 19 mm diameter tube and scanned over the entire length of the scaffold using a microCT system (µCT100 Scanco Medical, Bassersdorf, Switzerland). Scan settings were: voxel size 16 µm, medium resolution, 70 kVp, 114 µA, 0.5 mm AL filter, and integration time 500 ms. A cylindrical volume of interest was drawn around the entire scaffold. Analysis was performed using the manufacturer's evaluation software and a fixed global threshold of 22% (220 on a grayscale of 0–1000) was used to segment bone from non-bone. Total bone volume (BV, mm3), bone mineral density (BMD) and tissue mineral density of bone (TMD) were computed and calibrated to the manufacturer's hydroxyapatite (HA)

phantom. For histological analysis, harvested implants were fixed in z-fix, dehydrated, and embedded in paraffin for H&E staining.

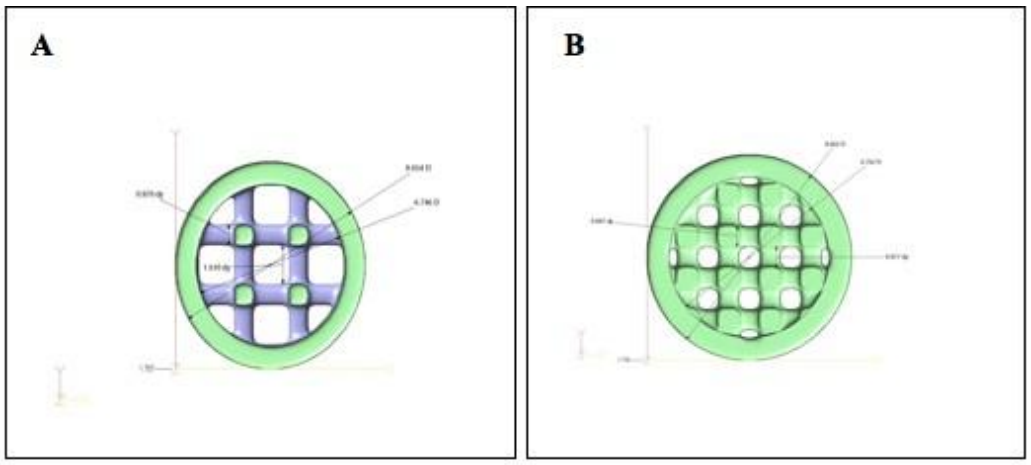
6.2.10 Statistical Analyses

Data are expressed as the mean value ± the standard error of the mean (mean ± SEM) and analyzed by one-way analysis of variance. Statistical significance was set at $p<0.05$.

6.3 Results

6.3.1 Characterization of Low and High Scaffold Porosity Designs

HA/TCP scaffolds with low and high porosity were designed in Interactive Data Language™ software (IDL) to have varying strut diameters of 1.6 mm and 0.870 mm, respectively Fig 6.1. The smaller diameter of 0.870 mm in the high porosity design allowed for a greater amount of void space and therefore, a greater number of pores. While the scaffolds were designed to have 30% and 50% porosity, the actual porosity of the built scaffolds for low and high designs was 26% and 58%, respectively. Other important parameters such as pore size - 600 um, pore shape - spherical orthogonal pores, and dimensions – 6 mm diameter x 3 mm height, were kept constant as to not introduce additional scaffold design influences on implanted cells (Fig. 6.1) Of note, the high porosity scaffolds had greater surface area (415 mm^2) as compared to low porosity scaffolds (344 mm^2).



C	Low	High
Porosity (actual)	26%	58%
Permeability m4/(N·s)	2.79E-07	1.43E-06
Pore Size	600 um	600 um
Strut Size	1.6 mm	0.870 mm
Dimensions	6 x 3 mm	6 x 3 mm
Surface Area	344 mm^2	415 mm^2

Figure 6.1 Low and high porosity scaffolds have different porosities, permeabilities, strut diameters and surface area. IDL generated images depicting theoretical design of A. Low and B. High porosity scaffolds. C. Table with important scaffold parameters such as porosity, permeability, pore size, strut size, dimensions, and surface area.

Experimental permeability was measured with a custom permeability chamber designed within our laboratory. Water flow through the chamber exerted a constant hydraulic pressure on the scaffolds, allowing permeability measurements to be taken by averaging the mass flow rate into and out of the scaffolds. Customized LabView software incorporated an equation derived from Bernoulli's equation (with a frictional loss correctional term) and Darcy's Law that was used to compute permeability. Measurements revealed that low porosity scaffolds (2.79E-07 m4/(N·s)) had significantly smaller experimental permeability than high porosity scaffolds (1.43E-06 m4/(N·s)) (Fig.

6.2), with a level of significance of $p \leq 0.05$. This confirmed that the two designs not only differed in actual porosity, but also had differences in permeabilities measured experimentally on the actual scaffold (n=6 for each design). Indicating that the cells implanted in the porous scaffold designs were subjected to different 3D scaffold microenvironments.

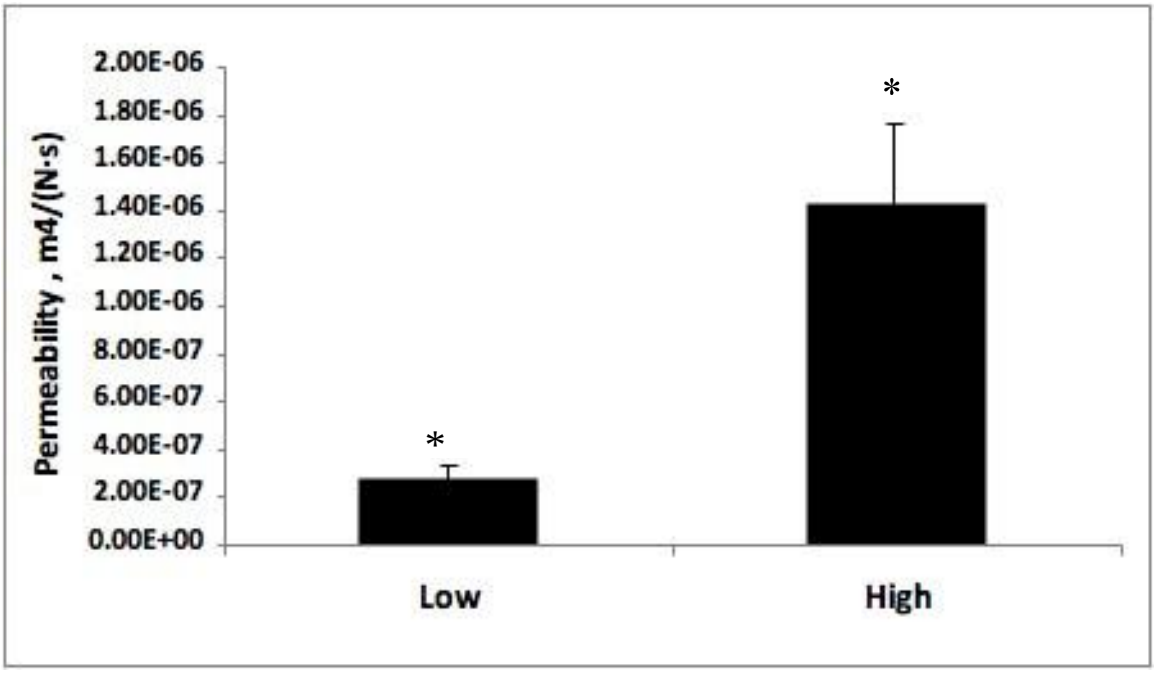


Figure 6.2 Quantified permeability of low and high porosity scaffolds. Using a custom permeability chamber developed in the STEG Hollister laboratory, experimental permeability was measured using a customized LabView program. Statistical significance was set at $p \leq 0.05$. For each design, n=6 scaffolds were measured in triplicate. Permeability of high porosity scaffolds was significantly greater than scaffolds with low porosity.

6.3.2 In Vivo Osteogenic Potential of Osteoprogenitors Isolated from hES-MSCs

After 4 weeks of subcutaneous implantation in HA/TCP scaffolds, quantitative PCR analysis was performed to determine if implanted cells differentiated toward osteoblasts

and began expressing osteogenic-related genes. Human specific primers to *alkaline phosphotase (ALP), bone sialoprotein (BSP),* and housekeeping gene *beta-Actin (ACTB, β-Actin)* were used to determine if genomic material obtained from scaffolds was of human origin. ALP, an enzyme that plays a key role in calcification and matrix mineralization, and BSP, a marker indicative of the matrix maturation phase were used because they are expressed at later stages of osteoblastic differentiation. As a control, β-Actin expression was not observed in the empty scaffold groups, but was observed in the unsorted, CD73/CD105, and hBMSC groups indicating the presence of cells within the scaffolds. Osteoblastic gene expression was then analyzed and normalized to β-Actin expression. In the low porosity design, low ALP expression was seen across all cell populations implanted. In comparison to the low porosity design for each cell population, there was a 1.5-fold increase in ALP in the unsorted population, a 6-fold increase for the CD73/CD105 population, and a 7.5-fold increase for the hBMSC population implanted in the high porosity design (Fig. 6.3).

