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Reconstructing the In Vivo Environment for the Development of Tissue-Engineered Constructs from Human Mesenchymal Stem Cells

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RECONSTRUCTING THE IN VIVO ENVIRONMENT FOR THE DEVELOPMENT OF TISSUE-ENGINEERED CONSTRUCTS FROM HUMAN MESENCHYMAL STEM CELLS

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To my parents…

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ABSTRACT

Human mesenchymal stem cells (hMSC) are obtained primarily from adult bone marrow and hold tremendous promise for use in a number of clinical applications. Currently, hMSC are being used in clinical trials to treat a number of diseases such as graft versus host disease and osteoarthritis, as well as to repair bone-tissue and regenerate cardiac muscle following myocardial infarcts. One approach combines hMSC with 3D scaffolds to develop tissue constructs, which can be directly implanted into the pathological site. However, little work has been done to understand the tissue-developmental patterns of stem cells in 3D constructs. Previous research has shown that cells cultured in 3D substrates exhibit markedly different contact structures and regulatory controls than cells grown on conventional 2D culture dishes. Furthermore, the spatial organization of cells has significant effects on their growth patterns and tissue formation. In this study, poly(ethylene terephthalate) (PET) matrices with defined pore structures were treated to achieve similar surface properties to conventional 2D tissue culture plastics. A depth-filtration method was used to uniformly distribute cells in the scaffolds, and cells were cultured for at least one month. In addition to comparing hMSC behavior in 2D and 3D systems, different 3D culture conditions were obtained by varying oxygen concentrations or applying media flow. Several experimental techniques were used to examine cellular characteristics, including cell growth, spatial organization, nuclear morphology, integrin expression, organization of their extracellular matrix (ECM) proteins and multi-lineage differentiation potential. HMSC cultured in 3D scaffolds proliferated significantly slower than 2D controls. However, they secreted and embedded themselves in an extensive ECM network, and demonstrated significant ability to organize their ECM proteins into aligned fibrils. They also regulated their expression of adhesion proteins consistent with the scaffold topography and ECM structure; thus indicating that cells in 3D can reciprocally interact with their surroundings and are capable of remodeling their microenvironment. Constructs grown at 2% oxygen exhibit a prolonged proliferation phase. This gives rise to constructs with similar ECM organization but higher cell-densities. HMSC also demonstrated improved ability to retain stem-cell

characteristics and exhibited enhanced differentiation ability when cultured under low oxygen conditions. Continuously perfusing hMSC constructs with fresh media allowed these cells to proliferate exponentially. However, in perfusion constructs, the ECM was disorganized, there was considerably less protein production, and there was a drastic change in the nuclear morphology from elongated to spherical. Hence perfusion systems facilitated superior cell growth, but affected cell phenotype as well as the physical and biological properties of the resulting constructs. Interestingly, while these cells had spherical nuclei, they proliferated extensively and showed enhanced ability to differentiate into bone-forming cells, contradicting proposed paradigms regarding the regulatory effects of cell-shape on the fate of adhesion-dependent cells. These studies approximate various elements of the native hMSC environment and emphasize the need for careful consideration of culture parameters in order to develop functional tissue-engineered constructs for clinical use.

CHAPTER 1

INTRODUCTION

1.1 OVERVIEW

The potential of stem cells to cure a plethora of debilitating diseases has been well publicized. Embryonic stem cells (ESC) in particular have been touted as a universal panacea for multiple pathologies including degenerative (e.g. Parkinson's disease), traumatic (e.g. spinal cord injuries) or genetic (e.g. Osteogenesis Imperfecta) diseases. However, there are a number or ethical, moral and legal concerns that restrict the funding for ESC research and limit their potential therapeutic use. Significantly less discussed in the public forum is the ability of adult stem cells to perform similar functions. Human mesenchymal stem cells (hMSC) derived from the adult bone marrow hold tremendous promise for use in a variety of clinical applications. HMSC are already being used in a number of small-scale clinical trials to treat patients who have suffered from heart attacks, osteoarthritis, or immune rejection following bone-marrow transplants and chemotherapy. These cells, which are present in low doses in bone marrow, can be harvested and utilized by applying a number of tissue-engineering approaches. Hence, significant amounts of energy are being expended to refine current tissue-engineering methodologies in order to expedite the widespread clinical application and availability of these cells.

Tissue engineering has employed three general approaches to achieve its goal of developing functional biological substitutes: isolated cells, tissue-inducing substances, or the combination of cells within three-dimensional (3D) scaffolds (Langer and Vacanti, 1993). In recent years, particular emphasis has been placed on using cells within matrices, since these can be engineered *in vitro* to develop constructs with proper tissue phenotypes, which can be directly implanted into *in vivo* defects. The interdisciplinary nature of this undertaking has resulted in significant advances in knowledge - and several paradigm shifts - in fields as diverse as embryology, cell biology, materials science and engineering. Yet, several fundamental questions remain: what are

the best cell sources, biomaterials and culture systems to be used, and can they be chosen and optimized for different clinical applications? This study touches briefly on these topics, but focuses primarily on the applicability of human mesenchymal stem cells (hMSC) to tissue-regeneration, and the effects of culture parameters on their tissue-development characteristics.

Friedenstien and coworkers (1976) were the first to investigate the characteristics of the colony-forming fibroblastic cells, which were isolated from the bone marrow by their selective adherence to tissue-culture plastics. Caplan (1991) called these cells 'mesenchymal stem cells' in reference to their high self-renewing properties and ability to form cartilage and bone. The term 'mesengenic process' (Caplan, 1994) was then coined to describe their step-wise progression into end-stage differentiated cells. HMSC are isolated primarily from the adult bone marrow where they act as supporting stroma for hematopoietic stem cells (HSC), and contribute to the HSC microenvironment by secreting ECM proteins, cytokines and growth factors (Haynesworth *et al*, 1996). However, their unprecedented multi-lineage capabilities, including differentiation into cells of ectodermal and endodermal origin, indicate that hMSC may hold significant promise for use in a variety of therapeutic applications. Already, hMSC are being used in clinical trials to treat osteogenesis imperfecta (Horwitz *et al*, 1999) and to regenerate myocardial tissue that has been damaged by ischemia (Pittenger and Martin, 2004). However, they may also be beneficial for treating other pathologies such as Parkinson's and Alzheimer's diseases, diabetes mellitus, and Duchenne's muscular dystrophy (DMD), as well as regenerating diseased or damaged tissues.

While hMSC may be used directly in some of these clinical applications, their ability to effectively repair or regenerate load-bearing mesenchymal tissues may be enhanced by their implantation into suitable scaffolds. These constructs may guide their organization and development, and influence the mechanical properties of the tissue constructs (Sharma and Elisseeff, 2004). However, relatively little is known about hMSC biology in 3D scaffolds. Traditional systems utilize flat, plastic dishes (2D culture) as substrates for culturing mammalian cells, and have been used to determine the *in vitro* characteristics of hMSC. These systems have two innate flaws: Firstly, they force cells to adapt to biologically and physically unnatural environments. Secondly, cells are unable to maintain proper interactions with each other and the

extracellular matrix (ECM), hence there is an absence of the tissue-context, which is a critical determinant of cell function. Thus, while 2D systems have been a useful tool for unearthing many enigmas in cell and molecular biology, they result in changes to cellular phenotype, so that many of the characteristics that identify hMSC in culture may not necessarily be reflective of their 3D or *in vivo* biology. Cell-cell and cell-matrix adhesions in 3D determine whether cells proliferate, migrate, differentiate, die, or maintain their function within a tissue (Bissell & Nelson, 1999). Therefore, to gain a better understanding of hMSC attributes, it is necessary to culture them in 3D constructs and observe their developmental processes.

A major challenge for developing cellular 3D constructs however, is the mass-transfer considerations, which limit the availability of nutrients to the innermost cells. Tumor spheroids larger than 1 mm in diameter become hypoxic at the core. Hence, cells at the center undergo necrosis (Sutherland *et al*, 1986). To avoid similar phenomena in tissue-constructs, bioreactor systems have been developed, which use forced-convection to minimize boundary layers and facilitate nutrient transfer into constructs. Bioreactors have several other advantages: they provide reproducible culture environments, facilitate uniform seeding patterns of cells, and can be used to provide specific mechanical stimuli, such as shear force or hydrostatic/hydrodynamic loading, which may enhance the phenotypic characteristics of differentiated cells (Martin *et al*, 2003).

In addition to 3D hMSC culture and bioreactors, another cell-culture parameter investigated was oxygen concentrations. Conventional systems expose cells to ambient (20%) oxygen conditions. The upper limit of oxygen tension experienced by cells in the body is 16% while bone marrow cells exist at average oxygen concentrations of approximately 5% (Lennon *et al*, 2001). Numerous reports have demonstrated that at lowered oxygen tensions (hypoxia), cells exhibit significant functional differences. Stem cells are particularly responsive to environmental changes in oxygen concentrations. For example, it is believed that HSC's *in vivo* differentiation and proliferation is spatially regulated along an increasing oxygen gradient (Cipolleschi *et al*, 1993). *In vitro*, murine and human HSC cultured under hypoxia maintain higher numbers of colony-forming cells and exhibit improved ability to repopulate marrow (Ivanovic *et al*, 2000a,

b). Hence, oxygen significantly impacts upon the maintenance of stem cell characteristics in culture.

Investigations of hMSC characteristics and capabilities are being pursued at a rapid pace, with hundreds of peer-reviewed publications each year. New methods are continually being reported for improving the *in vitro* isolation and expansion of hMSC, since obtaining sufficient quantities of functional cells is essential for realizing their clinical potential. One approach taken is to incorporate elements of their *in vivo* microenvironment into the design of culture systems in an attempt to maintain the unlimited, self-renewal capabilities of native cells. In this study, the effects of the spatial organization of the cells and the oxygen concentration in the culture system are investigated since these are considered as critical components of the hMSC niche. However, while elements of the cellular response to varying culture-conditions are evaluated, the focus of the study was to analyze how varying culture conditions affected the physiological characteristics of the resulting tissue structures.

1.2 DISSERTATION OUTLINE

This study can be sub-divided into the effects of: i) 3D matrices, ii) perfusion culture, and iii) hypoxia on hMSC growth and development. Chapter 2 is a comprehensive literature review of several relevant fields, and provides the backdrop against which the results of subsequent chapters are discussed. Advances in knowledge of hMSC biological characteristics are outlined, and the potential for their use in future therapeutic applications evaluated. This is followed by an analysis of 3D matrices from both cell-biology and tissue-engineering perspectives, which is supplemented with a brief overview of the role of bioreactor technology in engineering tissue-constructs. Lastly, a detailed review of studies done on hypoxia over the last 15 years, along with a report of the ubiquitous and indispensable role of oxygen on cellular and tissue functions is presented.

In the third chapter, developmental characteristics of hMSC grown in a 3D scaffold, and cultured in a tissue-culture plate are presented, and contrasted to the 2D analogy. Subsequently, similar

hMSC/polymer constructs were cultured in a perfusion bioreactor system. This facilitated comparisons between perfusion and static conditions on hMSC physiology, as well as the phenotype of the engineered tissue. The results of this study are detailed in Chapter 4.

Chapters 5 and 6 describe results of experiments conducted with hMSC under 2% oxygen tension. In chapter 5, again tissue-developmental characteristics in 3D matrices are analyzed and compared with those in control (20% O_2) static cultures. Chapter 6 however, looks at hMSC grown on 2D substrates and focuses on how oxygen tension affects their *in vitro* life-span and cellular characteristics.

The final chapter of the dissertation attempts to integrate the results from all of these studies into a coherent whole, emphasizing their significance, and the overall intellectual contribution to the field of tissue-engineering. Several ideas for future work, and expansion on these projects are presented.

CHAPTER 2

BACKGROUND AND LITERATURE REVIEW

2.1 HUMAN MESENCHYMAL STEM CELLS

2.1.1 What are hMSC?

The definition of a stem cell remained contentious for over three decades. Currently, a stem cell is defined simply as a cell with the ability to self-renew indefinitely *in vivo* while retaining the capacity to differentiate into at least one other specialized cell type (Watt and Hogan, 2000). HMSC are considered as stem cells since they can be expanded in culture for many passages and differentiated into osteogenic, chondrogenic, and adipogenic, lineages (Prockop, 1997; Pittenger *et al*, 1999; Heath, 2000). They are isolated primarily from the bone marrow, but similar multi-

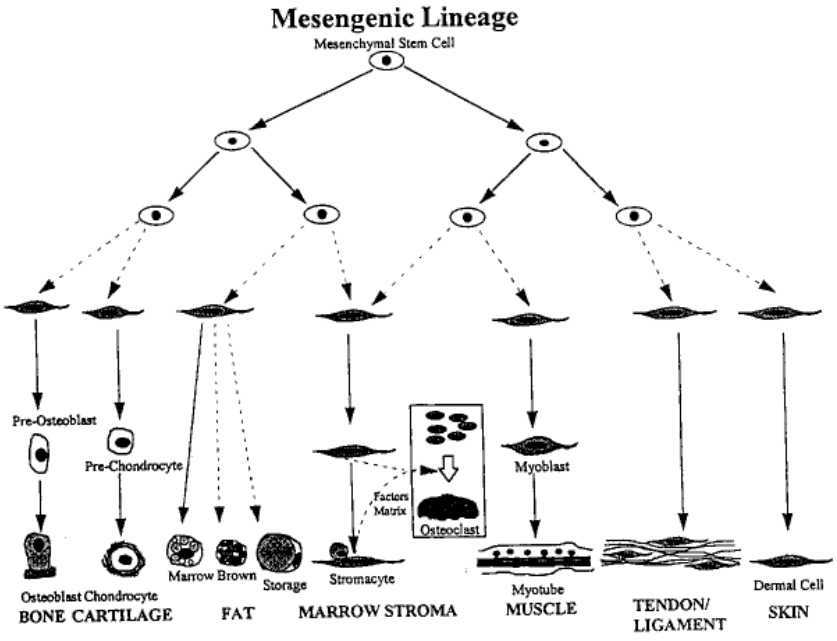


Figure 2.1 The Mesengenic Process (Haynesworth et al, 1998)

lineage progenitor cells have been discovered in most adult tissues (Dominici *et al*, 2001; Guilak et al, 2004), as well as in peripheral blood (Kuznetsov *et al*, 2001; Roufosse *et al*, 2004), umbilical cord blood (Romanov *et al*, 2003; Gang *et al*, 2004), human term placenta (Igura *et al*, 2004; Yen *et al*, 2005), and amniotic fluid (Fauza, 2004). Their easy extraction and extensive proliferation and multipotent differentiation abilities, along with a host of other characteristics, make hMSC very promising candidates for use in therapeutic applications. Despite continuing interest in their clinical potential however, there are significant gaps in current knowledge of hMSC biology, primarily because studies of hMSC characteristics take place outside of their tissue context. Hence, much of our knowledge of hMSC centers on what they can be induced to do in culture, rather than their actual *in vivo* functionality.

2.1.2 *In Vitro* Characterization of hMSC

2.1.2.1 Homogenous vs. Heterogenous Cell Population

Pittenger *et al*, (1999) isolated and characterized a homogenous population of hMSC from the bone marrow that exhibited fibroblastic morphology and multi-lineage differentiation potential. Even so, their results showed that only a certain percentage of cells differentiate when induced along a particular lineage. Other groups have presented evidence, which suggests that hMSC may be comprised of various cell sub-populations, differentiated by size and morphology, having diverse differentiation capabilities (Colter *et al*, 2001; Shur *et al*, 2002). Bruder *et al*, (1997) reported that the hMSC population lost the spindle morphology and became progressively broader and flatter as they approached senescence. Colter and coworkers (2001) observed both spindle and broad cells in early passage hMSC, but identified a third, small, round cell (termed RS-1) that had faster doubling times, longer life-spans and greater differentiation ability than the other two cell types. Shur *et al* (2002) used cell-size, but not morphology, to distinguish two sub-populations, and also found that the smaller cells were the proliferating progenitors. Hung *et al* (2002) passed cells through a 3 μm sieve, to separate the non-hematopoietic cells into two homogenous populations. In contrast, these authors found that the smaller cells were polygonal and had little self-renewal capabilities, while the larger cells were spindle-shaped, proliferated extensively and were multipotent. It is now widely accepted that hMSC isolated by traditional methods are heterogenous. Sekiya *et al* (2002) have reported culture conditions that select for the smaller, more primitive RS-1 cells observed in Colter's work. No characterization has been

able to distinguish sub-populations phenotypically, and aside from maintaining carefully controlled culture conditions, little else is done to further purify hMSC populations before performing *in vitro* or *in vivo* assays.

2.1.2.2 Surface Marker Profiles

Despite nearly 30 years of research, and considerable efforts to elucidate their biological properties, mesenchymal stem cells (MSC) isolated from various animal species remain enigmatic with respect to their phenotypic and functional characterization. Haynesworth et al (1992a) made the first attempt to characterize the hMSC phenotype based on their surface protein expression and their cytokine profile. These researchers were unable to discern a unique marker that conclusively distinguished hMSC from any other somatic cell types. Subsequent efforts have resulted in similar difficulty in identifying a characteristic marker exclusive to hMSC and in order to compensate for this, an extensive list of positive and negative markers are used (Pittenger *et al*, 1999; Conget and Minguell, 1999; Majumdar *et al*, 2003). However, there has not been consistency between different research groups with respect to the surface markers or cytokines expressed by the cells. This may be due in part to the variations in culture conditions, serum lots, or isolation methods, which affect the expression of some surface proteins, or may select for a phenotypically distinct population (see Table 2.1). Recently, Schieker *et al* (2004) described a spectral image analysis method where, instead of using only surface markers, they used simultaneous expression of collagen I, collagen IV, fibronectin and CD44, detected by immunofluorescence, as a criterion for identifying hMSC in culture. These proteins are not unique to hMSC, but are supposedly co-expressed only by early progenitors. However, the method targets hMSC that have been seeded and cultured on glass slides and detects proteins that are usually secreted into the extra-cellular spaces rather than confined in the cytoplasm. Hence, it is difficult to distinguish the source of secretion, and the applicability or value of this technique for identifying hMSC is yet to be validated.

The inability to use surface markers to conclusively define the hMSC phenotype has led to a need for different methods of identification. Researchers have relied on 'functional characterization', where the isolation procedure and the cells' ability to display multi-lineage differentiation properties are used to define the cells as hMSC. This has given rise to other

problems. Some groups have reported that cells with greater plasticity are co-extracted along with hMSC (Jiang *et al*, 2001; Reyes *et al*, 2001). These have been termed 'mesodermal progenitor cells (MPC)' or 'multipotent adult progenitor cells (MAPC)' to distinguish them from the original MSC, but this has been done without demonstrating definitive distinctions in markers, or proving that they are not artifacts of the *in vitro* culture system (Reyes & Verfaillie, 2001). Furthermore, hMSC extracted from adipose tissue or umbilical cord blood exhibit similar multi-lineage differentiation, but different surface-marker combinations (Gronthos *et al*, 2001). Hence, this technique, while indispensable in other applications, does not provide conclusive evidence for isolating or identifying an hMSC phenotype, and thus its usefulness in this case may be severely limited.

2.1.2.3 Growth Characteristics

HMSC can be extensively sub-cultured *in vitro*. HMSC can expand over a billion-fold in culture before undergoing senescence (Pittenger *et al*, 1999). They were shown to have a doubling time of 33h (Bonnet, 2003), but their proliferation rates are actually a function of time in culture, and several other culture parameters. Sekiya *et al*, (2002a) showed that proliferation potential is dependent on the initial cell seeding density. Various seeding densities were used ranging from 10 cells/cm^2 to 1000 cells/cm^2. Cells plated at the lower densities showed significantly increased doubling rates and underwent a 500-fold increase after 12 days in culture, as opposed to a 30-fold increase for cells plated at the highest density. Additionally, the cells plated at 10 cells/cm^2 retained a higher colony forming unit (CFU) efficiency after expansion indicating that there was a higher proportion of progenitor cells in these samples. These cells, like those in Bruder's culture, exhibited a progressive slowing in growth rates with culture time, accompanied by obvious morphological changes. However, hMSC maintain their potential to differentiate into osteoblasts after becoming senescent (Bruder *et al*, 1997), but lose their potential to differentiate into cells of other lineages (Sekiya *et al*, 2002a). Interestingly, HSC cultured in suspension cultures also proliferate quickly at the ultimate expense of their self-renewal abilities. It has been suggested for HSC, that maintaining these cells in a quiescent state via a p21 mediated mechanism (Kadareit *et al*, 2002) helps to maintain their undifferentiated phenotype in culture (Cheng *et al*, 2000). It is not yet known whether similar mechanisms exist for hMSC although a role for p21 in cell-survival has been reported (van der Bos *et al*, 1998).

In contrast to hMSC, the MAPC (Jiang *et al*, 2002) and marrow-isolated adult multilineage inducible (MIAMI) cells (D'Ippolito *et al*, 2004) do not exhibit any signs of senescence, regardless of the duration of *in vitro* culture. In fact, these cells express similarities with embryonic stem cells (ESC), by maintaining high telemorase activity. HMSC reportedly sustain normal telomerase activity during expansion (Pittenger *et al*, 1999), but immortalizing hMSC by overexpressing the telomerase reverse transcriptase gene allows indefinite *in vitro* life-spans (Simonsen *et al*, 2002) and maintains their multi-lineage differentiation capabilities (Abdallah *et al*, 2005).

2.12.4 Multipotent or Pluripotent Differentiation Characteristics

An integral characteristic of MSC is that they can form osteoblasts (Bruder *et al*, 1997; Jaiswal *et al*, 1997), chondrocytes (Mackay *et al*, 1998) and adipocytes (Pittenger *et al*, 1999) in culture when provided the appropriate biochemical cues. It has been further demonstrated that MSC can be directed along several other lineage pathways including skeletal (Ferrari *et al*, 1998) and cardiac (Pittenger and Martin, 2004) muscle, hepatocytes (Petersen *et al*, 1999) and neural cells (Woodbury *et al*, 2000). Furthermore, Dennis and Charbord (2002) reported that hMSC in culture progress naturally along a vascular smooth muscle lineage. The unexpected plasticity has fueled heated debates about somatic stem cell potential (Morrison, 2001). Reports that stem cells spontaneous fuse with fully differentiated cells to give the impression of trans-differentiation (Terada *et al*, 2002; Ying *et al*, 2002) increased skepticism about the limitations of existing experimental methodologies and conclusions of hyper-plasticity of stem cells (Hawley and Sobieski, 2002; Holden and Vogel, 2002). However, subsequent experiments have ruled out the possibility that MAPC plasticity is due to spontaneous fusion (Jiang *et al*, 2002), indicating that it may be possible for somatic cells to exhibit pluripotency. The fact that some tissues are still unable to regenerate begs the question of what are the *in vivo* functions of such cells.

Undifferentiated hMSC simultaneously express basal levels of genes characteristic of several differentiated species (Tremain *et al*, 2001). It has been suggested that the efficiency of hMSC differentiation is possible because of this constitutive expression, and that environmental conditions merely signal the cell to selectively upregulate transcription of a particular family of

genes (Zipori, 2004). While this has not yet been proven, differentiation studies highlight the importance of environmental factors on stem cell fate. Pittenger *et al* (1999) reported that differentiation of mesenchymal stem cells was affected by basal nutrients, cell density, spatial organization, mechanical forces, growth factors and cytokines. In culture, various hormones and chemicals are used to direct hMSC differentiation along different lineages. These are summarized in Table 2.2 Biochemical cues alone may not be sufficient for differentiation. HMSC differentiation into chondrocytes is not effective unless the cells are cultured in a cell pellet (Mackay *et al*, 1997), and adipogenic differentiation requires the two-dimensional cell layer to be fully confluent (Pittenger *et al*, 1999).

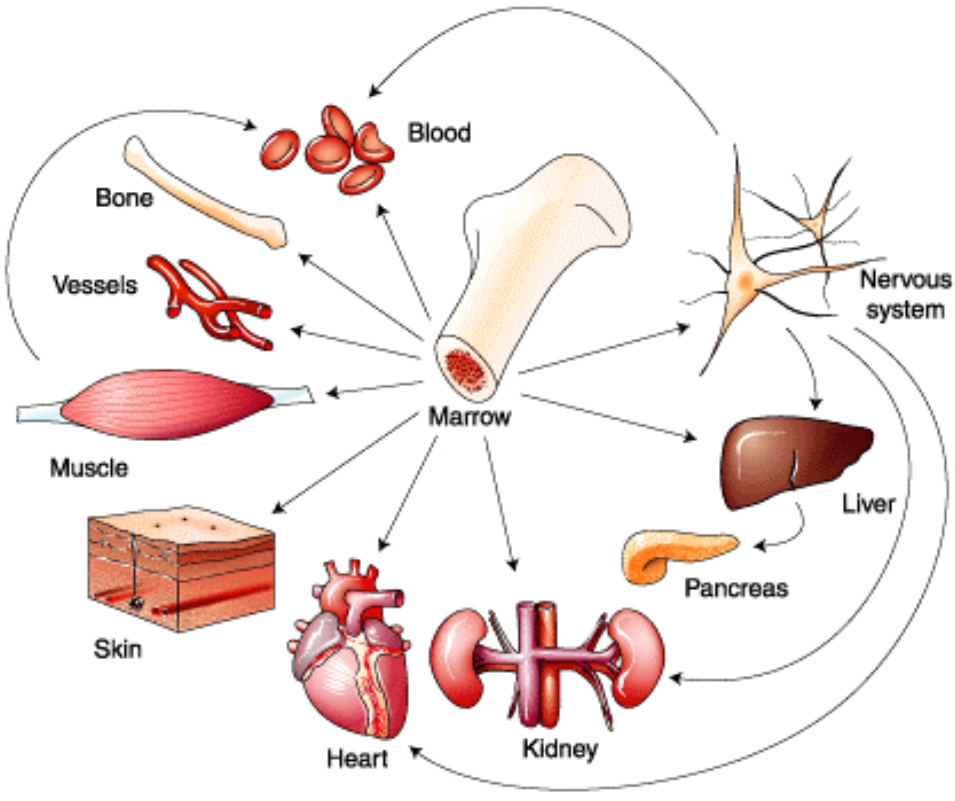


Figure 2.2 Plasticity of hMSC (Holden and Vogel, 2002)

2.1.3 *In Vivo* Characteristics of hMSC

2.1.3.1 Role of hMSC

In the bone marrow, hMSC occur at very low incidence (~ 0.001%) and typically reside in the G_0 phase of the cell-cycle (Kuo and Tuan, 2003). Although, hMSC demonstrate significant ability to differentiate in culture, little is known about their role in tissue maintenance or regeneration. Hematopoietic and epithelial stem cells are able to replenish their tissue compartments continuously, but an analogous role for hMSC has not been established. Despite the efficiency of hMSC differentiation into chondrocytes when induced, cartilage has limited ability to regenerate following injury. Similarly, both heart and brain tissues demonstrate no natural regeneration capacity even though hMSC can differentiate into cardiomyocytes and neurons (Javazon *et al*, 2004). A recent study showed that Sca-1/Ly-6A null mice with defective mesenchymal progenitors developed osteoporosis, implicating a possible role for MSC in maintaining bone tissue (Bonyadi *et al*, 2003).

2.1.3.2 Niche Concept

Stem cells were originally thought to be unique to continually renewing cell populations within the body, such as blood and epithelia. Most of the early studies on stem cells' *in vivo* characteristics were carried out using epithelial stem cells, where, it was found that a spatial relationship existed between the stem cells and their progeny (Hall and Watt, 1989; Jones *et al*, 1999). For example, epithelial stem cells residing within intestinal crypts, migrate toward the lumen as they differentiate into specialized cells that line the intestines (Potten and Loeffler, 1990). As stem cells remain within the crypts until they are ready to differentiate, it suggests that the crypt microenvironment provides important cues that maintain the cells in their primitive state. This gave rise to the concept of a stem cell '*niche*' (Watt and Hogan, 2000), or the idea that the compartment that the stem cells occupied was privileged. The niche integrates the effects of (i) secreted factors, (ii) interactions between neighboring cells and (iii) interactions between the cells and the extracellular matrix (ECM) via integrins. The molecular components of the niche have not been fully elucidated for various stem cell types, but it is believed that the stem cells' progression towards terminal differentiation is concomitant with their physical migration out of that niche, and has been well-characterized for epidermal and neural stem cells (Jensen *et al*, 1999; Gage, 2000). Stem cell migration and terminal differentiation take place

along a series of intermediate, transit amplifying steps, where progenitor cells with limited self-renewal and differentiation capacity are generated, so that several end-stage progeny are derived from a single stem cell. It is not fully clear what signals stimulate stem cells to emerge from their niche, or what controls their choice of lineage differentiation. Several signaling pathways are thought to be important, including NF-κB, Wnt and Sonic hedgehog. In addition to utilizing these molecular pathways, the expression of integrin β1 regulate differentiation in epidermal stem cells (Watt, 2001).

The stem cell niche concept is universal to all types of stem cells. Caplan (1991) reported a spatial distinction, with respect to the vasculature, between osteocytes, transit-amplifying cells and MSC. HSC and their progeny are also thought to be oriented by the vasculature, suggesting that oxygen, and possibly nutrient gradients, which decrease with increasing distance from the bone-marrow sinuses, are an important component of the HSC niche. MSC are also critical components of the HSC niche, secreting the necessary growth factors, and providing the physical interaction that maintains hematopoietic progenitors. Recent studies suggest that HSC may play a reciprocal role in facilitating MSC maintenance (Baksh *et al*, 2003). Hence, these two stem cell compartments may be very similar *in vivo*. Other work demonstrating that hypoxia increases the number of colonies generated by primary MSC indicates that oxygen may also be important in determining MSC fate (Lennon *et al*, 2001; D'Ippolito *et al*, 2004).

2.1.4 Clinical Potential of hMSC

2.1.4.1 Immune Characteristics and Application to GvHD

Aside from their proliferation and differentiation characteristics, hMSC are immune privileged; another trait that renders them suitable for therapeutic applications. Although hMSC express MHC I, and can be induced to express MHC II, they do not express co-stimulatory surface antigens that activate T-cell receptors. Also, hMSC are not passively non-immunogenic, but suppress T-cell function in co-culture (Aggarwal and Pittenger, 2004), inhibit leukocyte proliferation *in vivo*, and prolong the patency of skin grafts (Bartholomew *et al*, 2002). It is still debated whether the mechanistic basis of the immuno-suppression is physical (requires cell-cell contact) or chemical (cytokine secretion), but Aggarwal and Pittenger (2004) showed that co-

culturing MSC with T-cells, Natural Killer Cells and dendrytic cells changed the regulation of cytokine secretion by these cells and resulted in more tolerant cellular phenotypes.

Clinically, these attributes of hMSC have landed them a role in the treatment of graft versus host disease (GvHD); a debilitating disease with high mortality rates. In one clinical trial, hMSC from a third-party allogeneic donor was able to rescue a patient with stage IV GvHD, who had resisted all other available treatment methods (Le Blanc *et al*, 2004). Other clinical trials are currently in progress, but hMSC has been established as a very promising candidate, with no observed side-effects, for treating GvHD.

2.1.4.2 Myocardial Regeneration

The inability of cardiomyocytes to regenerate following myocardial infarction (MI), results in scar-tissue formation and leads to progressive heart failure. MSC derived from the heart or bone marrow generate functional cardiomyocytes in human and animal models of MI (Orlic *et al*, 2001a,b; Deb *et al*, 2003; Toma *et al*, 2002), and therefore show potential for use of hMSC in treatments for victims of coronary diseases (Rosenthal and Tsao, 2001). Clinical trials have confirmed that hMSC are able to successfully regenerate tissues in humans who suffered myocardial infarcts (Stamm *et al*, 2003, 2004; Menasché, 2004). In these trials, hMSC are injected directly into the infarct site and are able to engraft into the host tissue. While the results from these studies have been very promising, there is a dearth of knowledge about the mechanisms by which cells become engrafted into the host tissue, or how they are able to affect tissue regeneration.

2.1.4.3 Orthopedic Applications

HMSC have been used extensively in orthopedic applications to repair bone (Muschler *et al*, 2003; Quarto *et al*, 2001), cartilage (Ponticiello *et al*, 2000, Solchaga *et al*, 2002) and even tendon (Young *et al*, 1998; Awad *et al*, 2003) defects in animal models. Several researchers have cultured mammalian MSC in 3D scaffolds to enhance their osteogenic (Mizuno *et al*, 1997; Mueller and Glowacki, 2001; Yamada *et al*, 2003), and chondrogenic (Kavalkovich *et al*, 2002; Aung *et al*, 2002; Martin *et al*, 2001; Worster *et al*, 2001) differentiation capabilities. These scaffolds not only aid the differentiation process, but improve engraftment into host tissue when

surgically implanted. Results from animal studies showed that MSC-seeded scaffolds facilitated significant improvement in bone-tissue regeneration following treatment for critical-sized defects (Bruder *et al*, 1998a,b). Culturing MSC in collagen scaffolds prior to tendon insertion results in significant improvement in tendon repair. A novel non-invasive technique is being developed, which incorporates a gel, seeded with MSC, which can be directly injected into damaged cartilage before providing a cue that polymerizes the gel in-situ (Elisseeff, 2004).