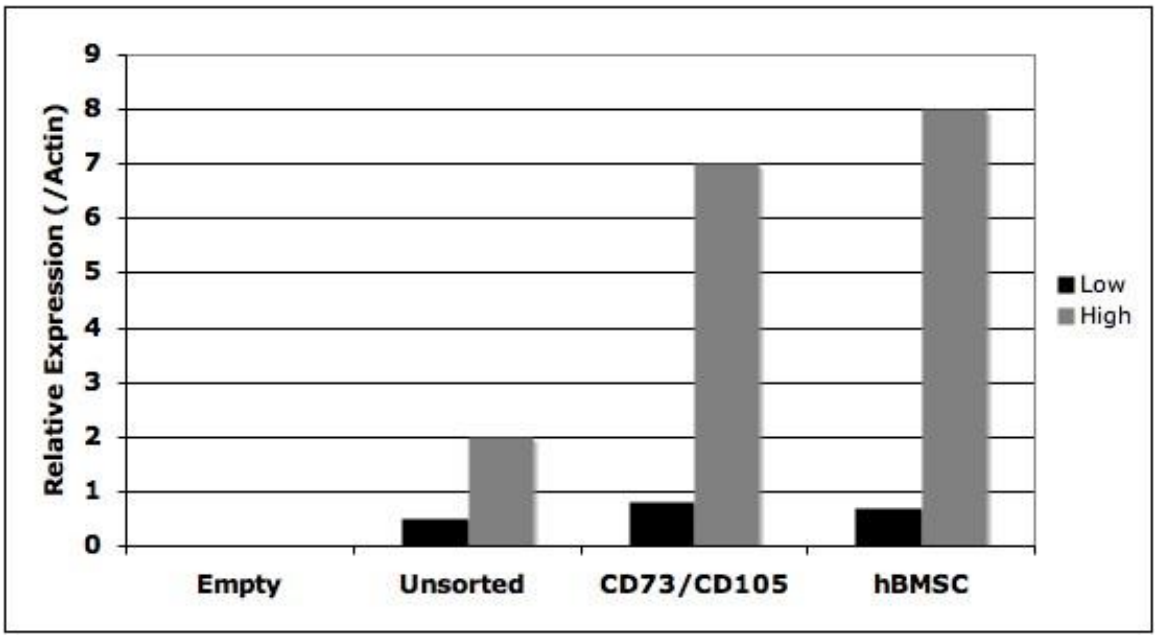


Figure 6.3 CD73/CD105 cells and hBMSCs express ALP in high porosity scaffolds. Upregulation of ALP mRNA expression for the CD73/CD105 and hBMSC populations in the high porosity design, as compared to the low porosity design, indicating osteoblastic differentiation**.** Samples (n=2) were analyzed by qRT-PCR and data was normalized to human β-*Actin* expression.

Expression of BSP was also analyzed and similar results were obtained. In the low porosity design, low BSP expression was seen across all cell populations implanted. In contrast for the high porosity scaffold design, a 3-fold increase in the unsorted population, an 8.5-fold increase in the CD73/CD105 population, and a 12-fold increase in the hBMSC population was observed after 4 weeks of implantation (Fig. 6.4). Based on these results, high levels of ALP and BSP mRNA expression were seen for the CD73/CD105 and hBMSC populations. Given the sample size of n=2, statistical significance was not able to be determined.

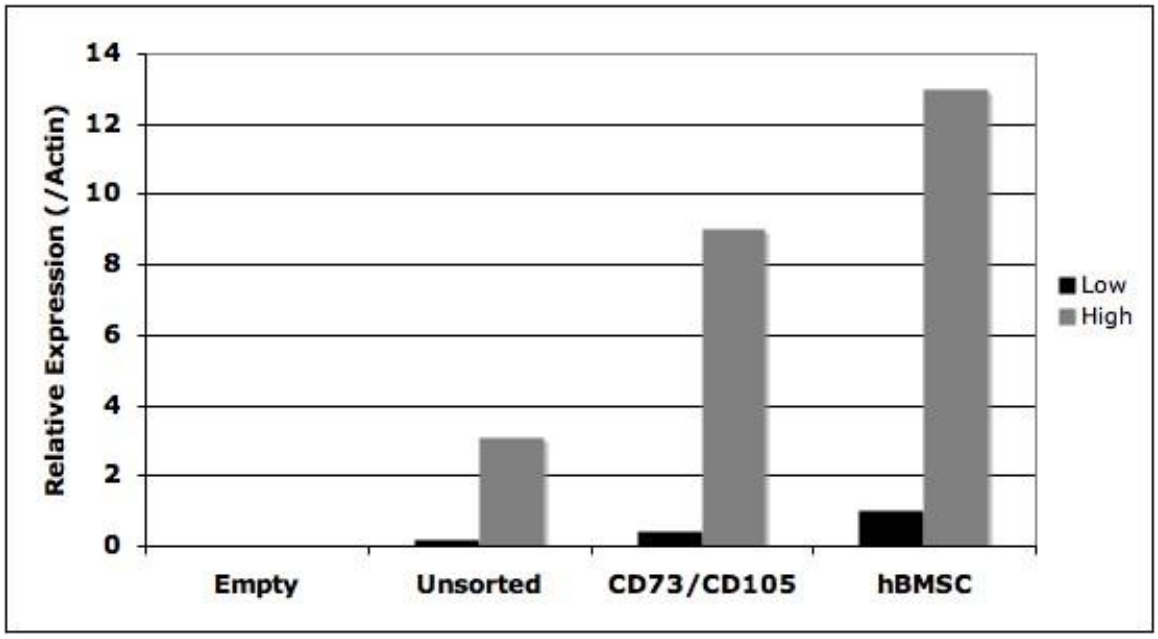


Figure 6.4 CD73/CD105 cells and hBMSCs express BSP in high porosity scaffolds. Upregulation of BSP mRNA expression for the CD73/CD105 and hBMSC populations in the high porosity design, as compared to the low porosity design, indicating osteoblastic differentiation**.** Samples (n=2) were analyzed by qRT-PCR and data was normalized to human β-*Actin* expression.

Histological evaluation of harvested porous HA/TCP scaffolds revealed bone formation within the pores of the high porosity design, whereas only fibrous connective tissue was seen within pores of the low porosity scaffold designs (Figs 6.5 and 6.6). Specifically, for the low porosity scaffolds, no observable bone was formed for any of the cell population groups, including the empty scaffolds. However, we noted the presence of adipose tissue within pores of the low porosity design implanted with the unsorted hES-MSC population (Fig. 6.5B).

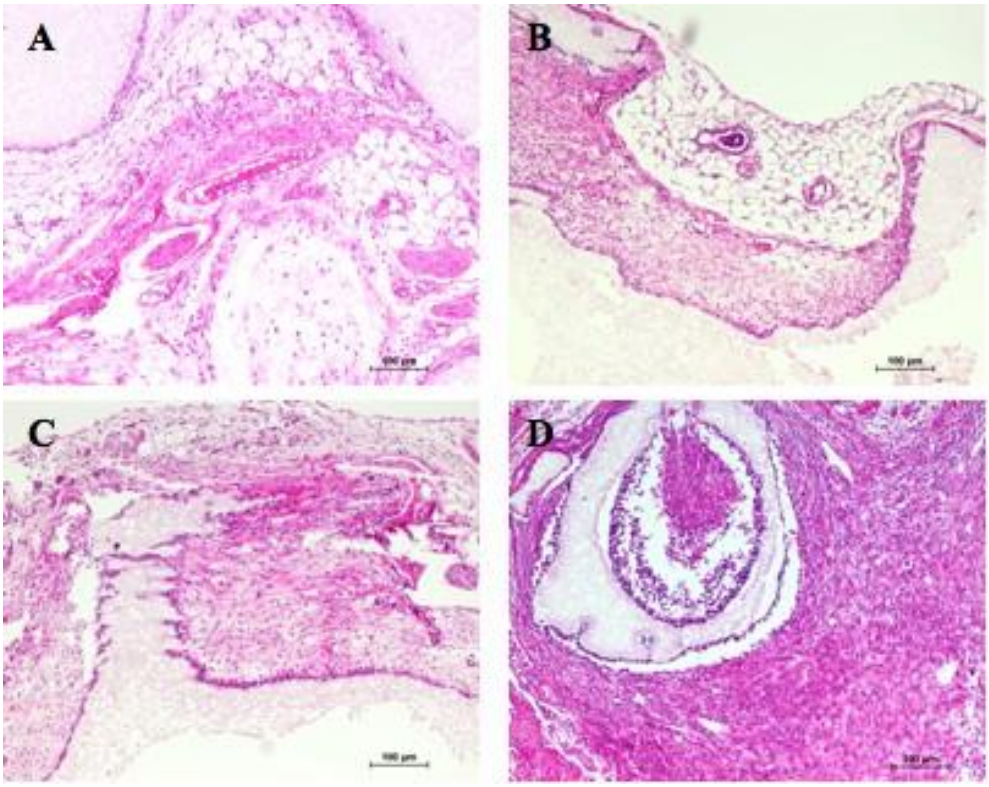


Figure 6.5 Connective tissue formation in low porosity scaffolds. Paraffin embedded sections were stained with H&E to determine tissue morphology in S30 scaffolds for A. Empty (negative control), B. Unsorted hES-MSC population, C. CD73/CD105+ osteoprogenitor populations, D. hBMSC population. No observable bone tissue or mineralized matrix was formed within low porosity scaffolds of any of the cell groups. Scale bar = 400 μm.

On the other hand, the high porosity designs exhibited mature bone formation by the CD73/CD105 and hBMSC populations. This was evidenced by the presence of osteocytes embedded within the calcified matrix, with osteoblasts surrounding the exterior of the small islands of bone formed within the pores (Fig. 6.6C and D). Although histology for the high porosity scaffolds implanted with CD73/CD105 and hBMSC populations revealed the presence of blood vessel invasion within the interior of the scaffold, there was no presence of well-developed marrow spaces indicating a lack of active hematopoiesis. Extensive cell attachment to the HA/TCP material and the lack of

marrow space development were the only similarities observed between the two scaffold designs.

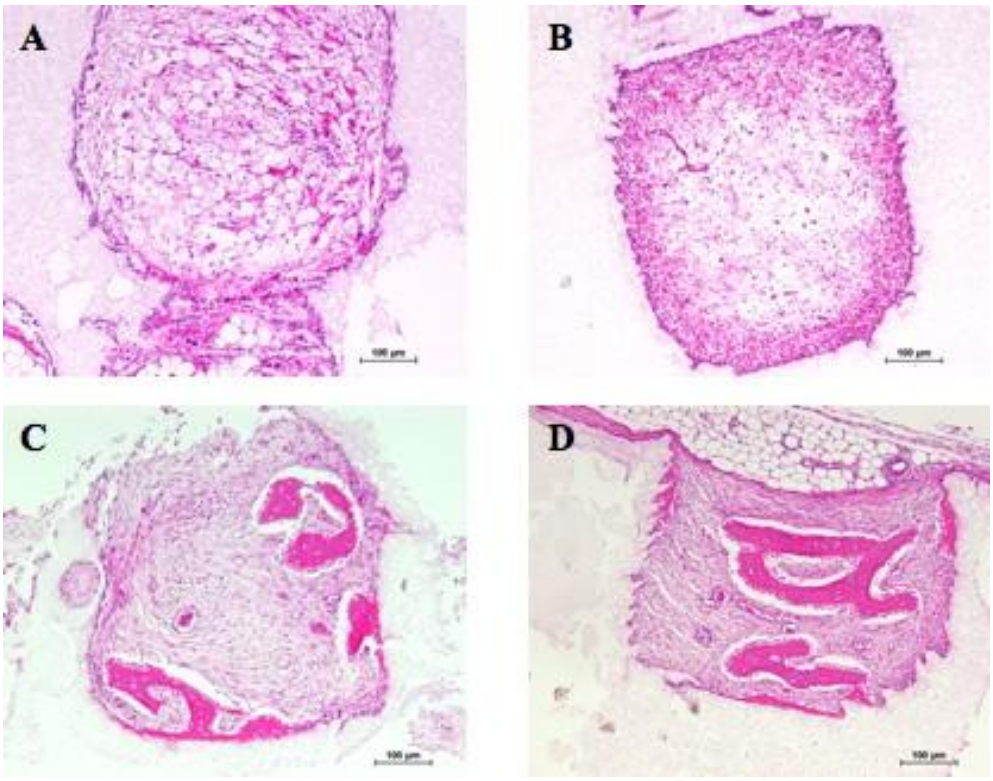


Figure 6.6 CD73/CD105+ and hBMSCs form bone *in vivo* in high porosity scaffolds. Paraffin embedded sections were stained with H&E to determine tissue morphology in S50 scaffolds for A. Empty (negative control), B. Unsorted hES-MSC population, C. CD73/CD105+ osteoprogenitor populations, D. hBMSC population. Histological bone was with osteocytes and proliferating osteoblasts was formed within pores of high porosity scaffolds for the CD73/CD105 and hBMSC groups. Scale bar = 400 μm.