2.1.4.4 Gene Therapy

HMSC use in gene-therapies is also promising owing to their rapid proliferation and amenability to genetic engineering (Prockop *et al*, 2003). HMSC huge expansion potential means that a group of cells transfected with a corrected gene can, within a relatively short time-span, produce sufficient cells with the functional gene that could be transplanted to repair a particular defect. The severity of bone defects such as osteogenesis imperfecta have been greatly mitigated by infusing allogeneic hMSC with a corrected gene into the circulatory system (Horwitz *et al*, 2002). HMSC in these experiments were able to home to and engraft in the bone and marrow stroma, and resulted in significant improvements in growth and bone formation in recipients. The potential of MSC for treating Duchenne's muscular dystrophy (DMD) was also indicated in dystrophic mice (Bittner *et al*, 1999; Gussoni *et al*, 1999). In this case, mice received bone marrow transplants, but MSC migrated and engrafted into the muscle tissue of dystrophic mice only. It has been found in a patient who received a bone-marrow transplant that bone marrow cells can home to and engraft in muscle tissue and persisted for years (Gussoni *et al*, 2002). This serendipitous finding provides further hope for using hMSC to treat DMD patients.

2.1.4.5 The Future of hMSC Therapy

In addition to these general areas, hMSC have been identified as possible therapeutic agents for several other pathologies. HMSC have been earmarked for trials in patients undergoing chemotherapy for breast cancer and bone marrow transplants since these cells enhance HSC engraftment and stimulate quicker recovery (Deans and Moseley, 2000). In spite of the existence of neural stem cells, hMSC have been chosen as possible candidates for neuronal repair (Mezey *et al*, 2003). HMSC infused into the brain of albino rats engrafted into the brain and migrated along pathways typical of neural stem cells (Azizi *et al*, 1998). The limitation of these methods

is that the cues required to direct differentiation *in vivo* are complex and are as yet very poorly understood. Current methods rely on the influence of local signals to direct hMSC differentiation after they migrate to the site of injury (Barry and Murphy, 2004). The ability to direct the *in vivo* differentiation of hMSC would contribute significantly to improving treatment methods. One of the ultimate goals also remains not only manipulating the *in vivo* differentiation pathway of MSC but also using them to generate complex composite tissues with more than one cell type (Caplan and Bruder, 2001).

2.2 THREE-DIMENSIONAL (3D) TISSUE-DEVELOPMENT

2.2.1 Cell Biology in Three Dimensions

Understanding the nature of cell-cell and cell-matrix interactions is critical for providing insight into myriad cellular processes and how these impact upon tissue functions. Conventional cell-culture methods investigate cellular characteristics on 2D substrates. While the benefits and gains in knowledge that have been achieved using this simple system cannot be overstated, it has been recognized that they result in anomalous cellular morphology and physiology (Geiger, 2001). Thus, their future role in the laboratory is limited. Recent studies have demonstrated that cells undergo different tissue development processes when grown in 3D scaffolds compared to conventional 2D cell-culture systems (Cukierman *et al* 2001). These authors showed that different adhesion complexes formed when cells were cultured on 3D *in vivo*-like, rather than 2D substrates, which affected the regulation of tyrosine phosphorylation and signaling processes. Similar research has demonstrated that the spatial organization of cells in 3D, their interactions with each other, and the architecture of the tissue all influence cell-fate decisions and apoptotic pathways (Boudreau *et al*, 1996; Santini *et al*, 2000; Zahir and Weaver, 2004). The ability to study cellular characteristics in 3D substrates has led to significant discoveries about cancer cell behavior (Weaver *et al*, 1997) and migration mechanisms (Wolf *et al*, 2003), as well as redefined the prior perception of the ECM as a static structural component of tissues (Boudreau and Bissell, 1998; Behonick and Werb, 2003).

Table 2.1 Surface Characterization of hMSC and hMSC-like Cells

Surface Antigen	MSCs	MAPCs	RS-1	PLAs	APCs
CD9					+
CD10		−	−		+
CD11a,b	−		−		−
CD13	+	+		+	+
CD14	−		−	−	−
CD18 integrin β2	−				−
CD29	+			+	+
CD31 PECAM	−	−	+/−	−	
CD34	−	−	−	−	+
CD44	+		+	+	+
CD45	−*	−	−	−	−
CD49b integrin α2	+	+			
CD49d integrin α4	−			+	+
CD49e integrin α5	+		+		+
CD50 ICAM3	−	−			−
CD54 ICAM1	+				+
CD56 NCAM				−	−
CD62E E-selectin	−	−		−	−
CD71 transferrin rec	+		+	+	
CD73 SH-3	+			+	
CD90 Thy-1	+	+	+/−	+	
CD105 endoglin, SH-2	+			+	+
CD106 VCAM	+	−		−	+
CD117	−	−			
CD133	−	(+)	−	−	
CD166 ALCAM	+				+
Others					
β2 microglobulin	+	+			
Nestin	+			+	
p75	+			+	
HLA ABC	+	−	+/−		+
HLA DR	− induc	−	−		−
SSEA-4	+	+			
TRK (A, B, C)	+		+		
Differentiation in vitro					
Osteo	+	+	+	+	+
Adipo	+	+	+	+	+
Chondro	+	+	+	+	
Neural	(+)			(+)	
Stromal	+	+			
Myoblast Sk	(+)	+		+	
Endotheliai	(+)	+			

Taken from Pittenger and Martin, 2004

Table 2.2 Differentiation Stimuli: Cellular and Molecular Markers

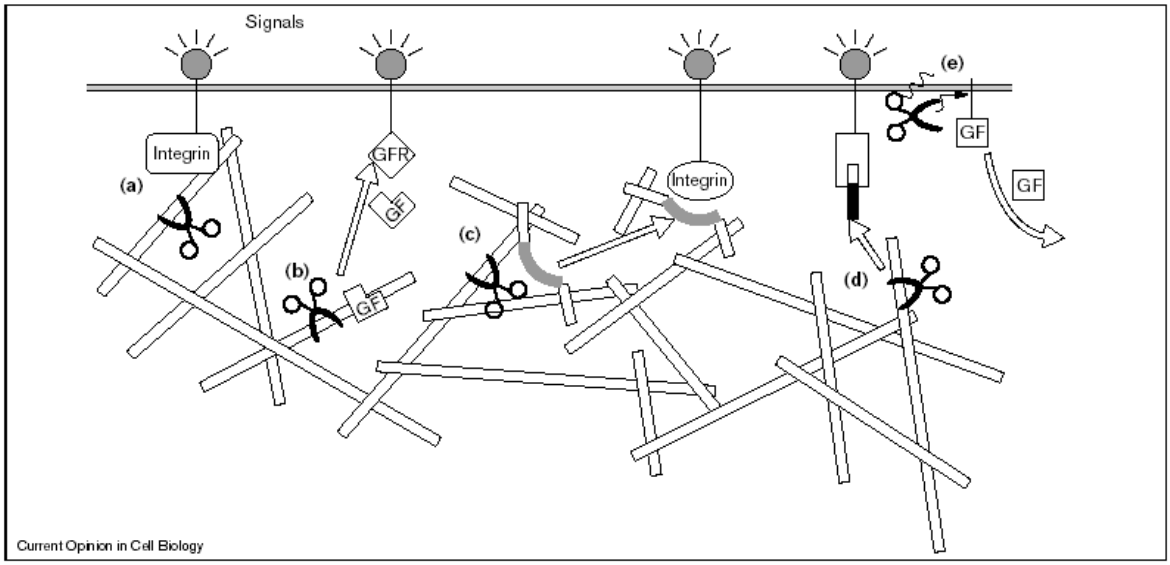
		Terminal phenotype identification markers	
Differentiation to:	Stimuli	Molecular	Cellular
Adipocytes	Dexamethasone + isobutylmethylxanthine Dexamethasone + isobutilmethylxanthine + indomethacin + insulin Dexamethasone + insulin	PPAR 2 C/EBPß aP2 Adipsin Leptin Lipoprotein lipase	Cytoplasmic lipid droplet accumulation
Chondrocytes	TGFß3 + ascorbic acid TGFß1 + ascorbic acid	Cbfa-1 Collagen types II and IX Aggrecan	Matrix enriched in proteoglycans and collagen types II and IX
Osteoblasts	Dexamethasone + ß-glycerophosphate + ascorbic acid	Cbfa-1 Bone/liver/kidney alkaline phosphatase Bone sialoprotein Osteopontin Osteocalcin Collagen type I	Mineralized matrix formation
Tenocytes	BMP-12	Collagen type II Proteoglycans	Improved biomechanical properties of implanted tendon
Hematopoietic supporting stroma	Hydrocortisone + horse serum Hemopoietic stem cell	n.d.	Maintain and support hematopoietic differentiation of $CD34^+$ cells Support osteoclastogenesis Support megakaryocytopoiesis and thrombocytopoiesis
Skeletal muscle cells	5-Azacytidine	MyoD Myf 5 and 6 MEF-2 Myogenin MRF4 Myosin	Multinucleated contractile cells
		Metavinculin Calponin h-Caldesmon Later smooth muscle actin	
Cardiac muscle cells	bFGF	GATA 4 and 6 Cardiac troponin I and C Sarcomeric-actin Slow twitch myosin ANP	n.d.
Astrocytes	DMSO + dexamethasone	Glial fibrillary acidic protein Intermediate filament	Integration into neonatal brain
Oligodendrocytes Neurons	PDGF + EGF + linoleic acid	Galactocerebroside Neurofilament Tubulin Blll Synaptophysin	

Taken from Minguell et al, 2001

2.2.2 Extracellular Matrix (ECM)

The ECM plays a central role in cellular organization in space, and bi-directional, signaling interactions between cells and the ECM are crucial for directing cell-fate and regulating gene transcription. The ECM is composed of various types of collagens, elastic fibers, glycosaminoglycans (GAGs), proteoglycans (PGs) and adhesive glycoproteins, such as fibronectin, laminin and vitronectin, all secreted by cells. It is the composition of the ECM, as well as its physical properties, which characterizes the various tissues of the body (Rosso *et al*, 2004). The ECM plays a significant role in sequestering and concentrating growth factors, setting up molecular gradients, and modulating the input to various signaling pathways in the cell (Figure 2.2). Upon integrating input signals, cells are able to secrete, degrade or re-organize ECM molecules accordingly (Streuli, 1999). This reciprocal flow of information is essential during development of multi-cellular organisms, where cells need to be directed to proliferate, migrate, differentiate or undergo apoptosis. It is also critical for maintenance of proper physiological function of cells, tissues and organs (Roskelley and Bissell, 1995).

Signaling between the cell and its environment is mediated by cell adhesion-complexes. These are comprised of transmembrane protein adhesion receptors (mainly integrins), structural proteins and adaptor proteins. Structural proteins anchor the adhesion machinery to the cytoskeleton, and adaptor proteins, are able to mediate signal transduction processes (Yamada and Geiger, 1997). There is significant diversity in the co-localization of molecular components that form the adhesion complexes. This is influenced by biochemical, physical and mechanical characteristics of the ECM or substrate, as well as other culture parameters (Zamir *et al*, 1999; Katz *et al*, 2000). Changes in composition of binding proteins confer specificity of the binding characteristics, which in turn affect morphology, migration, gene expression, proliferation and differentiation of cells (Yamada and Geiger, 1997; Zamir and Geiger, 2001b). Three classes of adhesion complexes have been identified, and these are discussed in the next section.



Some ways in which ECM remodelling can affect cellular differentiation. (a) Remodelled ECM directly alters cell–ECM interactions, affecting integrin signalling and integrin-mediated crosstalk with growth factors. (b) ECM remodelling releases bound growth factors (GF). (c) Exposure of cryptic sites (grey bands) within the ECM alters integrin signalling. (d) ECM remodelling releases bioactive ECM fragments (black bar). (e) Surface-bound ECM remodelling enzymes shed growth factor ectodomains. GFR, growth factor receptor.

Figure 2.3 Effects of ECM Remodeling (Streuli, 1999)

2.2.3 Cell Adhesion

Cell adhesion is essential for maintaining the existence of higher organisms, providing the link for cells to communicate with each other and their environment, thereby giving rise to tissues and organs. Extensive research has been done to characterize the components of cell-adhesion structures, and to elucidate the mechanisms of the molecular interactions that occur. Cell-cell binding structures facilitate specific tissue functions, such as conducting electrical impulses in the heart or brain, or maintaining the integrity of semi-permeable barriers in the skin or gut. Hence, they are very specific. There are also a variety of cell-ECM adhesion structures differing in size, stability, accessibility, function and molecular composition. Generally, protein receptors at these points on the cell's surface co-align elements of the extracellular matrix to those of the cell's cytoskeleton. The most intensively studied and best-characterized structures are the focal adhesions or focal contacts, but other adhesion complexes, including fibrillar adhesions, as well as several transient structures exist (Adams, 2001). A brief synopsis of integrins as well as focal and fibrillar adhesions follows, which summarizes the importance of these structures, as well as how they are differentially affected for cells cultured on 2D and 3D surfaces.

2.2.3.1 Integrins

Integrins were identified as the cell-surface adhesion receptors, which mediate cellular attachment to the ECM and, at times, to other cells. They are heterodimeric, transmembrane proteins comprised of non-covalently bound α and β subunits. Several variant α and β chains exist and different combinations of these confer binding specificity to particular ECM protein substrates (Hynes, 1992). Integrins must be anchored to the cytoskeleton to induce the conformational changes, which allow them to bind tightly to the ECM. This requirement forms the basis of an inside-out signaling mechanism, which regulates the affinity of integrins for the substrate and determines how tightly the integrins bind to the substrate (Horwitz, 1997). Upon binding the ECM ligand, changes occur in the cytoplasmic moiety, which may induce cytoskeletal re-arrangement (Rosso *et al*, 2004). Integrins can then modulate signal-transduction pathways, which influence cell survival, proliferation and gene expression, thus completing the bi-directional flow of information into and out of the cell (Hynes, 1999). Integrins' extensive influence over cellular processes makes them central in development and maintaining normal

physiology. Consequently, dysfunctional integrins may be a cause of certain diseases, rendering them as possible targets for therapeutic applications.

2.2.3.2 Focal Adhesions

The aggregation of bound integrins gives rise to focal adhesion complexes (Horwitz, 1997). FAs measure several square microns and keep the cell membrane separated from the substrate by distances of only 10 – 15 nm (Adams, 2001; Zamir and Geiger, 2001a). They exhibit considerable molecular complexity; being comprised of over 50 proteins (Zamir and Geiger, 2001b) many of which may exist in more than one form due to post-translational modifications. The known components of FAs can be categorized into cytoskeletal proteins, kinases, modulators of small GTPases, phosphatases and 'other enzymes' (Zamir and Geiger, 2001a). FAs are closely associated with the actin cytoskeleton, which it structurally links to the ECM forming the basis of signaling complexes. FAs are not known to exist *in vivo* (Adams, 2001; Yamada and Geiger, 1997) suggesting that they may be an artifact of cell-culture systems. One explanation put forth for the formation of FAs in cell-culture was that the presence of serum in growth media simulates a wound environment to which cells respond by forming tight adhesions and contracting. The rigid substrate translates these contractile forces into an isometric tension stimulating cells to generate stress fibers and proliferate (Burridge and Chrzanowska-Wodnicka, 1996). But FAs have not been detected in *in vivo* wound environments, so this explanation may be ultimately incorrect. Cukierman and co-workers (2002) have suggested an alternative explanation for the presence of FAs that will be discusses in 2.2.3.4.

2.2.3.3 Fibrillar Adhesions

Zamir *et al*, (1999) were the first to show that the adhesive contacts between cells and the *in vitro* substrates were not homogenous and coined the term 'fibrillar adhesion'. Fibrillar adhesions are an elongated variation of classical FA's. They differ primarily in their molecular composition and lack the tyrosine specific phosphorylation. Integrins comprise the transmembrane portions of both focal and fibrillar adhesions but they combine with different adaptor proteins. Although, integrins bind specific ECM proteins ligands, for foreskin fibroblasts in culture, it was seen that the $\alpha_v\beta_3$ integrin and its ligand vitronectin are present in focal adhesions whereas fibrillar adhesions contain the $\alpha_5\beta_1$ heterodimer bound to fibronectin

(Zamir *et al*, 1999; Cukierman *et al*, 2001). Functionally, fibrillar adhesions' lack of phosphotyrosine is related to their unresponsiveness to actomyosin-driven contractility. Therefore chemically disrupting the contractile forces led to a disassembly of focal, but not fibrillar adhesions (Katz *et al*, 2000).

2.2.3.4 Effect of Three-Dimensional Cell-Culture on Cell Adhesion

The research conducted by Zamir *et al* (1999) demonstrated that cells could vary their manner of interaction with the environment by modulating the number of focal or fibrillar contacts. This confirmed that mammalian cells have an innate adaptability of to their surroundings, but of greater importance, it showed that cells cultured on flat plastic surfaces inherently behave differently from cells growing *in vivo*. Cukierman and coworkers (2001) utilized a similar methodology to examine the types of adhesions formed if cells were allowed to grow in a physiological 3D substrate compared to a variety of other types of matrices. Using cultured fibroblasts, they were able to discover a unique adhesion complex formed when the cells were cultured in the 3D matrix. The molecular composition of the matrix differed from both focal and fibrillar adhesions and there was a concomitant change in the signal transduction pathway: Whereas focal adhesion kinase (FAK) and paxillin are phosphorylated in FAs, only paxillin remained phosphorylated in what the authors termed '3D adhesions'. The 3D adhesions were the influenced by the matrix topography (2D vs. 3D), pliability, and molecular complexity. Subsequent analysis suggested that the focal and fibrillar adhesions may be part of a maturation process on the way to achieving 3D adhesions (Cukierman *et al*, 2002).