6.4 Discussion

In the bone tissue engineering paradigm, the purpose of the scaffold is to provide an osteoinductive extracellular matrix (ECM) analog to support initial cell adhesion, growth and development of new bone. In this field, the major classes of materials used are natural polymers, synthetic polymers, ceramics, composite materials, and electrospun

nanofibers. In addition to selection of the appropriate material, the scaffold should possess design characteristics that elicit the desired biological response from the microenvironment. By optimizing design parameters and fabrication techniques, the ideal scaffold candidate should achieve the following: (i) to deliver progenitor cells or facilitate host cell recruitment via osteoconductive and osteoinductive material properties; (ii) to deliver important signaling molecules in a temporally and spatially controlled manner via growth factor incorporation and surface modification; (iii) to promote vascularization and tissue in-growth via changes in microporosity; (iv) to properly fit the shape of the anatomical defect via image and Computer-Aided Design (CAD) based scaffold design methods; and (v) to degrade into biocompatible byproducts at a rate that matches new tissue formation via selection of the optimal material composition (Giesen et al, 2004; Meinel et al, 2004; Hollister, 2005; Mao et al, 2006; Gersbach et al, 2007; Hutmacher and Cool, 2007; Hutmacher et al, 2007; Wang et al, 2007; Jones et al, 2009). To address the requirements of scaffolds specifically for bone repair, one must consider the delicate balance of imparting sufficient porosity, permeability, and pore architecture to facilitate vascular invasion, mass transport, and cell survival in the scaffold interior, while maintaining the ability to withstand load bearing conditions both long bone and craniofacial bone sustains during normal physiological function (Hollister, 2005; Hutmacher and Cool, 2007; Jones et al, 2007; Potier et al, 2007). These preclinical and clinical studies show that significant advancements have been made in the design of biomaterials to create replacements for damaged or pathological tissues, however, we must continue extensive investigation in this area of research to improve upon our understanding of disease progression and development of effective treatments.

Recent studies have investigated the interaction between ceramic scaffolds, macroporosity, and hBMSCs; however, there are no studies that have studied hESC differentiation and furthermore, osteogenic differentiation of MSCs derived from hESCs, in designed scaffolds for bone tissue engineering (Liu et al., 2007; Mankani et al. 2011). Two important studies previously demonstrated the ability of mesenchymal progenitors derived from hESCs to participate in the *in vivo* bone formation process in both a calvarial defect model and when implanted with HA ceramic particles subcutaneously (Arpornmaeklong et al., 2010; Kuznetsov et al., 2011). Therefore, we expanded upon these studies to investigate the importance of a designed 3D scaffold microenvironment on the osteogenesis of hESCs. To test the hypothesis that designed porous scaffold architecture influences hESC response and behavior, we fabricated both low and high porosity HA/TCP scaffolds. When the porous scaffolds for this study were designed, there was a wide range of tissue permeability and diffusivity coefficients to target for mass transport design. However, mass transport is constrained by the need to maintain pore connectivity to allow for contiguous cell seeding and/or migration (Hollister et al., 2008). Human trabecular bone permeability is measured to fall in the range of 0.003-0.11E-06 (Sandert et al., 2003; Hollister et al., 2008). Although our scaffold porosities did not impart a permeability that falls within the measured range of human bone tissue, the degree of cellular differentiation and amount of bone tissue formation in the high porosity design as compared to the low porosity design can be explained by the fact that the more porous scaffold allowed for cellular communication, cell migration, infiltration toward the center of the scaffold, and integration within the pores. This could also be due to the fact that a more porous scaffold facilitates greater oxygen, blood, and nutrient

diffusion into the scaffold.

Given this, we conclude that the CD73/CD105 osteoprogenitors differentiated into osteoblasts, and although at not extremely high levels, exhibited bone-specific gene expression. Furthermore, the appearance of islands of woven bone with osteocytes embedded within the matrix in the high porosity designs further confirmed that porosity does have an effect on hESC response to the biomaterial and to the *in vivo* microenvironment. It is noteworthy to mention that when comparing the different populations across scaffold design, the CD73/CD105 osteoprogenitors performed similarly to the hBMSCs. This was confirmed by ALP and BSP expression, increased bone volume, and histological evidence of mature bone, whereas the implantation of the unsorted populations did not lead to robust osteoblastic differentiation in either scaffold design. Therefore, we conclude that the CD73/CD105 subpopulation has enhanced *in vitro* and *in vivo* osteogenic potential. Overall, this research laid groundwork for future extensive investigation into 3D scaffold influences. Future work for this study includes investigating factors such as cell density, the length of the *in vitro* culture period prior to implantation, the type of gel used for encapsulation, and length of *in vivo* implantation. To evaluate cellular response, future studies could utilize qPCR to look at changes in gene expression, scanning electron microscopy (SEM) to examine morphological changes at various time points, and confocal microscopy to determine cellular distribution within the interior of the scaffold. Last, but certainly not least, staining with human mitochondrial antibody to confirm the human origin of the regenerated bone is a necessity.

Bone tissue engineering research has developed tremendously over the last two decades as a result of the individual strides made in the fields of developmental biology, stem cell biology, polymer chemistry, mechanical engineering, and biomedical engineering. The unique challenges faced by orthopedic and maxillofacial surgeons in the clinical setting has spurred investigation of effective tissue engineering strategies that involve isolated and enriched progenitor cells, sophisticated gene delivery methods, and complex biomaterial scaffold fabrication and design techniques. As we begin to understand how biomaterial properties influence hESC behavior, it will be possible to develop biomimetic scaffolds that contain incorporated signaling cues that induce cellular differentiation and ECM deposition, possess composite material properties that have the ability to generate hybrid tissue, and have tunable three-dimensional geometrical architecture that appropriately restores form and function to skeletal defects.

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CHAPTER 7

CONCLUSIONS AND FUTURE DIRECTIONS

7.1 Conclusions

Human embryonic stem cells (hESCs) present a potentially unlimited supply of cells that may be directed to differentiate into all cell types within the body and used in regenerative medicine for tissue and cell replacement therapies. An area of particular interest is stem cell transplantation for bone tissue regeneration. Current techniques used for bone tissue repair employ the use of auto- and allografting methods, however, these methods have inherent limitations that restrict their universal application. The limitations of these reparative strategies suggest that an alternative approach is required, and hESCs may provide a repository of cells for such an approach. One of the major gaps in our knowledge regarding hESCs is the lack of understanding the biological cues from the microenvironment that control and direct differentiation. Previous work has demonstrated that hESCs can be differentiated into osteoblasts, however, how to achieve directed differentiation still remains a pivotal question that remains unanswered. Therefore, we tested the hypothesis that controlling the local *in vitro* and *in vivo* microenvironments can direct osteoblastic differentiation of hESCs**.**

Overall, we demonstrated the importance of cell culture conditions in the *in vitro* microenvironment, and the importance of implantation site and scaffold design in the *in*

vivo microenvironment. First, we adapted a transwell co-culture system consisting of hESCs with human bone marrow stromal cells (hBMSCs), which demonstrated that pro-osteogenic soluble signaling factors secreted by hBMSCs into the *in vitro* cellular microenvironment directed differentiation of hESCs into osteoblasts. Secondly, we reproducibly derived mesenchymal progenitors from hESCs (hES-MSCs) that possess the characteristic hBMSC surface marker expression profile and are capable of undergoing differentiation along the mesenchymal lineage. Subsequently, via FACS analysis, we isolated multiple subpopulations of osteoprogenitors from within the hES-MSC population in order to identify candidate cells for biomaterial studies. Distinct osteoprogenitor cells were identified and when implanted *in vivo* in an orthotopic calvarial defect microenvironment, participated in the bone regeneration process. Lastly, we delivered osteoprogenitor hES-MSCs subcutaneously within designed hydroxyapatite/tri-calcium phosphate (HA/TCP) scaffolds to investigate the effects that porosity has on cell differentiation and overall bone tissue formation. We demonstrated that osteoprogenitors derived from hESCs survive and play a role in *in vivo* bone tissue formation within designed HA/TCP scaffolds with high porosity. Taken together, we conclude that from an *in vitro* microenvironment perspective the CD73/CD105 osteoprogenitor subpopulation is the best candidate for future cell-based tissue engineering studies that investigate biomaterial influence on hES-MSC differentiation. Furthermore, these cells in combination with high porosity HA/TCP scaffold design and implantation in an orthotopic bony microenvironment provides the optimal conditions for the osteogenesis of human embryonic stem cells.

Moving forward, it is vitally important that we continue to explore hESC biology in order to realize the potential of hESCs to cure diseases. The derivation of mesenchymal stem cells from human embryonic stem cells is an area of active investigation in that hESC-MSCs potentially offer insight into embryonic mesodermal development events, as well as provide information about underlying differentiation mechanisms and signaling pathways that have been unclear heretofor. In addition to elucidating the mechanisms by which hESC-MSCs differentiate, it is equally important to better understand how the 3D biomaterial microenvironment can be manipulated to direct and control this process. In general, stem cell research advances the knowledge and understanding of how an organism develops and how progenitor cells migrate from the stem cell niche to the site of damaged or diseased tissue. To improve upon the overall quality of human health, scientists must continue to work collaboratively with clinicians to drive translational "bench-to-bedside" research. To this end, extensive investigation into the xeno-free derivation, robustness, and non-tumorigenic safety of hESC-MSCs will be absolutely necessary as the field progresses toward the realization of clinical tissue engineering and regenerative medicine therapies.