2.2.3.5 Cell Adhesion and Cell Shape

Cell phenotype and gene-expression characteristics are also affected by the cell-shape. Most somatic cells cultured *in vitro*, need to adhere to a solid substrate in order to proliferate. If they detach from the substrate, they adopt spherical morphologies and either become quiescent or undergo apoptosis. Folkmann and Moscona (1978) showed in their seminal paper that cell proliferation, quiescence or apoptosis was regulated by the shape of the cell. However, they did not eliminate the possibility that differences in cell-fate were due to diverse expression or activation of integrins on the surface of cells. The dynamic connection between integrins and the cytoskeleton infers that integrin binding to, or dissociation from the ECM, may induce changes

in cell-shape. Hence, it is difficult to decouple the effects of integrin signaling from that of cell-shape. A subsequent experiment was able to use micro-patterned surfaces to distinguish the effects of cell-shape from the amount, density or activity of integrins on the cell-surface, and proved that apoptosis was governed by cellular morphology (Chen *et al*, 1997). These experiments were performed on rigid 2D substrates. In normal tissues, the ECM is the substrate, and plays a significant role in regulating cell-shape by providing resistance to tensile forces generated by cells (Ingber, 2003a; b). Thus, the physical rigidity of the ECM not only affects the molecular composition of the adhesion (Katz *et al*, 2000; Cukierman *et al*, 2001), but also affects cell-shape, adding another dimension of control – and complexity – to gene regulation.

2.2.4 Engineering Three-Dimensional Tissue Constructs

2.2.4.1 Biomaterials – Synthetic Polymers

The earliest scaffolds used for tissue-engineering purposes had two key requirements: biocompatibility and anti-thrombogenicity. Hence, many synthetic materials were used, such as poly(ethylene oxide) (PEO), polyurethane, and poly(ethylene terephthalate) (PET) (Langer, 1995). These polymers were non-immunogenic and non-degradable when implanted into the body, since their degradation by-products typically invoked inflammatory responses. Subsequently, new biodegradable materials like poly(glycolic acid) (PGA) and poly(lactic acid) (PLA), as well as derivatives and hybrids of the two were developed and used in suturing and other applications. (Gilding and Reed, 1979). Current tissue-engineering perspectives require implant materials to be biocompatible, biodegradable, and preferably biomimetic, hence shifting research focus to natural polymers, which provide biochemical, in addition to structural cues. However, synthetic materials are still advantageous for several applications. They are more reproducible, it is easier to control their topography and mechanical properties, and they provide less complex systems when studying the effect of cell organization on tissue-development. Additionally, asymmetric scaffolds can be produced, which may be useful for guiding the *in vitro* development of complex tissue structures (Sharma and Elisseeff, 2004).

2.2.4.2 Biomaterials – Natural Scaffolds

Several natural polymers have been used to develop cellular constructs, which may then be implanted into defect sites within the body to repair or regenerate tissue. Among these are *in*

vivo-derived, acellular ECM extracts (Badylak *et al*, 1999), chitosan (Francis-Suh and Matthew, 2000), hyaluronan (Campoccia *et al*, 1998), collagen gels (Mizuno *et al*, 1997) and Matrigel (Qian and Saltzman, 2003). Naturally occurring biomaterials are considered 'biomimetic' since they provide signaling input to the cells that may improve their particular differentiated phenotype. However, the poor mechanical properties of the unprocessed materials generally make them unsuited to TE applications. Cross-linking agents have been used to improve mechanical properties of chitosan, hyaluronan and small intestine submucosa (SIS) (Francis-Suh and Matthew, 2000; Schmidt and Baier, 2000; Gamini *et al*, 2002), but these give rise to cytotoxicity concerns. SIS, which is derived from porcine donors, has good mechanical properties (similar to native tissue), and has been used for many clinical applications in animal models, but the composition and quality cannot be controlled. Its use in humans is complicated by concerns about immunological responses to xeno-derived tissues. Clinical trials using SIS to repair rotator-cuff injuries have resulted in inflammation and a subsequent study has reported that a commercially available SIS product contains remnants of porcine cells and DNA (Zheng *et al*, 2005).

2.2.4.3 Biomaterials – Nano-fibrous scaffolds

An emerging paradigm in tissue engineering states that nano-fibrous materials are required to provide a 'true' 3D scaffold for cells (Zhang, 2004). The ECM fibers that interact with cells *in vivo* are several orders of magnitude smaller than the cells. Hence, cells are usually embedded in a complex, nanoscale fibrous network. Many tissue-engineering scaffolds have average fiber diameters on the order of 30 μm, and the pore spaces span up to 200 μm. Thus, in these scaffolds, individual cells generally interact with one fiber, which is likely to be perceived as a curved, 2D substrate. Nano-fibrous scaffolds are fabricated by electrospinning synthetic polymers (Li *et al*, 2005), or by self-assembly of protein monomeric molecules (Zhang, 2003). There is no currently no literature describing the effects of self-assembled scaffolds on cell growth. However, Li *et al*, (2005) showed that nano-fibrous scaffolds of poly(ε-caprolactone) (PCL) supported hMSC growth and chondrogenic differentiation. They have the added advantages of increased mechanical strength, compared to cell pellet chondrogenesis models, and the scaffolds can be tailored to fit into defect site.

2.2.4.4 Cell-Spatial Organization

Cells seeded into the 3D scaffolds assume different spatial orientations, determined by the topography of the scaffold. Ma and coworkers (2000) looked specifically at the effects of spatial organization on cell growth and function. Using a PET scaffold, they showed that human trophoblast cells cultured on 2D surfaces grew much faster and exhibited different growth kinetics to those grown on 3D matrices. However, when the pore sizes of the scaffolds were varied, it impacted significantly on the average sizes of aggregates formed by the cells. This in turn affected their patterns of growth and differentiation. In general, greater accessible polymer surface area in the scaffolds resulted in smaller cell aggregates and faster the proliferation rates but decreased differentiation. Luo and Yang (2004) also reported that hybridoma cells grown in similar 3D PET matrices exhibited decreased growth, but enhanced survival and antibody production.

HMSC and their progeny comprise an asymmetric cell population that is spatially oriented with respect to each other *in vivo* by local environmental cues. Hence, the microenvironment and spatial organization of stem cells within 3D scaffolds may inherently influence their proliferation and differentiation characteristics, and consequently, the properties of the resulting tissue-construct. Using the 3D topography, it may be possible to re-create elements of the cell niche, leading to the improved *in vitro* expansion of hMSC. In addition to this however, understanding the development of the ECM structure, how it is guided by the topography of the 3D scaffolds, and how this ultimately influences cell-fate and tissue-function is important for developing functional hMSC tissue-engineered constructs.

2.2.5 Bioreactor Systems for 3D Tissue Development

2.2.5.1 Effect on Construct Uniformity

The different types of bioreactors used for tissue-engineering applications include, but are not limited to, stirred flasks (Freed *et al*, 1994), rotating-wall vessels (Freed *et al*, 1998), hollow-fiber (Petersen *et al*, 2002), and perfusion systems (Bancroft *et al*, 2002). These systems are represented schematically in Figure 2.3 and show the flow path relative to tissue-constructs. The primary consideration in developing 3D cellular constructs is the ability to obtain a uniform distribution of cells throughout the scaffold. Static seeding of cells into 3D matrices results in

spatial heterogeneity, which compromises the functionality of the constructs. Using spinner-flasks to seed chondrocytes unto PGA scaffolds resulted in higher seeding efficiencies, and improved cellular distribution (Vunjac-Novakovic *et al*, 1998). However, this method still yielded non-uniform patterning of cells. There was a higher concentration of cells at the periphery of scaffolds compared to the core. Perfusion or depth-filtration methods utilize multiple passes of the cell-suspension through the matrices and enhance the uniform dispersion, seeding efficiency, and viability of cells (Li *et al*, 2001; Wendt *et al*, 2003).

Construct uniformity is also affected by transfer limitations of oxygen and nutrients to the innermost cells at high cell and tissue densities. Adequate transfer of oxygen through the tissue-culture medium to the cells is a concern even in conventional 2D tissue-culture (Randers-Eichhorn *et al*, 1996), but becomes a critical consideration when cells adopt 3D organizational structures. Engineered tissue-constructs are typically on the order of millimeters, and the lack of vascularity leads to hypoxic inner-regions. If this occurs, cells at the core do not develop proper phenotypes, and may even undergo necrosis (Martin *et al*, 2003). Simple methods to provide convectional media flow mitigate some of these problems. Freed *et al* (1994) showed that chondrocyte/PGA constructs cultured under static conditions had lower cell numbers, a thinner superficial zone, and less GAG content than constructs cultured in a similar system that was placed on an orbital shaker. Various reactor systems employ convectional transfer methods to mitigate the effects caused by diffusion limitation.

2.2.5.2 Bioreactor Environment Improves Cellular Functionality

The mechanical environment sustained by perfusion bioreactors significantly improve the functionality of tissue constructs. After using a spinner flask to seed cells into PGA scaffolds, Freed and coworkers (1998) cultured the constructs in a rotating, hollow-cylinder bioreactor, and obtained an improved cartilage phenotype. This same group attributed the superior structural and functional properties of the cartilage constructs to the mechanical parameters inherent in the reactor system (Pei *et al*, 2002). Other groups have used the reactor system to enhance cellular expression of the osteoblast phenotype. Bancroft *et al* (2002) showed that the rate of fluid flow affected the ECM expression and mineralization of osteoblast stromal cells grown in titanium meshes. In addition, the calcium deposition, alkaline phosphatase (AP)

activity, and cellular osteopontin content were increased in perfusion cultures relative to the static-culture constructs. In subsequent work, the group used culture media of different viscosities, but maintained the same flow-rates, to investigate the effect of shear-stress on bone formation (Sikavitsas *et al*, 2003). This model allowed the mechanical effects to be resolved from biochemical transport of nutrients to the cells, thus proving that the physical forces exert considerable influence on the tissue development (ECM deposition and mineralization) properties. In fact, fluid-flow was shown to be sufficient to induce osteoblast differentiation, even in the absence of dexamethasone (Holtorf *et al*, 2005). Importantly, the distribution of the ECM in these reactor scaffolds is uniform, whereas it is concentrated at the periphery of static culture scaffolds (Sikavitsas *et al*, 2005).

2.2.5.3 Description of In-house Perfusion System

The perfusion bioreactors used for inducing osteogenesis (Bancroft *et al*, 2002; Sikavitsas *et al*, 2003, 2005; Holtorf *et al*, 2005) force culture media through the 3D scaffold as shown in Figure 2.3d. The reactor system used to perform in-house experiments is described elsewhere (Zhao and Ma, 2005) and shown schematically in Figure 2.4 and perfuses media both above and below the cellular constructs. In this system, the resistance to flow through the scaffolds is prohibitive, therefore nutrient and metabolite transfer takes place primarily by diffusion, and has been modeled in this way (Zhao *et al*, under review). Hence, hMSC grown in these constructs have access to much greater supplies of nutrients than static cultures, but do not experience significant shear-stress, particularly at the core of the constructs. The constant flow of media at both surfaces ensure that there is no accumulation of cellular material at the lower surface of the construct giving rise to uniform tissue-development after extended time in culture. Additional advantages of the reactor design are the ability to seed cells within the system, thereby eliminating the need for transfer after seeding, and reducing the possibilities for culture contamination. The modular design allows several constructs to be cultured simultaneously (up to 15 constructs have been cultured at one time), and chambers can be independently removed at different time points.

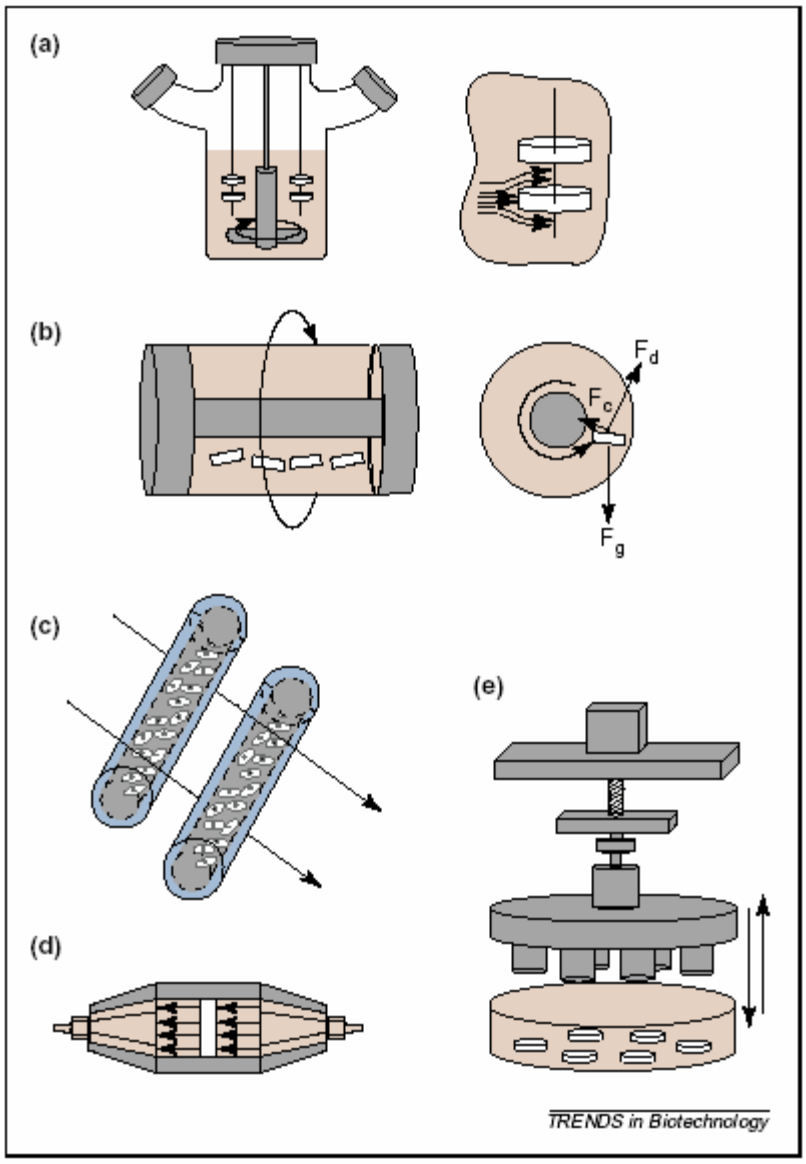


Figure 2.4 Representative Bioreactors used in Tissue Engineering Applications (Martin et al, 2003)

(a) Spinner-flask bioreactor. (b)Rotating-wall vessels (also known as microgravity bioreactors) balance the centrifugal (F_c), drag (F_d) and gravitational (F_g) forces, and provides a low shear-stress environment. (c) Hollow-fiber reactors. (d) Direct perfusion reactors allows interstitial flow through the cell-polymer constructs, enhancing nutrient transfer to construct core. (e) Biomechanical reactors, which can provide hydrodynamic forces.

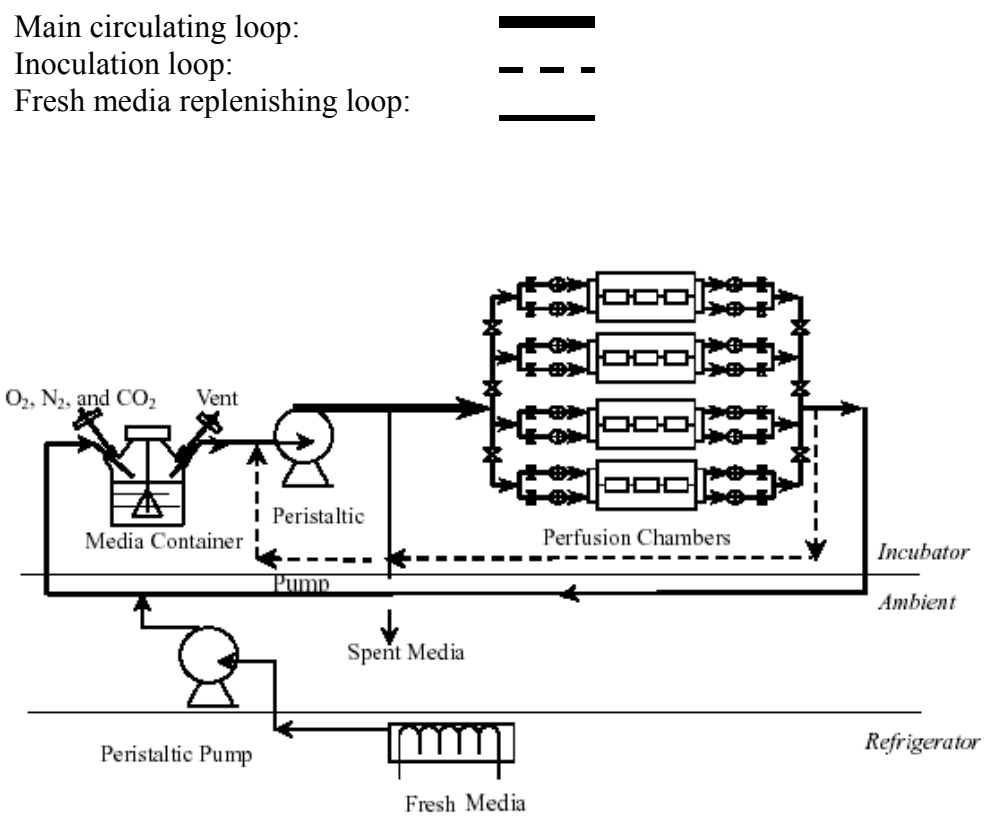


Figure 2.5 Schematic of Perfusion Bioreactor Used in Experiments (Zhao and Ma, 2005)

2.3 EFFECTS OF HYPOXIA

2.3.1 Role of oxygen in organ physiology

Oxygen's role as a metabolic and enzymatic substrate for higher organisms is well known. Mammals have developed cardiovascular, hematopoietic and respiratory systems as primary mechanisms to provide sufficient oxygen to all the cells of the body. In these cells, oxygen acts as an electron acceptor in the mitochondrial oxidative phosphorylation cascade, which is the primary mechanism for producing the energy required to carry out all cellular processes. These physiological systems are tightly regulated to ensure that oxygen levels remain within a narrow range of partial pressures: Too much oxygen results in the overproduction of reactive intermediate species, which are damaging to DNA and the cell membranes, and too little oxygen limits the cell's ability to produce the required levels of ATP for survival.

Hypoxia, (low levels of pericellular oxygen), results in a number of physiological responses. These include increased respiratory rates (Acker, 1989), increased red blood cell production via upregulation of erythropoietin (EPO) (Kantz, 1991), vasoconstriction of pulmonary vessels, vasodilation of vessels in other tissues and organs, and formation of entirely new blood vessels (Goldberg and Schneider, 1994). During hypoxia, cells change their metabolic pathway from oxidative respiration to glycolysis in order to conserve oxygen consumption and maintain ATP production levels. Hypoxia-induced upregulation of vascular endothelial growth factor (VEGF) (Ladoux and Frelin, 1993; Minchenko *et al*, 1994) suggests that VEGF plays a role in wound-healing, ischemia and tumor pathogenesis by stimulating the growth of endothelial cells and the formation of new blood vessels. Some adaptations to hypoxia aim to achieve a level of oxygen homeostasis within tissues and organs; others allow the tissues to adapt for a limited period to the prevailing low oxygen condition.

Extra-cellular oxygen tension also plays a major role during development of mammalian embryos. As fetuses grow, oxygen gradients develop, causing cells in various regions to experience hypoxic conditions. This not only stimulates the development of blood vessels via a VEGF-mediated response, but also acts as a signal to various cell types, stimulating them to proliferate, differentiate or undergo growth arrest or apoptosis (Iyer *et al*, 1998). In these experiments, an inability to respond to the hypoxic cue due to induced genetic defects resulted in

fetal mortality in rats. In embryology as well as pathophysiology therefore, oxygen's central role as a metabolic substrate may be secondary to its function as a signaling molecule, and the ability of cells to detect and respond to variations in the oxygen levels is critical to the survival and proper development of the organism. Hence, much effort has been expended to elucidate the mechanism(s) via which cells sense oxygen levels and transduce the signal to elicit a response.

2.3.2 Cellular O_2 Sensors

2.3.2.1 Heme Protein

For over a decade, researchers have speculated that a common oxygen sensing molecule exists to initiate a response to hypoxia. The major characteristic of the hypothetical oxygen sensor, apart from binding available oxygen molecules, is that it triggers a molecular, cellular and physiological response in the absence of oxygen. Several authors showed that the hypoxia-induced upregulation of EPO and other reporter genes share a common signaling mechanism (Goldberg and Schneider 1994; Gleadle *et al*, 1995a). This taken together with reports from other groups (Goldberg *et al*, 1988; Wang and Semenza, 1993; Ho and Bunn, 1996), suggested that the sensor is a heme protein. Research done to elucidate the oxygen-sensing mechanisms of unicellular organisms (reviewed in Bunn and Poyton, 1996) has shown that it is a heme protein that senses oxygen tension and initiates the signaling cascade in bacteria. However, there has not been a clear extrapolation of these results, via protein homology studies, to determine whether the same is true for mammals.

According to the heme hypothesis, the iron contained in the heme protein is locked into an 'oxy' form when it binds oxygen. In the absence of oxygen, the iron changes to the 'deoxy' conformation and triggers the hypoxic response. The evidence implicating a heme protein in mammals showed that replacing the iron in the heme moiety with a non-oxygen binding transition metal (cobalt) induced upregulation of EPO and VEGF, similar to when the cells were exposed to a hypoxic challenge (Goldberg *et al*, 1988; Goldberg and Schneider, 1994; Ho and Bunn, 1996). Similar results were found when iron-chelators were used to prevent the heme protein binding to oxygen (Wang and Semenza, 1993). Furthermore, studies showed that carbon monoxide (10% CO), which locked the heme protein in its 'oxy' conformation in the absence of oxygen, inhibited the induction of hypoxia response genes when the cells were subjected to 1%

oxygen tensions (Goldberg *et al*, 1988). These results provided strong evidence that the oxygen sensor was a heme protein. However, contradictory results created uncertainty about the universal nature of heme protein sensing. In a study done by Gleadle *et al* (1995b), they showed that diphenylene iodonium (DPI), inhibited the induction of VEGF, lactate dehydrogenase-A (LDH-A), glucose transporter-1 (GLUT-1) and EPO in response to hypoxia, but did not affect the induction of these genes when the heme iron was replaced by cobalt or was chelated to desferrioxamine (DFO). Since several hypoxia-induced reporter proteins were inhibited during hypoxia by DPI, it supported the idea that a common mechanism governed the induction of these different proteins. However, while the heme protein may have been involved in binding pericellular oxygen molecules, it did not appear to be the mediator of the cellular response since the induction of hypoxia reporter genes using cobalt and DFO was not inhibited by DPI. In fact the induction of reporter genes by cobalt and DPI may occur via a different signaling pathway to that of hypoxia or, as suggested by Gleadle and his coworkers, may occur downstream of the DPI block.

2.3.2.2 Oxidase Molecule

Since iodonium salts are inhibitors of flavoproteins, the influence of DPI on hypoxic gene regulation suggested that a flavoprotein may be involved in oxygen sensing. Flavoproteins reduce molecular oxygen to the $O_2^{-}\cdot$ radical via a redox cycling process (Cadenas, 1989). It is thought that the sensor is an oxidase molecule (similar to the NAD(P)H oxidase in macrophages) that converts molecular oxygen to reactive oxygen intermediates (ROIs), which then acts as signaling molecules (Acker, 1994a; b). Absorbance spectroscopy studies suggested that the sensor could be a b-type cytochrome, similar to the NAD(P)H oxidase (Görlach *et al*, 1993; Görlach *et al*, 1994). The protein was not inhibited by cyanide suggesting that it was not a part of the mitochondrial oxidative chain and was more likely located in the cell membrane. Experiments conducted by Fandrey *et al*, (1990) showed that inhibition of cytochrome P-450 led to a decrease in EPO expression while inducing cytochrome P-450 led to an increase in production of EPO. These results led to speculation that the reaction between cytochrome P-450 and cytochrome P-450 reductase caused molecular oxygen to be reduced to a superoxide. Although these results implicate an oxidase molecule along the signaling pathway, they run counter to the prediction of the model as do the results obtained by Gleadle *et al* (1995b) in using

DPI. A model involving the reaction of ROIs in the presence of oxygen indicates that ROIs would have an inhibitory effect on reporter gene expression, and the absence of ROIs would initiate gene upregulation. By inhibiting cytochrome P-450 or other flavoproteins with DPI, the amount of ROIs present in the cell should decrease and thus mimic hypoxia.

Fandrey and coworkers (1994) followed up prior experiments and showed that the effects of hypoxia on EPO is nullified by the addition of 1mM exogenous hydrogen peroxide (H_2O_2) to the growth media or by the addition of menadione or aminotriazole which increased endogenous H_2O_2 production. Substituting cobalt for iron in heme proteins also led to a decrease in intracellular hydrogen peroxide formation. Thus hydrogen peroxide levels or the amounts of other peroxide and superoxide molecules within the cell may be responsible for regulating reporter genes during hypoxic challenge.

2.3.2.3 Mitochondrial O_2 Sensor

Mitochondria, because of their central role in aerobic respiration, have also been considered as a logical site for oxygen sensing. However, because of the considerably low oxygen tensions that exist in the mitochondrion it was intuitively thought to be a suboptimal site for this purpose (Bunn and Poyton, 1996). Previously mentioned experiments indicated that the sensor resides in the plasma membrane of the cell and is uninhibited by cyanide, a powerful inhibitor of the mitochondrial respiratory chain (Görlach *et al*, 1993). However, the effects of DPI on inhibiting reporter gene expression during hypoxia could have been related to its effect on the mitochondria electron transport chain (Semenza, 1999). Rotenone and myxothiazol, other inhibitors of the mitochondrial chain also inhibited reporter gene expression during hypoxic stress (Chandel *et al*, 1998). Other experiments carried out by Chandel *et al* (1998) used Hep3B cells that had been depleted of mitochondrial DNA. These cells were not able to induce EPO, VEGF or glycolytic enzymes in response to hypoxia but they did induce these genes in response to $CoCl_2$ or DFO treatment. Subsequent research showed that by inducing a defect in the electron transport chain in mitochondria, there is a decrease in the HIF-1α's induction and DNA binding in response to a hypoxic challenge (Agani *et al*, 2000). These results suggest, not only that mitochondria are involved in oxygen sensing, but confirm that the signaling pathway for hypoxia differs from that activated when Co replaces Fe in heme proteins. It was also possible to re-evaluate the non-

inhibition of the hypoxic response by cyanide in the context of a mitochondrial sensor if it is taken into account that cyanide affects a different complex in the mitochondrial electron chain from the ones involved in peroxide formation.

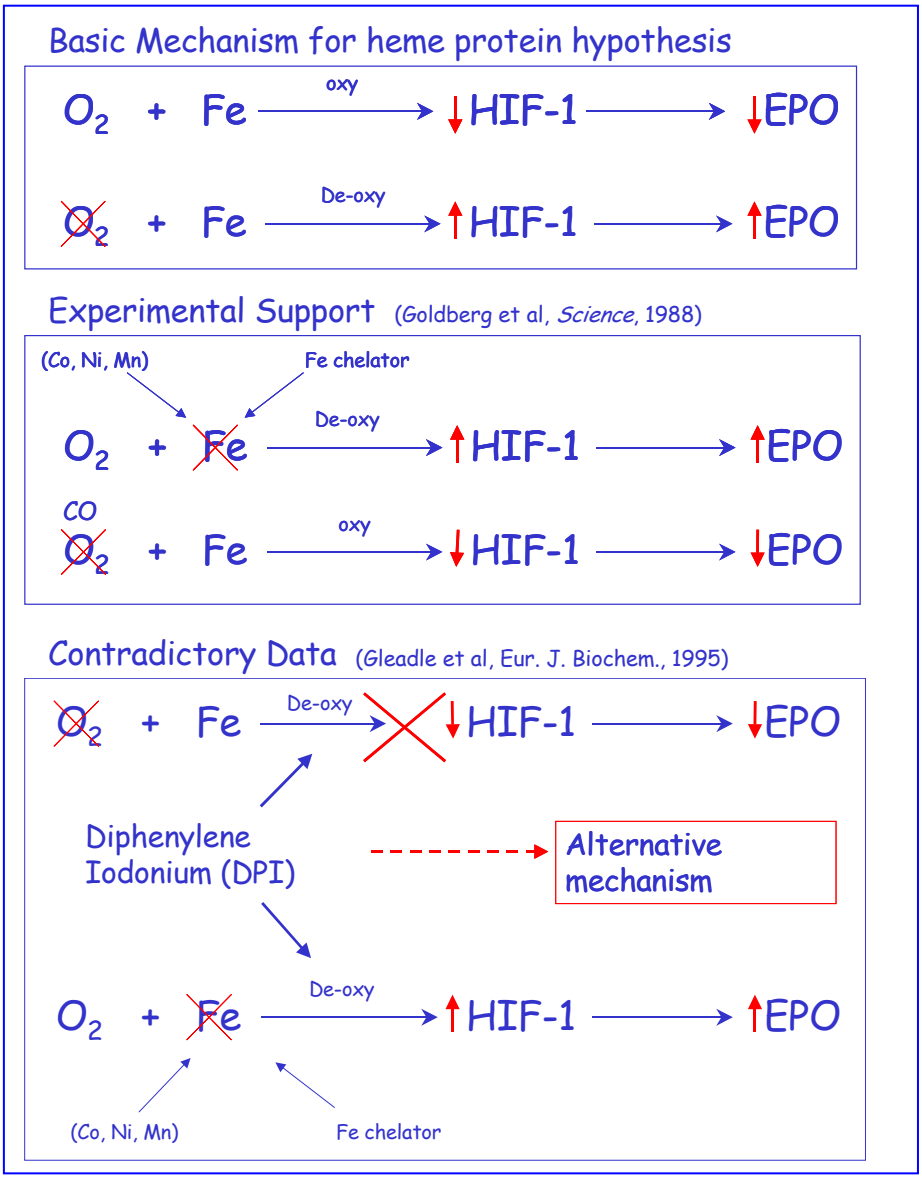


Figure 2.6a Schematic of Heme Protein Oxygen Sensor Mechanism showing erythropoietin (EPO) as the hypothetical universal reporter gene for hypoxia. Effects of hypoxic simulation is mediated by hypoxia-inducible factor-1 (HIF-1). Experimental results supporting and contradicting the mechanism are reported

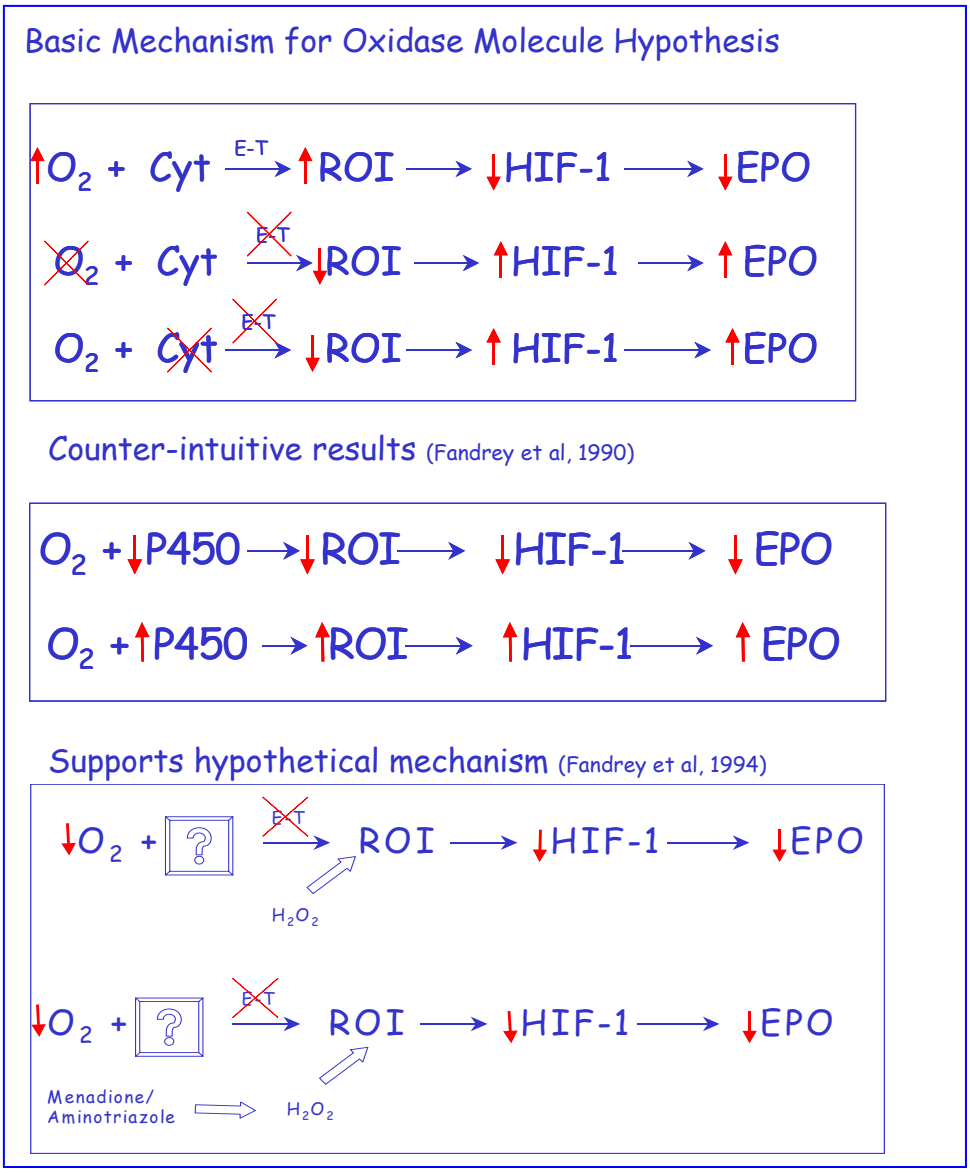


Figure 2.6b Schematic of Oxidase Molecule Oxygen Sensor Mechanism showing a cytochrome b (Cyt) sensor. Results of experiments investigating p450 as well as intermediate oxidizing species are reported