The long-term goal of this research is to further understand the biology of human embryonic stem cell development and more specifically, provide information about the effects the microenvironment has on the osteogenesis of hESCs. Many questions have arisen in terms of how we can improve upon *in vitro* culture, signaling cues, and scaffold design such that we can attain more directed cellular response, differentiation, and tissue formation. A few of these questions will be explored in the latter portion of this final

chapter. Nevertheless, utilizing the knowledge we have acquired on effects of the *in vitro* and *in vivo* microenvironments, we believe we have provided a platform for future studies aimed at developing hESC-based bone tissue engineering strategies.

7.2 Future Directions

7.2.1 Identification of candidate pro-osteogenic factors

In Specific Aim 1, as discussed in Chapter 3 of this dissertation, we demonstrated that soluble signaling factors secreted by differentiating hBMSCs directed differentiation of hESCs to osteoblasts. However, we did not pursue identifying candidate factors derived from the hBMSCs. We took a combinatorial approach to tissue engineering where the optimal cell source and biomaterial design were investigated, and one way to improve upon this investigation would be to study the effect pro-osteogenic factors has on tissue formation. It would be interesting to probe this avenue further in order to better understand the cues and molecular mechanisms that influence differentiation. And furthermore, once identified, these factors could be incorporated into biomaterials for delivery. Another interesting route would be to identify key factors, construct plasmids that express theses factors with a reporter gene, and infect cells in order to track hESC differentiation along the osteoblast lineage. Along this same line of thinking, we could investigate the possibility of achieving better bone formation through overexpression of identified pro-osteogenic factors within transfected hESCs delivered in a biomaterial *in vivo*.

One way to approach identifying candidate factors would be to use Oligo GEArray® Human Osteogenesis Microarray (SuperArray Bioscience Corporation). This particular microarray profiles the expression of 113 key genes involved in osteogenic differentiation, and contains genes functioning in the development of the skeletal system, growth factors mediating osteogenesis and related cell growth, and genes for extracellular matrix and cell adhesion molecules involved in bone development. Based on microarray data analysis of signal intensity, a comparison between the gene expression profiles of hES-MSCs and hBMSCs could be made in order to determine which genes have the highest expression at specific time points. We could use this information and select candidate pro-osteogenic factors for incorporation within polymer, ceramic, or polymer-ceramic composite scaffolds for the purpose of increasing hESC response to 3D biomaterials. Previous studies have performed high-throughput expressed sequence analysis to determine the gene expression profile of hBMSCs, and found that TGF-β, connective tissue growth factor, TGF-β-induced gene product, calphobindin II, smooth muscle protein, BMP-2, BMP-4, IL-6, and amongst others are expressed (Jia et al., 2002; Karadag et al., 2000). With this in mind, I propose these factors would be a great starting point to begin screening.

7.2.2 Scaffold design and material effects on cellular response in *in vitro* and *in vivo* microenvironments

Pore architecture plays an integral role in determining the rate and degree of bone formation. More specifically, the pore interconnectivity of the scaffold, which can be experimentally related to permeability, is widely accepted to contribute to osteogenesis

by facilitating cell and ion transport in and waste transport out of the scaffold, bone tissue and bone marrow invasion, and angiogenesis (Adachi et al., 2006; Hollister et al., 2005; Schopper et al., 2005) Since the internal pore architecture required for optimal osteoconductivity by HA/TCP has yet to be determined, it would be beneficial to explore the influence that pore interconnectivity has on the osteogenesis of hESCs.

Additionally from a material standpoint, ceramic-based materials have been combined with biodegradable polymers to be used for bone tissue engineering applications. Such composite materials can allow for additional drug delivery functionality and for fabrication of highly porous structures to allow for cellular infiltration when culturing stem cells inside of such scaffolds (Guarino et al., 2007; Lee and Shin, 2007; Rezwan et al., 2006). If given the opportunity, investigation into the behavior of hES-MSCs in response to polymer-ceramic materials could provide key information that would allow us to address the cranial defect model. Given the brittle nature of HA/TCP, it is not an ideal candidate for repairing anatomical defects in the craniofacial complex. Using a polymeric scaffold material such as HA-coated PCL or PPF, would allow for a much larger range of permeability and porosity designs that one could investigate.

Lastly, there are a quite a few key *in vitro* experiments and analyses that would offer great insight as to the interaction of the hESCs with the biomaterial. Cell density, the length of the *in vitro* culture period prior to implantation, and the type of gel used for encapsulation are a few parameters that I would examine. To evaluate cellular response, I propose using qPCR to look at changes in gene expression, scanning electron microscopy (SEM) to examine morphological changes at various time points, and confocal

microscopy to determine cellular distribution within the interior of the scaffold. In doing so, we could systematically determine the optimal cell culture conditions and parameters for developing novel and highly functional hESC-biomaterial constructs.

7.2.3 Clinical Grade Culture System

In order to make the use of hESCs more of a clinical reality, it is imperative that we try to overcome the limitations that animal-derived extracellular matrices and media products for cell culture maintenance poses. An exciting area of research deals with the development of alternative substrates for hESC culture, maintenance and expansion. To address this issue, a few reports showing synthetic cell culture substrates for hESCs that are devoid of xenogeneic components have recently been published (Brafman et al., 2010; Nagaoka et al., 2010; Rodin et al., 2010; Villa-Diaz et al., 2010). However, some of these substrates are based on recombinant proteins and/or peptides and pose certain problems such as difficulties in sterilization, propensity to degrade and the high cost of production (Hentschel et al., 2007; Huebsch et al, 2005).

Alternatively, it has been reported that culture dishes coated with synthetic polymers can be reproducibly fabricated, are inexpensive and easy to manipulate, and thus represent a viable system for hESC expansion. Specifically, the fully defined synthetic polymer coating made of poly[2-(methacryloyloxy)ethyl dimethyl-(3-sulfopropyl)ammonium hydroxide] (PMEDSAH), and in combination with human-cell conditioned or chemically defined medium, can support the long-term culture and self-renewal of undifferentiated human ES cells (Nandivada et al., 2011; Villa-Diaz et al, 2010). This culture system

makes use of a fully synthetic polymer as the structural motifs in cell-substrate interactions (i.e., no peptides, sugars, or proteins), and therefore provides a xenogeneic-free environment. Given these recent reports, it would be greatly beneficial to take this technology and apply it not only to the expansion of hESCs, but also to the derivation, expansion, and differentiation of hESC progeny. In the case of hES-MSC derivation, the ability to derive large quantities of progenitors in xenogeneic-free conditions would bring the field yet another step closer to developing clinically relevant hESC-based tissue engineering strategies.

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APPENDIX

APPENDIX
DERIVATION OF FUNCTIONAL MESENCHYMAL STEM CELLS FROM HUMAN INDUCED PLURIPOTENT STEM CELLS CULTURED ON SYNTHETIC POLYMER SUBSTRATES

Villa-Diaz, L.G.[1], Brown, S.E.[1], Liu, Y.[2], Ross, A.[3], Lahann, J.[3,4], Parent, J.M.[2], Krebsbach, P.H.[1]

[1]*Department of Biologic & Materials Sciences, School of Dentistry, and* [2]*Department of Neurology, Medical School,* [3]*Department of Biomedical Engineering,* [4]*Department of Chemical Engineering, University of Michigan, Ann Arbor, MI, 48109.*

ABSTRACT

Human induced pluripotent stem (iPS) cells may represent the ideal cell source for research and applications in regenerative medicine. However, standard culture conditions that depend on the use of undefined substrates and xenogeneic medium components represent a significant obstacle to clinical translation. Recently, we reported a defined culture system for human embryonic stem (ES) cells using a fully defined synthetic polymer coating, poly[2-(methacryloyloxy)ethyl dimethyl-(3-sulfopropyl)ammonium hydroxide] (PMEDSAH), in conjunction with xeno-free and defined culture medium. Here we tested the hypothesis that iPS cells grown in this defined culture system can be differentiated into mesenchymal stem cells (iPS-MSCs). Human iPS cells were cultured on PMEDSAH and differentiated into functional MSCs, as confirmed by expression of characteristic MSC markers (CD166+, CD105+, CD73+,

CD44+, CD34- and CD45-) and their ability to differentiate *in vitro* into adipogenic, chondrogenic and osteoblastic lineages. To demonstrate the potential of iPS-MSCs to regenerate bone *in vivo*, the newly derived cells were induced to osteoblast differentiation for 4 days and transplanted into calvaria defects in immunocompromised mice for 8 weeks. MicroCT analysis and histology demonstrated *de novo* bone formation in the calvaria defects for animals treated with iPS-MSCs, but not for the control group. Moreover, positive staining for human nuclear antigen and human mitochondria monoclonal antibodies unambiguously confirmed the participation of the transplanted human iPS-MSCs in the regenerated bone. These results confirmed that human iPS cells grown in a defined and xeno-free system have the capability to differentiate into functional MSCs with the ability to form bone *in vivo*.

INTRODUCTION

Induced-pluripotent stem (iPS) cells and embryonic stem (ES) cells have the ability to undergo self-renewal and differentiate into every cell type in the body[1-3], and therefore represent a potential renewable cell-source for cell therapies and regenerative medicine. Both pluripotent stem cell sources can further give rise to progenitor cells, such as mesenchymal stem cells (MSCs), that themselves have the capability to differentiate into mesoderm derivatives, such as bone, fat, cartilage, tendon and muscle[4-6]. In addition, MSCs have important immunomodulatory and engraftment-promoting properties [for review 7]. While MSCs can be isolated from bone marrow[8], adipose tissue[9], umbilical cord blood[10], umbilical cord stroma[11], placenta[12] and other tissues and organs, the harvesting procedures are invasive, expensive and laborious. Direct derivation of

MSCs from pluripotent stem cells represents an effective alternative to obtain larger populations of progenitor cells that are needed for cell therapies and/or regenerative medicine.