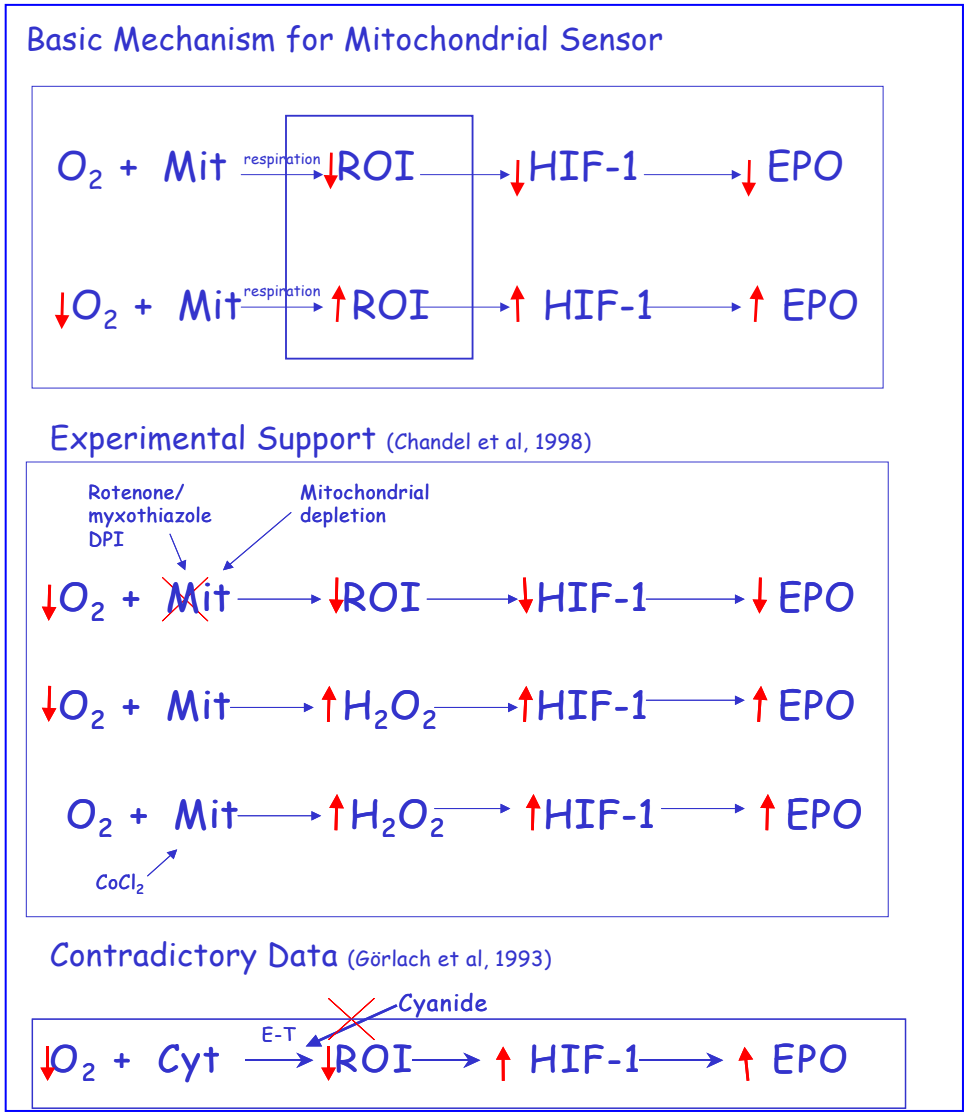


Figure 2.6c Schematic of Mitochondrial Sensor Mechanism results supporting and contradicting the mechanism are reported

2.3.2.4 Phosphorylation Mechanism

An alternative hypothesis is that cells utilize a phosphorylation mechanism to activate a second messenger and trigger the hypoxic response. The heme protein oxygen sensor in bacteria is known as Fix L, which acts as a kinase under hypoxic challenge and phosphorylates the transcription factor Fix J (Bunn and Poyton, 1996). The occurrence of a PAS domain on both Fix L and HIF-1 (the hypoxia-induced transcription factor in mammalian cells) led to speculation that a similar mechanism might take place in mammals. However, there has been no experimental data detecting the binding of the heme protein to HIF-1 suggesting that this mechanism is not utilized by mammalian cells (Wenger, 2000). Still, using a specific kinase inhibitor (genistein) reduced the DNA binding activity of HIF-1, and conversely, cells transformed with v-Src oncogene (having constitutive phosphorylation activity) increased HIF-1 activity, and consequently, upregulated the expression of downstream genes induced by this transcription factor (TF) (Wang *et al*, 1995b). Thus, circumstantial evidence suggests that this mechanism is utilized in mammalian cells.

2.3.2.5 Summary

There is data that supports as well as contradicts several distinct hypothetical mechanisms for putative oxygen sensors. The heme proteins bind oxygen and their signaling pathway may 'cross-talk' with the hypoxia pathway, but they do not appear to be *the* oxygen sensor. Data from different experiments used to implicate an oxidase as the oxygen sensor is contradictory. It appears that for this model of oxygen sensing, it has not yet been agreed whether ROIs are inducers or inhibitors of the hypoxic response. For example, Chandel *et al* (1998) claims that mitochondrial upregulation of hydrogen peroxide occurs during hypoxic stress suggesting that ROI formation may be necessary for the hypoxic response, while, as mentioned previously, Fandrey and coworkers (1994) have shown that induction of endogenous peroxide formation leads to inhibition of the hypoxic response. The concept of the mitochondria as an oxygen sensor is tied to the oxidase hypothesis since several oxidases are involved in the mitochondrial electron transport chain. Some bits of evidence have been used to both support and reject its involvement as well, stressing the need for clarity.

In summary, despite extensive work done to illuminate the oxygen sensor in higher organisms, the data obtained after a decade of intense research remained inconclusive, suggesting that oxygen sensing in higher organisms is not a simple, linear process. Oxygen is the most critical element for survival in mammals, and it is possible that oxygen sensing may be very complex with several inputs from various interconnecting signal transduction pathways being synthesized to modulate the response. Data already demonstrates possible communication between the hypoxic pathway and the pathway for CO and nitric oxide (NO) sensing (Semenza, 1999) which may complicate experimental observations. Thus, it has been necessary to work backward from what was already known (the hypoxia inducible factor (HIF) family of proteins is always involved in inducing reporter genes) in order to discover the unknown.

2.3.3 Hypoxia-inducible Factor – 1

2.3.3.1 HIF-1: Mediator of Hypoxic Response

Although the mechanism(s) via which cells sense and respond to hypoxia had not yet been fully elucidated, it had been convincingly demonstrated that the cellular responses were mediated by the HIF family of proteins (Wenger, 2000). HIF-1 was identified and isolated by Semenza and Wang (1992) due to its binding an enhancer region in the 3'-flanking region of the EPO gene in response to hypoxia. It has since been shown that HIF-1 also binds the enhancer region of VEGF (in its 5'-flanking region), GLUT-1, GLUT-3, GAPDH and a plethora of other genes during hypoxic stress (Semenza, 1999). HIF-1 protein is upregulated during hypoxia and when hypoxia is simulated by culturing cells in the presence of cobalt chloride to replace the iron in the heme proteins, and knocking out the gene for the HIF-1α subunit prevents cells from responding to hypoxic stress. These data indicate that HIF-1 plays a central role in the mechanism for eliciting cellular responses to hypoxia.

2.3.3.2 Biology of HIF-1

HIF-1 is a heterodimeric transcription factor composed of HIF-1α and HIF-1β subunits, two basic helix-loop-helix (bHLH) PAS (Per-AhR/ARNT-Sim) domain proteins with molecular masses of approximately 120 kD and 91 kD respectively (Wang *et al*, 1995a). HIF-1β was identified previously as the aryl hydrocarbon receptor nuclear translocator (ARNT) and is common to several transcription factors whereas HIF-1α gives the HIF-1 protein its unique

properties. The expression levels of HIF-1β remain fairly constant but HIF-1α levels are dependent on the prevailing oxygen tension (Semenza, 1998). HIF-1α is inversely regulated by extracellular oxygen concentrations such that a decrease in oxygen concentrations leads to an exponential increase in HIF-1α expression levels. The expression of HIF-1α doubled when oxygen concentrations decreased from 20% to 6%, but increased up to 10 times when oxygen decreased from 6% to 0.5% (Jiang *et al*, 1996). Thus, at physiological oxygen tensions, the HIF-1 response to oxygen is very sensitive, allowing delicate control of gene-regulation with variations in oxygen tension.

The regulation of HIF-1 levels takes place at multiple sites. Although HIF-1 is reported to be constitutively expressed in cells and tissues, there is an increase in HIF-1 mRNA stability (Weiner *et al*, 1996; Yu *et al*, 1998) as well as data indicating post-translational modifications which enhance HIF-1α protein stability (Huang *et al*, 1996) in response to hypoxia. The HIF-1α subunit is rapidly degraded under normoxic conditions with a half-life of about five minutes (Wang *et al*, 1995a), but is stabilized under hypoxic conditions where it has a half-life of approximately thirty minutes (Huang *et al*, 1998). The degradation occurs via a ubiquitination mechanism (Salceda and Caro, 1997) after being tagged with the von Hippel-Lindau (pVHL) tumor-suppressor protein (Cockman *et al*, 2000).

2.3.3.3 Regulation of HIF-1Activity and the Elusive Oxygen Sensor

Attempts to increase understanding of how pVHL binding to HIF-1 targets HIF-1 for degradation has unexpectedly shed light on the oxygen sensing mechanism of cells. It is known that under hypoxic conditions, HIF-1 is stabilized via an oxygen-dependent degradation (ODD) domain (Wenger, 2000). However, during normoxia, pVHL is able to bind HIF-1α's ODD when the proline at position 564 (P^{564}) of HIF-1α is hydroxylated (Ivan *et al*, 2001; Jaakkola *et al*, 2001; Yu *et al*, 2001). Subsequent studies showed that, like the previously known collagen prolyl-4-hydroxylase, this newly discovered hydroxylase required Fe^{++}, oxoglutarate, ascorbate and most notably, diatomic oxygen (Fedele *et al*, 2002). Oxygen is rate-limiting in the reaction and in the absence of oxygen, these proteins are unable to hydroxylate P^{564}. The subsequent binding of pVHL to the ODD during normoxia targets HIF-1 for ubiquitination. The dependence

of these hydroxylases on oxygen and their direct effect on HIF-1 levels imply that they may be the formerly elusive cellular oxygen sensor.

In addition to the stability of HIF-1α being increased during hypoxia, its transcriptional activity is also regulated. HIF-1α contains two transcriptional activation domains (TADs) (Semenza, 1998), one N-terminal (NAD) and the other C-terminal (CAD), and the two are separated by an inhibitory domain (ID) (Jiang *et al*, 1997). While the NAD is contained within the ODD of HIF-1α, (and thus correlates with protein stability), the CAD's activity is regulated by the ID according to the prevailing oxygen tension (Fedele *et al*, 2002). The regulation of transcriptional activity takes place via a mechanism related to that, which increases protein stability i.e. oxygen-dependent hydroxylation of an asparagine residue in HIF-1α (N^{803}) and HIF-2α (N^{851}). This hydroxylase is also dependent on Fe^{++} and 2-oxoglutarate. In the presence of the hydroxylated asparagine group, CAD is unable to bind the cyclic AMP response element binding protein (CREB) binding protein (CBP)/p300 complex, a process which is observed under hypoxia (Kallio *et al*, 1998), and is thus unable to bind to the enhancer sequence of target genes (Fedele, 2002). Thus, two distinct hydroxylation mechanisms allow a two-step regulation of HIF-1α activity according to the availability of oxygen. These hydroxylases have presented themselves as the long-elusive oxygen sensors but it is not possible to determine whether this may be the only mechanism via which cells sense oxygen or totally discount previously mentioned hypotheses.

2.3.3.4 HIF-1 as Transcription Factor

After stabilization at low oxygen tensions, HIF-1α rapidly translocates to the nucleus. In the nucleus HIF-1α couples with its counterpart HIF-1β. Dimerization of the HIF-1α and HIF-1β subunits is required for HIF-1 to bind DNA and initiate transcription of oxygen dependent genes. HIF-1 binds to the hypoxia response element (HRE), a sequence-specific (5'-RCGTG-3') binding site of previously mentioned target genes including glucose transporters, EPO, VEGF and inducible nitric oxide synthetase (iNOS) (Wenger, 2000; Semenza, 1998; Ebert *et al* , 1995). HIF-1's global influence over various cellular adaptations implicates its role in affecting a number of fundamental cellular processes including adhesion, proliferation, differentiation, glycolysis and apoptosis (Ramirez-Bergeron and Simon, 2001; Desplat *et al*, 2002; Lash *et al*,

2001; Iida *et al*, 2002; Carmeliet *et al*, 1998). HIF-1 has been shown to be present in all tissues *in vivo* and additionally plays an essential role during embryonic development of the organism.

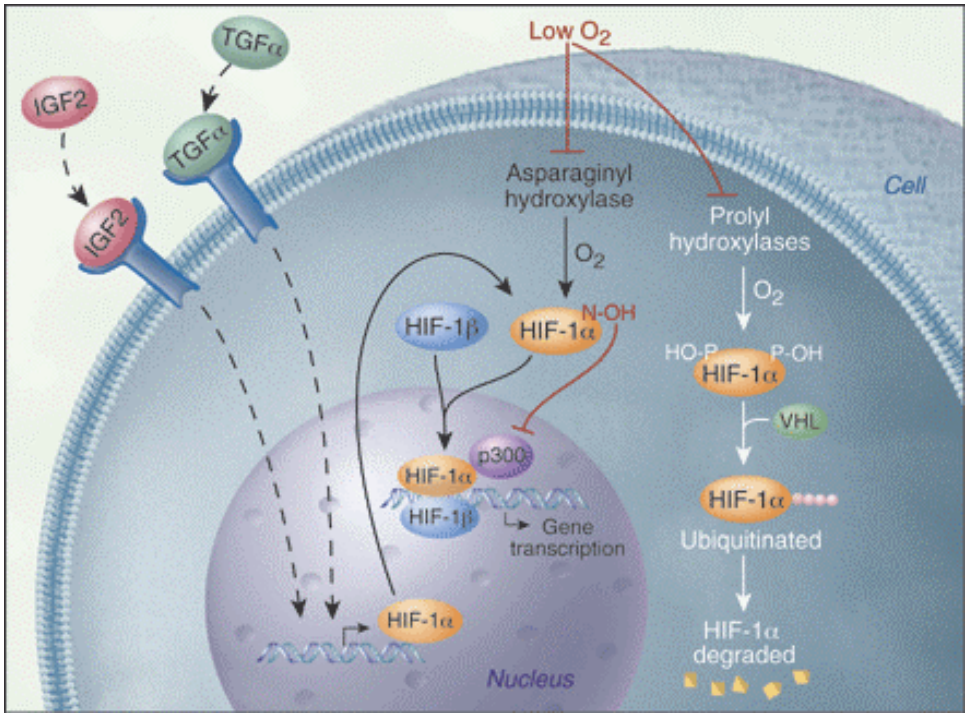


Figure 2.7 Putative Oxygen Sensing Mechanism (Marx, 2004)

2.3.3.5 Role of HIF-1 in Development

The role of HIF-1 in development is of particular interest for tissue engineering applications. In gene-knockout studies, both Arnt$^{-/-}$ or Hif-1a$^{-/-}$ mice failed to develop to full term (Kozak *et al*, 1997; Iyer *et al*, 1998) despite the fact that both subunits may dimerize with other partners (Semenza, 1998). Although the effects of the individual deletion of these two subunits were distinct, there were similarities in the developmental defects caused by deleting either gene: both

resulted in abnormalities in the formation of the neural tube and general vascularization (Semenza, 1998). The absence of HIF-1β resulted in vascular defects to the yolk sac, embryo and placenta, whereas the absence of HIF-1α led to the degeneration of the cephalic vasculature. HIF-1$\alpha^{-/-}$ cells also resulted in death of the cephalic mesenchymal cells and myocardial hyperplasia, which occluded the chambers. Culturing HIF-1$\alpha^{-/-}$ embryonic stem (ES) cells showed that the VEGF reporter gene response is not induced during hypoxia in these cells (Iyer *et al*, 1998). The mRNA of VEGF, glucose transporters and glycolytic enzymes are lower in HIF-1$^{-/-}$ ES cells and these cells exhibit decreased proliferation rates (Iyer *et al*, 1998). However, it was found that the VEGF mRNA expression actually increased in HIF-1$\alpha^{-/-}$ embryos as compared to wild-type embryos suggesting that the vascular defects that occur during embryogenesis are not associated with deficient VEGF. Instead, it is proposed that the developmental anomalies may be caused by the apoptotic effects on the surrounding mesenchyme (Kotch *et al*, 1999).