Like human ES cells, human iPS cells will need to be cultured in clinically compliant conditions, if broader translation into clinical practice is intended. Although co-culture with human feeder cells represent a xeno-free option for the *in vitro* expansion of pluripotent stem cells[13], such human feeder cell environments are undefined, may contain pathogens and will require expensive and labor-consuming screening. Similarly, extracellular matrix coatings made of undefined animal derived proteins such as matrigel, vitronectin, fibronectin or laminin are also expensive, may be immunologically incompatible with humans, have batch to batch variation, and will require extensive pre-transplant screening.

To overcome some of the limitations of human feeder cells or animal-derived extracellular matrices, synthetic cell culture substrates for pluripotent stem cells that are devoid of xenogeneic components have recently been developed[14-19]. Some of these substrates are based on recombinant proteins and/or peptides and thus are hampered by well-known problems of polypeptide matrices such as difficulties in sterilization, propensity to degrade[20] and the high cost of production[21].

Alternatively, cell culture coatings based on synthetic polymers can be reproducibly fabricated, are inexpensive and highly manipulable, and thus represent a

valuable option to expand pluripotent stem cells. Recently we reported the development of a fully defined synthetic polymer coating made of poly[2-(methacryloyloxy)ethyl dimethyl-(3-sulfopropyl)ammonium hydroxide] (PMEDSAH), that in combination with human-cell conditioned, or chemically defined medium supports the long-term culture and self-renewal of undifferentiated human ES cells[19, 22]. This pluripotent culture system makes use of a fully synthetic polymer as the structural motifs in cell-substrate interactions (i.e., no peptides, sugars, or proteins) and therefore provides a xenogeneic-free environment.

In this study, we tested the hypothesis that patient specific iPS cells can continuously proliferate (15 passages) on PMEDSAH in an undifferentiated state and yet will be capable of subsequent lineage-specific differentiation as well as regeneration of clinically relevant craniofacial skeletal defects. Importantly, we also demonstrate that human iPS cells cultured in this clinically compliant culture system can be directed toward differentiation into functional MSCs *in vitro* and bone formation *in vivo*.

MATERIALS AND METHODS

Generation of induced-pluripotent stem (iPS) cells

Retroviral vectors carrying *Klf4*, *Sox2, Oct3/4* and *c-Myc* were generated by transient co-transfection (using Addgene plasmids 17217, 17219, 17220, and 17226, and VSV-g envelope plasmid 8454) into Clontech GP2-293 packaging cells. Viral supernatant was harvested after 60 h, filtered and concentrated. Human fibroblasts were cultured in

DMEM + 10% fetal calf serum (FCS) with 1x non-essential amino acid supplement (Invitrogen, Carlsbad, CA). To generate iPS cells, two rounds of viral transduction of 30,000 fibroblasts were performed and cells were incubated with virus for another 48 h. After 4 d, cells were passaged on irradiated MEFs in fibroblast medium, and the following day switched to hES cell-medium, which consists of Dulbecco's modified Eagle Medium (DMEM)/F12 (Invitrogen), 20% knockout serum replacer (Invitrogen), 1 mM L-glutamine (Invitrogen), 1x non-essential amino acid supplement (Invitrogen), 0.1 mM β-mercaptoethanol (Sigma), and 4 ng/ml human recombinant fibroblast growth factor 2 (FGF2; Invitrogen). Cells were cultured in dedicated incubators set at 37°C/5% CO_2. The iPS colonies were manually picked and passaged. Immunohistochemistry was used to confirm expression of Nanog, stage-specific embryonic antigen (SSEA)-3/4, Oct3/4, and alkaline phosphatase. Culture of H7-hES cells (WA07, WiCell Research Institute; NIH Registration Number 0061) was performed as described above for human iPS cells.

Illumina Microarray

Total RNA was purified from iPS cells, parental fibroblasts and the H7-hES cells with the RNeasy Mini kit (Qiagen; Valencia, CA) and DNAse-I treatment. A total of 400 ng of RNA was amplified and labeled with the Total Prep RNA amplification kit (Ambion; Austin, TX) and 750 ng of biotin-labeled cRNA was used to hybridize to Illumina HumanHT-12 v4 Expression BeadChip. After washing, chips were coupled with Cy3 and scanned in an Illumina BeadArray Reader (Illumina, Inc., San Diego, CA). Un-

normalized summary probe profiles, with associated probe annotation, were output from BeadStudio.

Culture of iPS cells in defined culture conditions

Human iPS cells were cultured on PMEDSAH coated plates with human-cell-conditioned-medium (hCCM, GlobalStem, Inc., Rockville, MD) supplemented with 4 ng/ml of FGF2, as described previously[19, 22]. Briefly, PMEDSAH coated plates were pre-incubated with hCCM for at least 48 h at 37°C in 5% CO_2 atmosphere before use. Twenty-four h before passaging onto PMEDSAH coated plates hES cell-medium was replaced with hCCM, and passaging was mechanically performed using a sterile pulled-glass pipette or the StemPro EZPassage Disposable Stem Cell Passaging Tool (Invitrogen). Cells were observed every 48 h using a Leica stereomicroscope and differentiated cells were removed mechanically using a sterile pulled-glass pipette, followed by replacement of cell culture medium.

Derivation, culture and characterization of MSCs

To induce differentiation of iPS cells into mesenchymal stem cells (MSCs), embryoid bodies were formed and cultured in suspension for 7 days with hCCM in low-attachment culture dishes. Subsequently, approximately 70 embryoid bodies/well were plated onto 0.1% gelatin-coated 6-well plates in the presence of MSC growth medium (α-MEM, 10% FBS, 200 mM L -glutamine and 10 mM nonessential amino acid supplement and 8 ng/ml FGF2). Cells were cultured for up to 2 weeks to reach confluence, then trypsinized and passaged at a ratio of 1: 3. Cells were continually passaged in T-75

flasks until a homogeneous fibroblastic morphology appeared.

Characterization of MSCs involved analysis of cell surface antigens and functional differentiation assays *in vitro*. Cell surface antigen profiling was performed using fluorescent-activated cell sorting (FACS). MSCs were harvested using trypsin 0.25% EDTA, and after neutralization, single cell suspensions were washed in cold BSA, 0.5% (w/v) (Sigma) in DPBS and incubated at a concentration of $1X10^6$ cells/ml in 1 μg/ml unconjugated goat anti-human IgG (Invitrogen) on ice for 15 min to block nonspecific binding. Samples ($2.5X10^5$ cells) were then incubated on ice with the optimal dilution of fluorochrome-conjugated monoclonal antibody (mAb) in 1 μg/ml unconjugated goat anti-human IgG in the dark. All mAbs were of the immunoglobulin G1 (IgG1) isotype. The following conjugated antibodies were used in the analyses: allophycocyanin (APC)-conjugated antibodies against CD44 (fluorescein isothiocyanate (FITC)-conjugated mAbs against CD29, CD90, and CD45, phycoerythrin (PE)-conjugated mAbs against CD49a, CD49e, CD73, CD166 and CD105. All antibodies were from BD Pharmingen (TM, San Jose, CA) except for CD105 which was supplied by eBioscience (San Diego, CA). After 30 min incubation, cells were washed twice with ice cold 0.5% BSA in DPBS. Nonspecific fluorescence was determined by incubating cells with respective fluorochrome conjutages raised against antihuman IgG1. At least 10,000 events were acquired for each sample using a FACSCalibur instrument (Becton Dickinson, San Jose, CA) and cell flow cytometry data were analyzed using CELLQUEST software (Becton Dickinson).

For functional differentiation, human iPS-MSCs at passages 6–7 were induced to differentiate into adipogenic, chondrogenic, and osteogenic lineages in cell-specific culture medium. For osteogenesis, the cultures were incubated in DMEM that was supplemented with 15% FCS, 100 U/ml penicillin, 100 μg/ml streptomycin, 12 mM L-glutamine, 10 mM β- glycerophosphate (Sigma, St Louis, MO), 1 nM dexamethasone (Sigma), and 0.5 μM ascorbate-2-phosphate (Sigma). Media was changed 2 times per week for 3 weeks. Cells were fixed with 10% formalin for 20 minutes at RT and stained with Alizarin Red, pH 4.1 (Sigma) for 20 minutes at RT.

For adipogenesis, the cultures were incubated in DMEM that was supplemented with 15% FCS, 100 U/ml penicillin, 100 μg/ml streptomycin, 12 mM L-glutamine, 5 μg/ml insulin (Sigma), 50 μM indomethacin (Sigma), 1×10^{-6} M dexamethasone (Sigma), and 0.5 μM 3-isobutyl-1-methylxanthine (IBMX; Sigma). Media was changed 2 times per week for 3 weeks. Cells were fixed with 10% formalin for 20 minutes at RT and stained with Oil Red (Sigma) in ethanol (Sigma) for 20 minutes at RT.

For chondrocyte differentiation, the cultures were incubated in DMEM that was supplemented with 15% FCS, 100 U/ml penicillin, 100 μg/ml streptomycin, 12 mM L-glutamine, 5 μg/ml insulin(Sigma), 50 μM indomethacin (Sigma), 1×10^{-6} M dexamethasone (Sigma), and 0.5 μM 3-isobutyl-1-methylxanthine (IBMX; Sigma). Media was changed 2 times per week for 3 times. Cells were fixed with 10% formalin for 20 minutes at RT and stained Safranin O (Sigma) in ethanol (Sigma) for 20 minutes at RT.

Preparation of iPS-MSCs for implantation

iPS-MSCs were harvested using 0.25% trypsin-0.53 mM EDTA (Gibco), and cell pellets were re-suspended in 5 mg/ml human plasma fibrinogen (Sigma) at a concentration of $2x10^6$ cells per 40 µl. The fibrinogen cell suspension was then pipetted directly into 6 x 3 mm cubic pieces of Gelfoam (Pharmacia & Upjohn Co, NY, NY). Following absorption by the Gelfoam sponges, 5 µl of human thrombin (200 unit/ml; Sigma) was added. Gelation of the fibrin was observed and placed immediately on ice until implantation.

Surgical procedure and cell transplantation in craniofacial defect model

All procedures were approved by the University of Michigan Committee on the Use and Care of Animals. Four 5 week-old female immunocompromised mice (N:NIH-bg-nu-xid; Harlan Sprague Dawley, Inc., NC) were anesthetized with intraperitoneal injections of ketamine, (Ketaset, 75 mg/kg, Fort Dodge Animal Health, IA) and xylazine, (Ansed, 10 mg/kg, Lloyd laboratories, IA). A semilunar scalp incision was made from right to left in the post-auricular area, and a full-thickness flap was elevated. The periosteum overlying the calvarial bone was completely resected. A trephine was used to create a 5-mm craniotomy defect centered on the sagittal sinus and the calvarial disk was removed. One gelfoam sponge with or without human iPS cells was inserted in the calvaria defect per animal. The incisions were closed with 4-0 Chromic Gut suture (Ethicon/Johonson&Jhonson, NJ). All mice were killed 8 weeks after the implantation.

Radiology, histology and microCT analyses

Radiographic analysis was performed immediately after the calvaria were harvested with the use of a microradiographic apparatus (Faxitron X-ray Corporation, IL). MicroCT was performed to quantify bone volume and mineral density. The calvaria were immediately fixed in aqueous buffered zinc formalin, Z fix (Anatech, MI). Specimens were embedded in 1% agarose and placed in a 34 mm diameter tube and scanned over the entire length of the calvaria using a microCT system (μCT100 Scanco Medical, Bassersdorf, Switzerland). Scan settings were: voxel size 12 μm, medium resolution, 70 kVp, 114 μA, 0.5 mm AL filter, and integration time 500 ms. The center of the defect was visually identified and a cylindrical volume of interest (5 mm diameter) was drawn centered around the defect. Analysis was performed using the manufacturer's evaluation software and a fixed global threshold of 23% (230 on a grayscale of 0–1000) was used to segment bone from non-bone. Total bone volume (BV, mm3) and tissue mineral density of bone (TMD) were computed and calibrated to the manufacturer's hydroxyapatite (HA) phantom.

For histological analysis, calvaria were decalcified with a 10% EDTA solution for 2 days, dehydrated with gradient alcohols and embedded in paraffin. Coronal sections 5 μm in thickness were cut and stained with hematoxylin and eosin. Subsequently, the sections were deparaffinized and rehydrated, followed by antigen retrival treatment, blockage of endogenous peroxidase, 2.5% horse serum and avidin/biotinin. Then sections were reacted with mouse anti-human nuclei monoclonal

antibody and mouse anti-human mytochondria monoclonal antibody (Millipore), and signals were amplified with ImmPRESS reagent (VectorLabs, Burlingame, CA) and imaged with ImmPACT DAB substrate (VectorLabs).

Quantitative real-time reverse-transcriptase polymerase chain reaction (qRT-PCR)

Total RNA was extracted using Trizol (Invitrogen) and 1mg of RNA was reverse transcribed into cDNA using Superscript III Reverse Transcriptase (Invitrogen). Two μl of the diluted reverse transcribed cDNA (RT reaction, 1:5 in RNase free-water) were amplified in a 30 μl PCR assay volume, using the TaqMan Gene Expression Master Mix (Applied Biosystems, Foster City, CA, USA), target primers and Probe (unlabeled PCR primers and a FAMTM

dye-labeled TaqMan minor groove binder (MGB) probe) (Applied Biosystems). Gene expression was measured by qRT-PCR on an ABI Prism 7700 Sequence Detection System (Applied Biosystems). Levels of expression of target genes were correlated to standard concentrations and normalized to levels of beta-actin as an endogenous reference. Subsequently, the expression levels of investigated genes were normalized to the expression levels of control samples and reported as fold changes.

Cytogenetic analysis

Karyotype analysis of was performed at Cell Line Genetics (Madison, WI). Chromosomes were prepared using standard protocols and measurements were performed using the GTL-banding method on at least 20 metaphase preparations.

Statistical analyses

All experiments were performed in triplicate and data were expressed as the mean value ± the standard error of the mean (mean ± SEM) and analyzed by one-way analysis of variance. The levels of statistical significance were set at $p<0.05$.

RESULTS

Derivation of iPS cells and culture in xeno-free conditions

Human fibroblasts were reprogrammed into iPS cells by overexpression of *Oct3/4* (also known as *POU5F1), Sox2, Klf4* and *c-Myc* genes individually packaged into retroviral vectors. After 10-14 days in culture on irradiated MEFs, hES cell-like colonies formed and proliferated (Fig 1A-C). The morphology of emerging iPS cell colonies resembled the distinctive characteristic morphology of undifferentiated hES cell colonies with well-defined borders and a high nucleus:cytoplasm ratio[3] (Fig. 1B-C). Histochemical analysis revealed continuous expression of Oct3/4, Nanog, SSEA-4 and alkaline phosphatase after 25 passages, showing the proper maintenance of pluripotency of the iPS cell lines on MEFs (Fig. 1D-G). Global gene expression analysis showed that *Nanog, Oct3/4* and *Sox2* from iPS cells co-localized in scatter plots with the gene expression patterns of H7-hES cells, while differing from the parental skin fibroblast cell line (Fig. 1H), further confirming successful cellular reprogramming.

To establish cultures in fully defined and xeno-free conditions, human iPS cells were transitioned to PMEDSAH-coated plates with human cell-conditioned medium

(hCCM), as previously described for human ES cells[19, 22]. PMEDSAH-coated plates continuously supported attachment, proliferation, self-renewal and maintenance of pluripotency of undifferentiated human iPS cells. These findings were confirmed by rigorous characterization performed every 5 passages, including karyotype analysis (S-Fig 1A), expression of Oct3/4, Sox2 and SSEA-4 (Fig. 2), and formation of embryoid bodies (EB) expressing markers of all three germ layers (data not shown).

Derivation of mesenchymal stem cells (MSCs) from human iPS cells

The derivation of human iPS-MSCs was performed following protocols used for the differentiation of human ES cells into MSCs[5]. Selected colonies were collected to form EBs, while other colonies were maintained on PMEDSAH-coated plates for further propagation as undifferentiated iPS cells. Initially, a heterogeneous cell population developed from EBs and by passage 2, greater than 90% of the total cell population acquired a fibroblast-like morphology that matched the described morphology of derived human ES-MSCs[4, 5]. Gene analysis by qRT-PCR demonstrated that *Oct3/4, Sox2*, and *Nanog* expression was downregulated in the derived iPS-MSCs relative to parental human iPS cells (Fig 3A). Similarly, the expression of *c-Myc* and *Klf4*, used in the reprogramming of human fibroblasts into iPS cells was downregulated in the derived iPS-MSCs. As an initial characterization to confirm the derivation of MSCs from the iPS cells, fluorescence activated cell sorting (FACS) analysis of cell surface markers present in hMSCs was performed at passages 1, 5, and 9. The immunophenotype of the iPS-MSCs was consistent over all time points studied (CD166+, CD105+, CD73+, CD44+,

CD34- and CD45-) (Fig 3B). The derived iPS-MSCs also maintained a normal karyotype over 12 passages (S-Fig 1B).

To verify the multilineage differentiation capacity of the iPS-MSCs, *in vitro* differentiation directed toward adipogenic, chondrogenic, and osteogenic lineages was performed. Adipogenic differentiation of iPS-MSCs was demonstrated by a 5-fold increase in *peroxisome proliferator activated receptor gamma (PPAR-γ)* mRNA in iPS-MSCs treated with adipogenic factors as compared to controls (S-Fig 2). Chondrogenic differentiation was observed after iPS-MSCs were cultured as micromass pellets in medium containing chondrogenic supplements for four weeks. Safranin O staining of chondrogenic pellets revealed chondrocyte-like cells residing in lacunae surrounded by a well-defined glycosamnioglycan (sGAG) and proteoglycan-rich extracellular matrix (Fig 4A). Gene analysis by qRT-PCR demonstrated increased mRNA levels of genes related to chondrogenesis, such as *aggrecan (ACAN), collagen type II alpha 1 (COL2A1), perlecan (HSPG2), glypican 3 (GPC3)* and *syndecan 2 (SDC2)* in chondrogenic pellets compared to iPS-MSCs cultured in 2D and with growth medium (Fig 4B).

Osteogenic differentiation of iPS-MSCs was induced after cells were cultured in medium containing osteogenic supplements for four weeks. By the end of the differentiation assay, calcium deposition was observed in iPS-MSCs cultured with osteogenic medium, but not in cells in growth medium (Fig 4C). In addition, increases in mRNA levels of genes related to osteogenesis such as *bone morphogenic protein 2 (BMP2), collagen type I alpha 1 (COL1A1), osteocalcin (BGLAP), alkaline phosphatase*

(ALP), bone sialoprotein 1 (SPP1), osteonectin (SPARC) and *distal-less homeobox 5 (DLX5)*, was observed in mineralized iPS-MSCs compared to proliferating iPS-MSCs (control group) (Fig 4D). To demonstrate the specificity of iPS-MSC commitment under defined culture conditions, the expression of genes related to chondrogenesis was evaluated in iPS-MSCs directed to osteogenesis, and vice-versa (S-Fig 3). As expected, the expression of chondrogenic-related genes was not changed in osteogenic samples in relation to iPS-MSCs in growth medium. In contrast, a significant increase in expression of *DLX5* and *SSP1* was observed in chondrogenic samples, while other bone-related genes did not change compared to control iPS-MSCs.

In vivo osteogenic potential of human iPS-MSCs derived iPS cells cultured on PMEDSAH

To verify the capability of human iPS-MSCs to regenerate bone *in vivo,* cells treated with osteogenic factors for 4 days were transplanted into calvaria defects of immuncompromised mice. After 8 weeks, animals were euthanized and specimens were analyzed by microCT and histology. MicroCT analysis demonstrated a 4.2 fold increase in volume of new bone formed in calvariae transplanted with iPS-MSCs compared to controls. New bone formation was observed within the central region and on the margin of the defect adjacent to the mouse calvaria. Histological evaluation identified osteocytes surrounded by woven bone, proliferating osteoblasts lining the exterior of the newly formed bone, as well as small bone marrow cavities within the newly formed bone (Figure 5A). Fibrous connective tissue filled out the rest of the defect.

To determine the contribution of the transplanted human iPS-MSCs in new bone formation, specific anti-human monoclonal antibodies that do not cross-react with murine cells were used. Positive staining for human nuclear antigen and human mitochondria were observed in osteocytes embedded in the newly-formed bone, but not in the surgical margins of the native mouse bone or in the fibrous tissue that filled the defect (Fig 5B-D).