2.3.3.6 Other Members of the HIF Family

HIF-1 is very well-characterized and is the best understood protein in the hypoxia-inducible factors family of proteins. Other members include HIF-2 and HIF-3. The function and expression pattern of HIF-3α is still unclear (Wenger, 2000). HIF-2α shares 48% amino acid homology and has similar structure and functional domains with HIF-1α. Consequently, it is regulated via the same hydroxylase mechanisms described for HIF-1α and, although the downstream target genes of HIF-2α have not yet been identified, it is believed that it has distinct functions from HIF-1α *in vivo* (Fedele *et al*, 2002). Gene knockout studies have been done with HIF-2$\alpha^{-/-}$ mice (Tian *et al*, 1998), which showed that the embryos develop normal circulatory systems (unlike HIF-1$\alpha^{-/-}$ embryos) but still did not survive the gestational term. Other studies suggested a role in vascular remodeling (Peng *et al*, 2000) and lung maturation (Compernolle *et al*, 2002) during embryonic development. The latter study showed that some mice survived to birth but died shortly after as a result of a deficiency of lung surfactant. Thus, even though much work remains to be done to elucidate the exact target of HIF-2, it is clear that its role in embryogenesis is different from that of HIF-1.

2.3.4 Hypoxia in Cell-Culture

2.3.4.1 Effects on Cellular Functions

Despite the wide-ranging effects of extra-cellular oxygen levels on cells, its role in affecting cellular physiology has been generally overlooked in the *in vitro* culture of mammalian cells. Cell-culture is typically performed in ambient oxygen conditions (20% O_2) whereas cells *in vivo* experience oxygen tensions within the range of 4 – 7% O_2 (Lennon *et al*, 2001). When hypoxic conditions are employed *in vitro*, researchers typically observe significant changes in cell behavior. Over three decades ago Packer and Fuehr (1977) showed that when cells are cultured under hypoxic conditions (10% O_2) *in vitro* they are maintained for longer periods. Other groups have reported increased proliferation rates when cells are cultured under hypoxic conditions (Lennon *et al*, 2001; Ma *et al*, 2001). A variety of other responses to hypoxia have been reported in the literature. These observed effects are wide-ranging since researchers are testing the effects of hypoxia on the in-vivo functions of particular cell-types. Thus, murine MSC (mMSC) increased their secretion of VEGF and basic fibroblast growth factor (bFGF), upregulated matrix metalloproteinase (MT1-MMP) and displayed a concomitant increase in migration and angiogenesis (Annabi *et al*, 2003). Human dermal fibroblasts (HDF) increased VEGF expression, showed a transient increase in collagen I production and decreased expression of MMP3 (Steinbrech *et al*, 1999), rat lung fibroblasts increased post-translational collagen synthesis (Horino *et al*, 2002), human placental trophoblasts decreased their expression of 17β-estradiol (Ma *et al*, 2001) and HDFs grown in 3-D spatial orientation increased MMP-1 expression (Kan *et al*, 2003). While it may be possible to link oxygen tension to cell-type specific functions there are some common themes that prevail irrespective of cell-type. These include changes in the metabolic pathways, cell death and cell differentiation.

2.3.4.2 Changes in Cellular Metabolism

Cells exposed to hypoxic stress try to maintain their ATP production rates by switching to the glycolytic pathway. Glycolysis is the first step of the oxidative pathway where glucose is broken down to pyruvate and each glucose molecule yields two ATP molecules. When oxygen is limiting, cells can no longer produce ATP via the oxidative respiratory pathway. Thus, in order to maintain their levels of ATP production, which is critical for survival, cells uptake larger

amounts of glucose and upregulate enzymes involved in glycolysis. The particular enzymes upregulated depend partially upon the limiting step for the particular cell/tissue type. Thus Bashan *et al* (1992) showed that culturing L6 muscle cells under 3% O_2 resulted in a 650 % increase in glucose uptake compared to normoxic cells over 2 days. This corresponded with a significant increase in GLUT1 (but not GLUT4), an effect that was reversible upon return to normoxic conditions. A similar effect was observed with human umbilical vein endothelial cells (HUVEC) cultured at 2 % oxygen for 96 hours (Loike *et al*, 1992). These cells demonstrated increased glucose consumption and lactate production and upregulated transcription and translation of GLUT1. Subsequent experiments with bovine aortic endothelial cells (BAEC) and bovine pulmonary artery endothelial cells (BPAEC) under 3% O_2 indicated that these cells upregulated their expression of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) relative to cells cultured under normoxia (Graven *et al*, 1994). Other enzymes such as phosphoglycerate kinase 1 (PGK-1) and lactate dehydrogenase A (LDH-A) involved in metabolism are regulated in HepG2 (liver) cells during hypoxia (Firth *et al*, 1994). Recent research suggests a connection between metabolic and apoptotic pathways (Plas and Thompson, 2002; Downward, 2003) since proteins have been identified that are common to both pathways. The results suggest that a decrease in the ability of cells to carry out glycolysis, and consequent decline in ATP levels, increases their inclination to undergo apoptosis. Much more research is necessary, to determine how relevant these studies are to the hypoxic condition where there is a significant increase in metabolic rates and a concomitant HIF-1 mediated increase in apoptosis. Additionally, Malhorta and colleagues (2001) showed that during hypoxia the presence or absence of glucose facilitated two independent signaling pathways for induction of apoptosis.

2.3.4.3 Cell-Survival: Implications for Cancer Treatment

Hypoxia influences cell-cycle regulation via two independent mechanisms: HIF-1 dependent regulation of p53, p21 and Bcl-2 and HIF-1 independent regulation of p27 (Carmeliet et al, 1998). These authors generated HIF-1$\alpha^{-/-}$ ES cells and implanted these and wild-type ES cells into nude mice to generate HIF-$\alpha^{-/-}$ and HIF-$\alpha^{+/+}$ tumors. The HIF-$\alpha^{-/-}$ tumors grew more quickly as a result of fewer cells undergoing apoptosis mediated by the HIF-1 induced p53/p21 pathway. Iida *et al* (2002) showed that by liposome-mediated transfection of endothelial cells with HIF-1α induced p21 expression and caused cells to be arrested in G_1. These data indicate

that HIF-1 expression may inhibit the growth of tumors. HIF-1 also influences the expression of chemokine receptors important for tumor metastasis (Poellinger and Johnson, 2004) such that the upregulation of HIF-1 inhibits the expression of these receptors. Thus HIF-1's role in tumor development and metastasis has led to speculation that it may be a possible therapeutic target. However, the influence of hypoxia on tumor progression was shown to be dependent on their *in vivo* environment (Bluow *et al*, 2003) introducing additional complexity.

2.3.4.4 Hypoxia is a Niche Parameter

Hypoxia may also have a unique role in affecting the differentiated states of different cell types. Pfander *et al* (2003) showed that, in addition to helping to maintain ATP levels of chondrocytes under hypoxia, HIF-1α also influenced cellular synthesis of extracellular matrix by upregulating collagen II and aggrecan synthesis. One particularly interesting series of hypoxia studies has been done on murine and human hematopoietic stem cells (HSC) (Ivanovic *et al*, 2000a; b; 2002; Desplat *et al*, 2002). The authors showed that the CD34+ population of cells underwent decreased proliferation when cultured under hypoxic conditions yet retained a greater ability to give rise to colonies and repopulate marrow. This suggests that HSC are maintained in a quiescent, undifferentiated state under low oxygen conditions and indicates that oxygen concentrations may be a critical component of the HSC niche. The suggestion that O_2 concentrations may be a contributing factor to stem cell niches is of particular importance since the focus of this study are hMSC, which also reside within the bone-marrow and are closely associated with HSC. Previous reports on hypoxic studies of rat MSC indicate that hypoxia increases their proliferation rates as well as their potential for osteoblastic differentiation (Lennon *et al*, 2001). In contrast, murine MSC showed no difference in proliferation when cultured in hypoxic as opposed to normoxic conditions (Annabi *et al*, 2003). Hypoxia has been used as an isolation parameter for selecting a primitive population of MSC-like cells from bone marrow (D'Ippolitto *et al*, 2004), however, to date, there has not been any literature on the effects of hypoxia on hMSC when cultured *in vitro*.

2.4 STEM-CELL TISSUE ENGINEERING

HMSC are a viable alternative to embryonic stem (ES) cells for treating a panoply of diseases: They are free from ethical and moral concerns, exhibit reproducible characteristics, are able to regenerate or repair various tissue types, migrate to the sites of injuries, do not form teratomas when implanted *in vivo*, and can be used in conjunction with various tissue-engineering strategies such as gene-therapy, developing *in vitro* tissue constructs, injectable gels, or can be delivered by simple intravenous infusion. An important consideration is that, one of the main strategies of tissue engineering has always been to mimic the relevant characteristics of the system that it is trying to replace or repair (Langer and Vacanti, 1993; Langer, 1995). Many hMSC studies have been considered 'biology out of context', hence, understanding how various components of the hMSC niche affect their differentiation and tissue-development may provide useful engineering strategies. Apart from differences in cell-cycle, hMSC *in vivo* differ from their cell-cultured counterparts in their spatial organization and access to nutrients and oxygen. It is our goal to observe the effects of these parameters on the quality, development and differentiation potential of hMSC grown *in vitro*.

CHAPTER 3

HMSC TISSUE DEVELOPMENT IN 3D PET MATRICES

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3.1 INTRODUCTION

Adult human mesenchymal stem cells (hMSC) are prime candidates for many cell-based tissue-engineering applications because of their plasticity and easy accessibility. These cells, which are harvested and isolated from the bone marrow, can be induced to differentiate under defined culture conditions into osteoblasts (Cheng *et al*, 1994; Bruder *et al*, 1997), chondrocytes (Kavalkovich *et al*, 2002; Majumdar *et al*, 2001), and adipocytes (Nakamura *et al*, 2003), as well as mature cells of other germ layers (Orlic *et al*, 2001a; Reyes *et al*, 2001; Jiang *et al*, 2001; Spees *et al*, 2003). Despite such potential, their use in therapeutic applications is restricted by our limited understanding of their behavior in three-dimensional (3D) tissue constructs. This is mainly due to conventional cell-culture approaches, which investigate cellular characteristics on two-dimensional (2D) substrates resulting in anomalous cellular behavior, morphology and physiology (Geiger, 2001). Though several researchers have cultured mammalian mesenchymal cells in various 3D culture systems, this has been done mainly in order to enhance their osteogenic (Mizuno *et al*, 1997; Mueller and Glowacki, 2001; Yamada *et al*, 2003) and chondrogenic (Kavalkovich *et al*, 2002; Aung *et al*, 2002; Martin *et al*, 2001; Worster *et al*, 2001) differentiation capabilities. However, understanding the structural and functional roles of these 3D constructs is important in developing engineered MSC tissue constructs that are required for many clinical applications.

Recent studies have suggested that cells undergo different tissue development processes when grown in 3D scaffolds compared to conventional 2D cell-culture systems. Cukierman *et al* (Cukierman *et al*, 2001, 2002) showed that the adhesion complexes differed when cells were

cultured on 3D *in vivo*-like substrates. This affected the regulation of tyrosine phosphorylation, impacting signaling and other cellular processes. Additionally, others have shown that variations in cell spatial organization within a matrix influence both their proliferation and function (Ma *et al*, 1999a, 2000). This relationship between cells and their immediate environment has given rise to the concept of the cell niche, which acknowledges the complexity of the interplay among a particular cell, its neighbors and the extracellular matrix (ECM) environment (Potten and Loeffler, 1990; Jensen *et al*, 1999; Watt and Hogan, 2000). The niche is particularly important in cells that exhibit population asymmetry, such as hMSC, since the communication between the cells and their progeny is critical in influencing progenitor fate and the development of the tissue. Undifferentiated hMSC secrete several ECM proteins (Prockop, 1997) as well as various cytokines (Haynesworth *et al*, 1996), and thus play an integral role in maintaining their specific microenvironment. Understanding the development of this ECM environment and its influence on MSC proliferation and differentiation potentials is important in developing functional MSC tissue constructs.

The objective of this study is to investigate 3D MSC tissue development in non-woven poly(ethylene terephthalate) (PET) scaffolds by examining MSC plasticity, the expression and organization of the ECM microenvironment, and maintenance of multilineage potentials upon induction. HMSC were seeded in 3D non-woven PET fibrous scaffolds, which is hydrolyzed to give it similar properties to those of conventional culture dishes. The scaffolds are highly porous (~85%) with large specific surface area, providing sufficient surface area for cell adhesion and space for cells to grow and develop an ECM microenvironment. The cells were cultured over an extended period and it was found that the growth kinetics of the cells in 3D matrices differed from 2D growth characteristics reported in literature. Compared to 2D culture, hMSC also displayed dissimilar levels and patterns of expression of several ECM proteins (including collagen type I and type IV, fibronectin and laminin) and adhesion molecules (integrins and paxillin). The cells were cultured for over one month in the matrices and induced to differentiate into the osteoblastic and adipocytic lineages. These results showed that hMSC grown in 3D scaffolds display tissue development patterns distinct from their 2D counterpart verifying that three dimensionality is an important factor influencing hMSC tissue development.

3.2 MATERIALS AND METHODS

3.2.1 Reagents

Cell culture media, FBS and Penicillin/Streptomycin were obtained from Life Technologies (Rockville, MD). Antibodies to ECM proteins and paxillin were obtained from ICN Biomedical (Costa Mesa, CA). All other reagents were obtained from Sigma (St. Louis, MO) unless otherwise stated.

3.2.2 Cell Culture

Bone marrow aspirates of about 2 ml were drawn from healthy donors ranging in age from 19 to 49 years under an Institutional Review Board-approved protocol. Plastic adherent nucleated cells were separated from the aspirate and cultured as previously described (Sekiya *et al*, 2002b; DiGirolamo *et al*, 1999). These cells were expanded using α-MEM with 10% FBS and 1% Penicillin/Streptomycin at 37°C and 5% CO_2. Cells were seeded onto the PET matrices via depth filtration. Approximately 5 x 10^6 cells were suspended in 20 ml of media and filtered through four matrices in a stack (see Li *et al*, 2001 for schematic). The cell suspension passed through the matrices four times to increase the overall efficiency of seeding. The PET matrices were then transferred to 12-well plates, with one matrix per well in 3 ml of media. The media was changed every 2 days.

3.2.3 DNA Assays

A DNA standard was prepared by dissolving salmon testes DNA in TEX (10 mM Tris, 1mM EDTA, 0.1% Triton X-100) and its concentration confirmed using light at 260 nm from a UV spectrophotometer (μQuant, Biotek Instruments). Serial dilutions were prepared and used to construct a standard curve for each assay. Cells in the PET matrices were lysed by placing the entire scaffolds in TEX with 0.1mg/ml proteinase K (Fisher Scientific, Pittsburgh PA) at 50°C overnight. Samples of cell lysate (100 μL) were placed in duplicate or triplicate in to a 96-well plate and 100 μL of Picogreen (Molecular Probes, Eugene, OR) added to each well. The plate was incubated at 37°C for 10 min in the dark and then read on a fluorescent plate reader (Fluoro Count, Packard).

3.2.4 Scanning Electron Microscopy (SEM)

PET matrices containing the cells were removed from the wells, washed in PBS and fixed in 2.5% glutaraldehyde in 0.1M sodium cacodylate buffer (Electron Microscopy Sciences, Fort Washington, PA) at pH 7.4 for 2 - 4 hours. The cells were then rinsed in buffer and post-fixed in 2% osmium tetroxide in 0.1 M cacodylate buffer (Electron Microscopy Sciences, Fort Washington, PA) for 2 hours. After rinsing, the cells were dried using a series of graded ethanol washings followed by critical point drying. The dried samples were sputter coated using palladium with gold plating and then observed using a JSM 840 scanning electron microscope.

3.2.5 Immunocytochemistry Staining

Cells were washed with PBS and fixed with 4% paraformaldehyde for 30 min, blocked and then incubated with the primary antibody overnight at 4^{o}C. Cells were blocked using PBS with 1% BSA and incubated with secondary antibody conjugated to FITC (Jackson Laboratories, West Grove, PA) for 1 hour at room temperature. Cells were stored in TBS and viewed using a Zeiss 510 confocal microscope or an Olympus IX70 fluorescent microscope.

3.2.6 Flow Cytometry

For integrin analysis, cells were harvested, washed and incubated with primary antibody for 1 hr followed by incubation with the secondary antibody conjugated to a fluorescent probe for 30 min. The fluorescence was recorded by collecting 10000 events on an Epics XL flow cytometer (Beckman Coulter). For paxillin, the cells were first fixed and permeabilized using IntraPrep Permeabilizing solutions (Beckman Coulter) followed by a similar staining procedure.