DISCUSSION

The use of MSCs in regenerative medicine has advanced significantly, as demonstrated by numerous stem cell-based clinical trials (www.clinicaltrials.gov) in phase I and phase II currently under way to treat human conditions, such as bone defects, wound repair, myocardial infarction, stroke, diabetes and graft-versus-host-disease [for review 23]. While MSCs can be isolated from several patient tissues, the cell harvesting procedures are invasive, expensive and laborious. Furthermore, the *in vitro* expansion capacity of isolated MSCs is limited and extensively cultured primary cells may take on phenotypic characteristics that are not consistent with the behavior of natural MSCs *in vivo*. Thus, iPS-MSCs represent an important alternative source of primary cells. iPS cells have gained attention because they possess pluripotency, self-renewal and differentiation properties, which are similar to ES cells without sharing the ethical concerns associated with hES cells. However, current practices to maintain human iPS cells and human ES cells in an undifferentiated state typically rely on undefined and xenogeneic components that ultimately impede our ability to use these stem cells to treat debilitating human diseases.

Here, we demonstrated that human iPS cells can proliferate in an undifferentiated state on PMEDSAH-coated plates, a synthetic polymer coating devoid of xenogeneic contamination. The cells can be subsequently differentiated into functional MSCs with *in vivo* bone regeneration capabilities. PMEDSAH-coated plates supported the expansion of undifferentiated human iPS cells. After 15 passages on PMEDSAH-coated plates, iPS cells maintained the expression of transcription factors and cell surface markers associated with pluripotent stem cells, as well as a cell/colony morphology and normal karyotype[3]. Most importantly, iPS cells cultured on PMEDSAH maintained their pluripotent character. Thus, PMEDSAH-coated culture substrates in combination with human-cell conditioned medium represents a clinical-grade culture system free of xenogeneic contamination for the expansion of human iPS cells.

Going beyond the current state-of-art[6], human iPS cells cultured on the fully synthetic PMEDSAH substrate under clinically compliant conditions were used to derive MSCs and transplanted *in vivo* where they not only survived, but contributed to *de novo* bone formation. The derived iPS-MSCs expressed similar levels of markers present in hMSCs[24], while qRT-PCR revealed that genes associated with pluripotency and reprogramming markers were no longer expressed once the cells were directed to differentiate. This fact, coupled with the critical observation that no teratomas formed in mice treated with transplanted human iPS-MSCs, indicated a reduced tumorigenic risk of this progenitor population compared to undifferentiated iPS cells. The derived human iPS-MSCs were able to differentiate *in vitro* into adipogenic, chondrogenic and osteogenic lineages. Interestingly, the chondrogenic and osteogenic differentiation of the

derived human iPS-MSCs was more pronounced than adipogenic differentiation. Similar observations have been made for different populations of *in vivo* derived MSCs indicating a variability that depends on the origin of the cell population[25, 26]. It remains to be determined whether the differences in differentiation levels are inherent to the nature of the derived iPS-MSCs, or influenced by the origin of the parental iPS cells as dermal fibroblasts. In fact, epigenetic memory has been suggested for iPS cells depending on their origin[8, 27, 28]. However, a significant up regulation of *PPAR-γ* in cultures treated with adipogenic medium does suggest the potential for iPS-MSC differentiation towards adipogenesis. In addition, effective chondrogenic differentiation of human iPS-MSCs was achieved, as confirmed by histological and gene expression analyses of chondrogenic pellets. Robust deposition of proteoglycans was observed in pellets of iPS-MSCs treated with chondrogenic factors, as indicated by the intensity of the safranin 'O' staining. Significant expression of extracellular matrix-related genes present in cartilage suggests the maturity of the chondrogenic pellets obtained from human iPS-MSCs. The *in vitro* osteogenic differentiation of human iPS-MSCs was also robust, as indicated by calcium deposition and upregulation of genes related to osteogenesis in cultures treated with osteogenic medium. Taken together, the derived human iPS-MSCs described here meet the specifications of a defined MSC population as proposed by the International Society for Cellular Therapy[29]: (1) adherence to tissue culture plastic under standard culture conditions; (2) an immunophenotype similar to that of human bone marrow stromal cells with low expression of HSC markers; and (3) the ability to undergo *in vitro* differentiation along the osteogenic, chondrogenic and adipogenic lineages.

To examine the possible use of derived human iPS-MSCs in cell therapies and regenerative medicine, cell-transplantation assays were performed in immunocompromised mice with the goal of developing bone *in vivo*. Histological and microCT image reconstruction confirmed the formation of new bone within the calvaria defects of mice treated with transplanted human iPS-MSCs after 8 weeks. Randomly distributed and unorganized bone fragments were found within the calvarial defects. Furthermore, positive immunostaining of osteocytes for monoclonal antibodies to human nuclei and human mitochondria confirmed the participation of transplanted human iPS-MSCs in the regenerated bone. Although the clinical critical size defect was not completely healed, the fact that human iPS-MSCs participated in bone regeneration *in vivo* in a calvarial defect mouse model suggests these cells became functional MSCs and osteoprogenitors that can be used as a tool for future cell transplantation studies that investigate craniofacial bone repair in response to disease and trauma.

In summary, human iPS cells can be cultured on the synthetic polymer coating, PMEDSAH, in a fully defined and clinically-compliant system under xeno-free conditions. iPS cells maintained on this substrate have the capacity to differentiate into functional MSCs both *in vitro* and *in vivo.* Future work investigating factors such as the ideal number of transplanted cells, survival and distribution, *in vitro* osteogenic induction prior to transplantation, and even more importantly, the optimal cell carrier with osteoconductive properties, is necessary to reveal the role that transplanted cells can play in regenerating bone. Additionally, although not a particular goal of this work, it is important to determine whether virus-free and transgene-free iPS cells can be derived on

PMEDSAH-coated plates. Taken together, the PMEDSAH culture system and efficient iPS-MSC derivation on this synthetic substrate provides a unique platform for the future design of cell-based strategies for bone regeneration.

ACKNOWLEDGMENTS

The authors thank W. King for his insightful comments and critical reading of the manuscript, to S. Hongli, M. Pressler, M. Lynch and M. Dzaman for their support performing surgical procedure and cell transplantation in craniofacial defect model, qRT-PCR, microCT analysis and immunohistochemistry, respectively. This research was supported by the NIH grants R01 DE016530, R01 NS065450, F31 DE019760 and the NIDCR T32 Tissue Engineering and Regeneration Training Program.

Figures

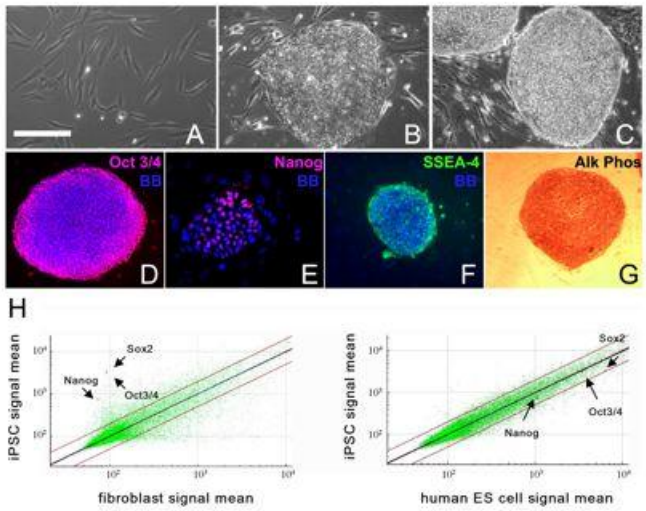


Fig 1. Characterization of human iPSC lines. Phase contrast microscopic images show human fibroblasts (**A**), an undifferentiated iPS cell colony at passage 30 (**B**) derived from reprogramming of the fibroblasts in panel A, and an H7 human ES cell colony at passage 45 (**C**). Immunocytochemistry of iPS cells cultures showing expression of the pluripotency markers Oct3/4 (**D**), Nanog (**E**), and SSEA-4 (**F**) and histochemical staining for alkaline phosphatase (Alk Phos; **G**). Bisbenzimide (BB, blue) was used to visualize cell nuclei in **D-F**. Scale bar: 100 μm in **A** and **E**, and 200 μm in **B-D**, **F** and **G**. (**H**) Scatter plots of microarray data showing global gene expression patterns of human fibroblasts, human iPS cells of the same genetic background, and human ES cells (H7). The position of individual pluripotency genes labeled in the panels is indicated with red dots.

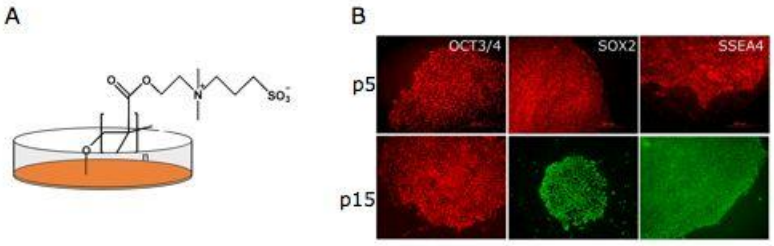


Fig 2. Culture of human iPS cells in a clinical compliant culture system. (**A**) An Schematic representation of a PMEDSAH-coated plates that serves as feeder-free chemically-defined substrate. The chemical structure of PMEDSAH is depicted, showing its carbonyl and sulfonate groups that contribute to the zwitterionic nature of the polymer. Serum-free human cell conditioned medium supplemented with FGF2 provide the soluble components. (**B**) This clinical compliant culture system supports the long-term growth of human iPS cells, as shown here at passage (p) 5 and 15 colonies expressing undifferentiated markers (OCT3/4, SOX2 and SSEA4) of pluripotent stem cells.

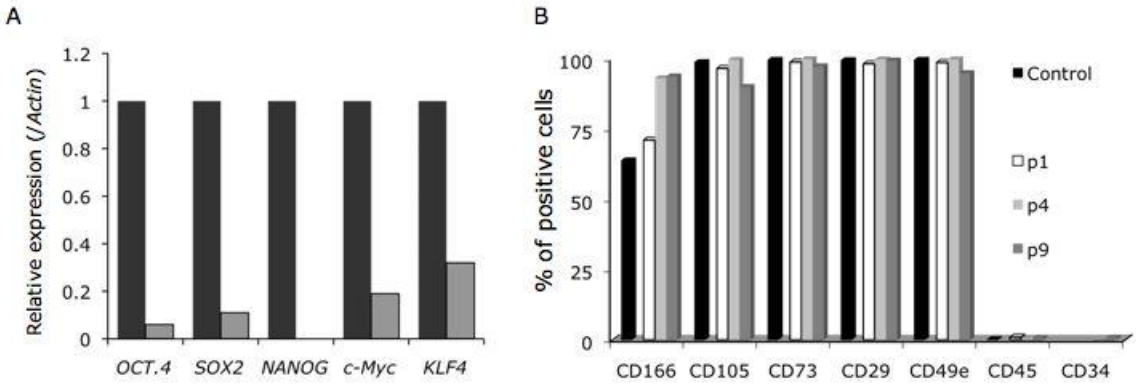


Fig 3. Characterization of mesenchymal stem cells (MSC) derived from human iPS cells cultured in a clinical compliant system. After 5 passages of culture in a clinical complaint culture system, human iPS cells were differentiated into MSCs. (**A**) The expression of reprogramming factors (*OCT4, SOX2, c-Myc* and *KLF4*) used to generate the iPS cells was analyzed in the differentiated MSCs (p1; light-grey columns) by qRT-PCR, showing low levels of mRNA compared to undifferentiated iPS cells (dark-grey columns). Data was normalized to human β-*Actin* expression. *NANOG* expression was evaluated as well. (**B**) The expression of characteristic MSC cell-surface markers was analyzed by flow cytometry at different passages (1, 4 and 9). As control, human bone marrow cells were used.

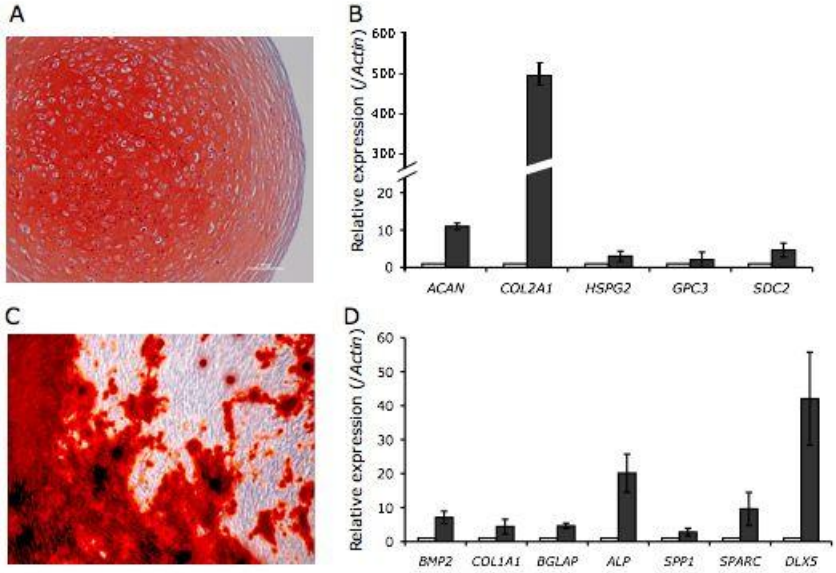


Fig 4. *In vitro* differentiation of human iPS cell-derived mesenchymal stem cells (MSC). At passage 5, human iPS cell-derived MSCs were induced to differentiated into chondrogenic, osteogenic and adipogenic lineages. (**A**) Induction into chondrogenic lineage was confirmed by histology and staining of proteoglycans by Safranin 'O' staining in pellets, and (**B**) up-regulation of chondrogenic-related genes: *aggrecan* (*ACAN*), *collagen type II alpha-1* (*COL2A1*), *perlecan* (*HSPG2*), *glypican 3 (GPC3)* and *syndecan 2 (SCD2)*. (**C**) Osteogenic differentiation was confirmed by Alizarin Red Staining indicating calcium deposition in mineralized cultures and (**D**) up-regulation of osteogenic-related genes: *bone morphogenetic protein 2* (*BMP2*), *collagen type I alpha-1* (*COL1A1*), *osteocalcin (BGLAP), alkaline phosphatase (ALP), bone sialoprotein 1 (SPP1), osteonectin (SPARC)* and *distal-less homeobox 5 (DLX5)*. Adipogenic induction was detected by up-regulation (5.48 ± 0.7 fold-increase; S-Fig 2) of *peroxisome proliferator activated receptor gamma* (*PPAR-γ*). Cells were cultured in differentiation medium (dark columns) and compare to control cells cultured in MSC-grow medium (white columns). Samples were analyzed by qRT-PCR, data normalized to human β-*Actin* expression and results shown are the mean and SEM of three experiments.

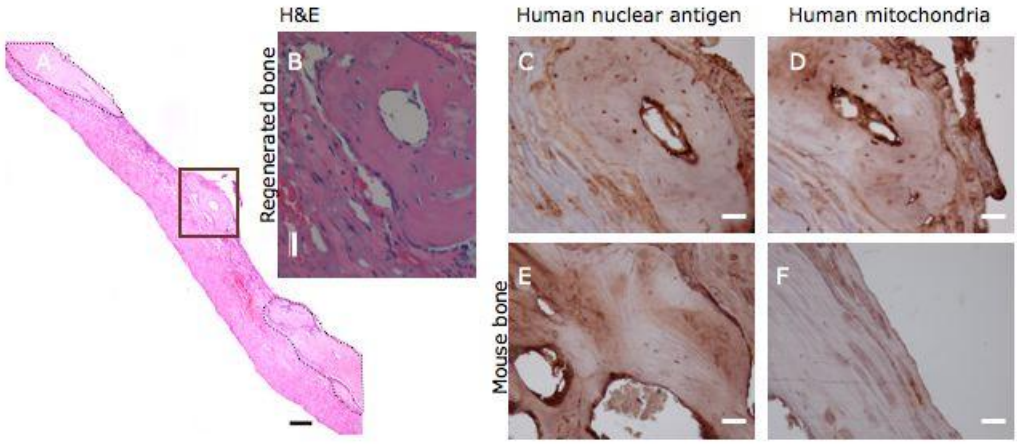
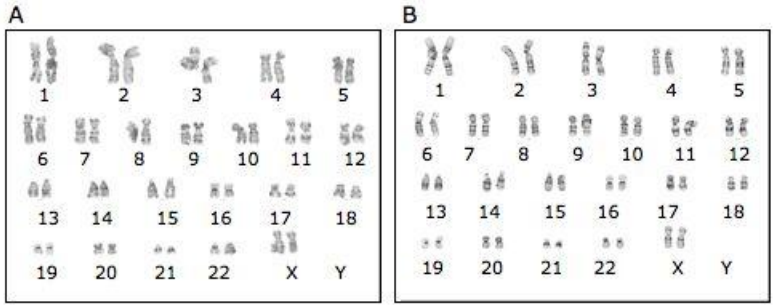
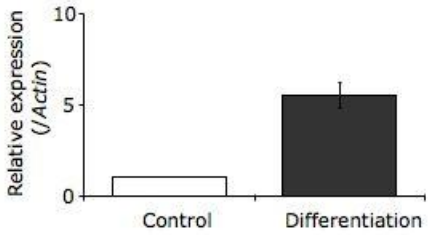


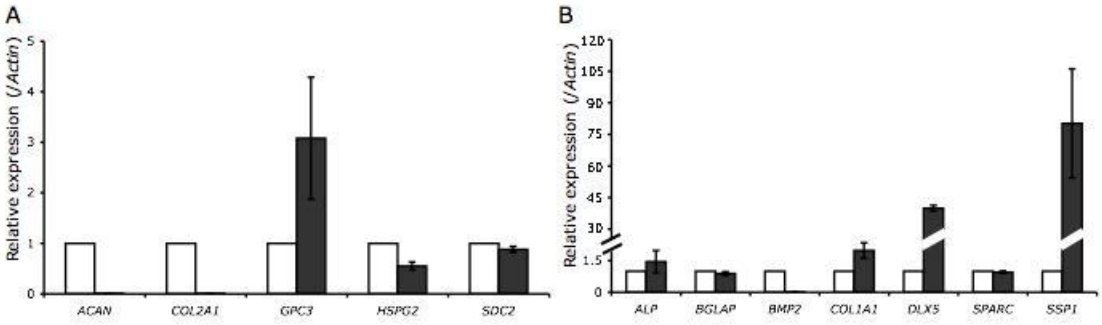
Fig 5. *In vivo* bone formation by human iPS cell-derived mesenchymal stem cells (MSC). After 4 days of culture in osteogenic medium human iPS cell-MSCs were transplanted into a calvaria defect in SCID mice. Eight weeks later animals were euthanized and skulls processed for microCT analysis, followed by decalcification and preparation for histology. **A**) Reconstructed image stained with H&E showing an isolated ossicle (highlighted area in brown box and in expanded images B-D) apart from the limits of the calvaria defect (delimited by dashed lines and in expanded images E and F). Immunostaining with human nuclear antigen (**B** and **E**) and human mitochondria (**C** and **F**) antibodies confirmed that the origin of the regenerated bone was from transplanted human iPS-MSCs. Scale bar in **A**= 100 μm, while in **B-F**= 20 μm.



S-Fig 1. Karyotype analysis of human iPS cells and derivative mesenchymal stem cells (MSC). Cytogenetic analysis performed on twenty G-banded metaphase cells from human iPS cells cultured in a clinical compliant system at passage 10 (**A**) and hiPS cell-derived MSCs at passage 12 (**B**), demonstrated an apparently normal female karyotype.



S-Fig 2. *In vitro* differentiation of hiPS cell-derived mesenchymal stem cells (MSC) into adipogenic cells. Adipogenic induction was detected by up-regulation of *peroxisome proliferator activated receptor gamma* (*PPAR-γ*). Samples were analyzed by qRT-PCR, data normalized to human β-*Actin* expression and results shown are the mean and SEM of three experiments.



S-Fig 3. Expression of chondrogenic and osteogenic related genes in *in vitro*-differentiated human iPS-MSCs. Q-RT PCR was used to analyzed the expression of genes-related to chondrogenic differentiation of human iPS-MSCs directed towards osteogenic differentiation (**A**), and osteogenic-related genes in cells induced to chondrogesis (**B**). White columns show the control group: human iPS-MSCs cultured in growth-medium, while dark columns indicate cells induced to chondrogenic (**A**) and osteogenic (**B**) differentiation. *Aggrecan* (*ACAN*), *collagen type II alpha-1* (*COL2A1*), *perlecan* (*HSPG2*), *glypican 3 (GPC3), syndecan 2 (SCD2), bone morphogenetic protein 2* (*BMP2*), *collagen type I alpha-1* (*COL1A1*), *osteocalcin (BGLAP), alkaline phosphatase (ALP), bone sialoprotein 1 (SPP1), osteonectin (SPARC)* and *distal-less homeobox 5 (DLX5).* Data was normalized to human β-*Actin* expression and results shown are the mean and SEM of three experiments.

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