3.2.7 Osteoblast Differentiation

Cells were grown in α-MEM media containing 10% FBS, 100 nM dexamethasone, 10 mM sodium-β-glycerophosphate and 0.05 mM ascorbic acid-2-phosphate. From day 3, the cells were assayed to determine alkaline phosphatase (AP) activity. For this, cells were digested with 500 μL lysing buffer (PBS with 1% Triton X-100, 0.5% sodium deoxycholate, 0.1 % SDS, 0.1 mg/ml phenylmethylsulfonylfluoride, 3% aprotinin), 50 μL of cell lysate was then added to 50 μL of substrate and 50 μL alkaline buffer. The mixture was placed in 37^{o}C water bath for 15 minutes

after which the reaction was stopped using 100 μL of 0.5 N NaOH solution. P-nitrophenol was used to construct a standard curve. The amount of p-nitrophenol liberated was determined using an absorbance reader with a 405 nm filter. Cells were also assayed using the von Kossa staining technique to detect any mineralization of the ECM. Matrices were removed from the wells and washed. The cells were fixed in 10% formalin solution for 1 hour then washed in silver nitrate under a bright light for approximately 15 minutes or until the calcium nodules turned black. The cells were differentiated in 5% sodium thiosulfate, washed and allowed to dry.

3.2.8 Adipocyte Differentiation

Cells were induced by treating with adipogenic induction (AI) medium (high glucose DMEM supplemented with 10% FBS, 0.2 mM indomethacin, 0.5 mM isobutyl-1-methyl xanthine, 1 μM dexamethasone and 5 μg/ml insulin). After 2 days, the AI media was removed and replaced with adipogenic maintenance (AM) medium (high glucose DMEM supplemented with 10% FBS and 10 μg/ml insulin). This treatment was continued in a cyclic fashion. Differentiation into adipocytes was detected using the Nile Red assays, which stained lipid vacuoles. Stock Nile Red solution was prepared in acetone at a concentration of 1mg/ml following the protocol described by Greenspan *et al* (1985). The cells were fixed in 1.5% glutaraldehyde in PBS and then washed with PBS. Stock Nile Red solution was diluted in PBS at a ratio of 1:100 and the cells were incubated in this diluted solution for thirty minutes. The cells were viewed using a Zeiss 510 confocal microscope.

3.3 RESULTS

3.3.1 Cell Seeding & Growth Kinetics

Approximately 55% of the cells filtered through the PET matrices adhered to the matrices. The matrix at the top of the stack retained the highest proportion of cells. The number of cells in each successive matrix in the stack decreased, with the matrix at the bottom retaining the lowest proportion of cells (Figure 3.1). The cells were maintained for over one month in culture and their growth and metabolism were monitored over this period. The cells maintained a very high metabolic rate throughout the culture period and for up to 7 weeks after plating as measured by glucose consumption and lactate production (data not shown). However, the cells generally did

not proliferate to a large extent and the total number of cells in all four matrices increased only 260% after one month in culture (Figure 3.2a). This proliferation was seen to be dependent on seeding density: the top matrix retained approximately 0.9 x 10^6 cells after filtration and this value increased 215% after one month whereas approximately 0.4 x 10^6 cells were seeded onto the bottom matrix and increased 414% to 1.73 x 10^6 cells during the same culture period (Figure 3.2b).

3.3.2 Production of extra-cellular matrix proteins

When hMSC are grown in normal 2D tissue culture environments, there is minimal production of ECM proteins (Figure 3.3a). In stark contrast, when these cells are seeded onto the 3D PET matrices and allowed to grow under static conditions, the cells express large amounts of ECM proteins and organize them into an extensive fibrous network. SEM pictures of these cells grown in 3D confirm these observations and also demonstrate various morphologies adopted by the cells in the scaffolds (Figure 3.3b, c).

We investigated this protein network to determine which ECM proteins are expressed in 3D culture. The expression of collagen I, collagen IV, laminin, and fibronectin, but not collagen II, was detected using antibodies conjugated to fluorescent probes. Interestingly, whereas collagen I and laminin were confined almost exclusively to the cytoplasm of the cells when grown in 2D, these proteins are expressed abundantly in 3D culture and organized into distinct fibrils. Similarly, collagen IV is highly upregulated when the cells are grown in 3D, and displays a matted pattern. The amount of fibronectin may not differ significantly from 2D to 3D, however, the fibrils are more structured in the PET matrix (Figure 3.4).

3.3.3 Cell Adhesion Molecules

The expression of integrins is related to the differential expression of ECM proteins. Flow cytometry showed that compared to 2D, the $\alpha_2\beta_1$ integrin is expressed to a greater extent by cells in 3D PET than on 2D surfaces (Figure 3.5). Conversely, the expression of $\alpha_V\beta_3$ integrin is almost non-existent in the matrix, whereas it is expressed in about 40% of the cells grown in a regular tissue-culture flask. $\alpha_5\beta_1$ expression, while high in both 2D and 3D culture is slightly reduced in 3D PET matrices. Cells in 2D ubiquitously express paxillin (Figure 3.6a). However,

for the cells grown in PET, the paxillin expression appears much more localized and concentrated to fewer attachment points (Figure 3.6b). Flow cytometry data indicates 25% reduction in paxillin expression when cells are cultured in 3D (Figure 3.6c).

3.3.4 Osteoblast Differentiation

The cells were induced into osteoblasts after one month in culture. Alkaline phosphatase activity increased in the cells that were induced as compared to the control (uninduced) cells as early as three days after treatment (Figure 3.7). The AP activity of these cells steadily increased up to day 12 after treatment, by which time it was approximately five times higher than the control cells. At day 25, the AP activity of the induced cells was significantly reduced, but still remained at twice the level of the control. The von Kossa stains revealed significant mineralization of the ECM within the PET scaffolds of induced cells as opposed to uninduced cells (Figure 3.8). This indicates that while the cells are not spontaneously differentiating into osteoblasts, they maintain a high potential to differentiate when induced along that lineage.

3.3.5 Adipocyte Differentiation

Cells were first induced to differentiate in 2D culture so that morphology and lipid vacuole formation could be observed. From as early as 3 days after induction, it was obvious that the cell morphology was markedly different from uninduced hMSC. Lipid vacuoles were apparent in induced cells and confirmed by Oil Red O staining (data not shown). Nile Red was used to stain lipid vacuoles in 3D cultures and detected using confocal microscopy. For cells grown in 3D scaffolds, the Nile red assay demonstrated extensive adipogenesis, evidenced by lipid vacuole stains, in cells induced to become adipocytes, whereas the uninduced cells stained negative (Figure 3.9).

3.4 DISCUSSION

3.4.1 Seeding and Growth

Uniform cell distribution within the 3D scaffold is important for cells to utilize available surface area and to support subsequent tissue development in space within the scaffold. Depth-filtration has been shown to successfully achieve this goal in non-woven PET matrices (Li *et al*, 2001). A

correlation was observed between the number of cells seeded in a matrix and the position of that matrix in the filter stack. This phenomenon is characteristic of depth-filtration processes that are likely to be governed by the strong adherence of hMSC to the treated polymer surface. The variation of seeding density affected proliferation potential but not growth patterns. Earlier studies (Bruder *et al*, 1997) reported that hMSC exhibit three phases of growth: lag, log and plateau. A similar but exaggerated phenomenon was observed for the cells grown in 3D. In 2D the lag phase lasted for 2 days followed by approximately 4 – 6 days of exponential growth and attainment of a plateau by day 9. In 3D culture, the lag phase persisted for about 5 days with 15 – 20 days of linear growth, followed by a decrease in growth-rate around day 25. On average, 2D cells increased to more than five times their original number after 9 days in culture whereas they only increased 2.6 times after a month in 3D scaffolds. As in 2D, the cell proliferation in the scaffold was dependent on their original seeding density. A maximum fourfold expansion was obtained in the matrices with the lowest initial seeding density but two-fold expansion when the initial seeding density doubled. Thus, even though growth patterns remain similar, the cells in 2D exhibited a markedly higher proliferation potential. The hMSC are directed to secrete large amounts of ECM proteins and to create an intricate ECM network in 3D scaffolds rather than proliferate to fill the pore spaces. The structure of the non-woven 3D scaffold controls the architecture of the ECM network and spatial patterning of cells, which in turn influence cell proliferation potential. This conclusion for the profound effect of the 3D scaffold on cellular physiology is supported by the observation that hMSC grown in 3D scaffolds also have different integrin expression patterns than 2D controls.

3.4.2 Microenvironment

ECM proteins are important components of tissue, and their expression and organization are critical for the proper functioning of native tissue and engineered tissue constructs. HMSC cultured in tissue-culture flasks express collagen I, fibronectin, laminin, and collagen IV (Prockop, 1997). On 2D surfaces, however, the expression of these proteins is not related to their *in vivo* function, and thus they display arbitrary organizational patterns. Collagen I expression in 2D culture, for example, is found only in the cytoplasm of cells. On 3D scaffolds collagen is strung out into thin, highly structured fibrils, similar to their organization in differentiated tissues such as ligaments, tendons and bone (Wang *et al*, 2003). Similar

distinctions are seen for laminin in 2D and 3D culture. Collagen IV and fibronectin, while not confined to the cytoplasm in 2D, display very little structure. In 3D culture however, their fibrils are also more organized. The ability of cells to remodel the ECM, and reciprocally, the amenability of the ECM to being remodeled in the 3D matrix, significantly affects cell-cycle regulation (Grinell, 2003) and influences the growth kinetics of hMSC in PET. In 3D PET matrix, the ECM fibrillar alignment, which is influenced by cell orientation (Wang *et al*, 2003), is generally directed by the orientation of neighboring fibers of the PET matrix. This phenomenon is a significant aspect of 3D culture systems and indicates the possibility of directing cells to form the desired tissue architecture by configuring the 3D structure of the scaffold.

The amount of expressed ECM proteins is also upregulated in 3D culture. ECM proteins influence a plethora of cellular activities including cell survival, proliferation, differentiation and tissue development. Laminin, known mainly for its role in embryogenesis, has been implicated in cell survival, proliferation and induction (Eckblom *et al*, 2003). Collagen IV plays a role in cell adhesion and migration (Marneros and Olsen, 2001), and fibronectin, a major component of the ECM of fibroblast-like cells, has been shown to be the most effective ECM molecule in promoting adhesion of hMSC to tissue culture surfaces (Tsuchiya *et al*, 2001). The increased expression of laminin, collagen IV and fibronectin are likely to be connected to their functions in increasing cell survival and adhesion. Prior studies have found that, under 2D tissue culture conditions, cells exhibited progressive cell-matrix adhesion patterns suggesting the existence of a maturation process for cells to develop their microenvironment closer to their *in vivo* conditions (Cukierman *et al*, 2001). When cells are grown in 3D PET scaffolds that have similar surface properties as tissue culture-ware, the *in vitro* maturation process is greatly enhanced as evidenced by the extensive ECM network formation and the distinct integrin expression patterns. This phenomenon suggests that three-dimensionality itself is an important factor contributing to *in vitro* tissue development.

Three-dimensional scaffolds have been reported to selectively facilitate chondrogenic differentiation of bovine MSC (Martin *et al*, 2001), and studies have shown that growing rat MSC on 3D collagen I substrates induced their differentiation into osteoblasts (Mizuno *et al*,

1997; Mizuno *et al*, 2000). Therefore it is necessary to determine whether the extensive ECM network formation is accompanied by hMSC differentiation. In spite of the high collagen I content of the ECM secreted by the cells in our study however, there was no indication of the cells in the matrices spontaneously differentiating along the osteoblastic lineage without stimulation. The AP activity of these cells remained significantly lower than that of cells induced to become osteoblasts with dexamethasone. Collagen II is expressed exclusively by chondrocytes, which produce significant amounts of ECM proteins and glycosaminoglycans (Tsuchiya *et al*, 2001; Carlberg *et al*, 2001). We tested the expression of collagen II to examine whether hMSC spontaneously differentiate into chondrocytes when grown in the 3D scaffold. Immunocytochemistry stains of collagen II were negative indicating that the hMSC did not spontaneously differentiate along this pathway. These results showed that the formation of an ECM network in a 3D matrix is a part of hMSC tissue development process prior to and independent of differentiation.

3.4.3 Cell Adhesion

Integrins mediate bi-directional signaling interactions between the cells and their surroundings and regulate cell-cycle and tissue development (Hynes, 1992). Cell adhesion apparatus, formed by integrins and adaptor proteins, link the ECM and cytoskeleton and is a powerful regulator of tissue morphogenesis. Cukierman *et al* (2001) showed that the molecular composition of the adhesion structures may differ and is affected by the composition, three-dimensionality and pliability of the substrate. Cells grown in the 3D PET scaffolds may develop distinct cell adhesion apparatus that affects the subsequent tissue development processes. The effects of the 3D scaffolds and the ECM secretions on the expression of various integrins were therefore investigated. An increased expression of $\alpha_2\beta_1$ was observed. This protein binds collagen I and is reported to have a role in organizing its structure (Klein *et a*l, 1991; Schiro *et al*, 1991). The elevated $\alpha_2\beta_1$ expression by hMSC in the 3D matrix is believed to contribute to the highly organized collagen fibrils observed in the 3D scaffolds. The $\alpha_5\beta_1$ integrin is a fibronectin receptor and, like fibronectin, is strongly expressed in both 2D and 3D culture. There is, however, a small but statistically significant decrease in its expression in 3D culture, which may exert critical influence on cell proliferation and survival capabilities (Giancotti and Ruoslhati, 1999; Damsky and Ilić, 2002). $\alpha_V\beta_3$ integrin expression is significantly lower in 3D. $\alpha_V\beta_3$

integrin is a promiscuous receptor that is weakly expressed by hMSC (Majumdar *et al*, 2003) and mature fibroblasts. It is a component of 2D focal adhesions but not a part of the usual *in vivo* 3D adhesion complexes (Cukierman *et al*, 2001). It is possible that the lack of its expression in 3D may be related to the lack of focal adhesions characteristic of 2D adhesions. The expression of $\alpha_V\beta_3$ in endothelial cells is related to cell proliferation and survival (Meerovitch *et al*, 2003) and hence, the decrease in its expression may be linked to the decreased proliferation of hMSC in 3D scaffolds. The integrin expressions of hMSC grown in 3D matrices further indicate that they possess 3D adhesion characteristics distinct from classical 2D adhesion complexes.

Other proteins are involved in the adhesion/signaling complex. Paxillin is an intracellular protein confined to adhesion points on the inner surface of the cell and it is one of several adaptor proteins that connect integrins to the cytoskeleton. It functions mostly as a signal transducer, recruiting and binding several signaling and structural proteins (Turner, 1998). The decrease in the amount of paxillin expressed in 3D culture is perhaps a consequence of reduced attachment points in the 3D system. The large available surface area in 2D facilitates many points of attachment as shown in Figure 3.6. Paxillin is a component of both focal and 3D adhesion complexes. Therefore when cells are grown in 3D, its expression may not simply be downregulated, but paxillin may also be associated with different protein complexes. Variation in paxillin levels and its colocalization with different integrins in 3D would also indicate changes in the regulatory mechanisms of cells (Cukierman *et al*, 2001; Cukierman *et al*, 2002). The change in the amount of ECM proteins expressed combined with dissimilar integrins and paxillin expressions suggest a variation in the manner hMSC influence and interact with their environment when cultured in 3D scaffolds. The cells are able to create their own microenvironment when cultured in 3D by using ECM proteins to form a mesh and embedding themselves within that network. Consequently, the numbers and possible types of adhesion complexes formed under these conditions vary and the cellular responses differ compared to cells grown in 2D. With the changes in the expression of integrins and paxillin, it is not surprising that hMSC display different growth kinetics in the 3D matrix despite the large surface area for cell attachment and growth.

3.4.4 Multi-lineage Potential

Despite much research done on characterizing hMSC (Majumdar *et al*, 2003; Haynesworth *et al*, 1992a,b; Pittenger *et al*, 1999; Shur *et al*, 2002), no distinctive marker or combination of markers has been found that unambiguously identifies them. This is further complicated by the occurrence of cells with similar multi-lineage capabilities but varying phenotypes (Reyes *et al*, 2001; Jiang *et al*, 2002). Hence, the ability of the cells to differentiate along osteoblastic and adipogenic lineages at the end of the culture period, was taken as evidence of its progenitor status. Despite being cultured in the matrices for extended periods of time and expressing large amounts of collagen I, these cells did not become restricted to the osteoblastic lineage and, in fact, remained in an undifferentiated state. More importantly, the cells retained their potential to become osteoblasts when induced, expressing very high alkaline phosphatase activities and showing extensive mineralization of their matrices. The AP activity of the cells in 3D was significantly higher than that reported for 2D induction (Bruder *et al*, 1997) suggesting that cells grown in 3D scaffolds may display greater differentiated characteristics when induced along a particular lineage than cells grown in 2D culture. Furthermore, when induced to become adipocytes, Nile Red stains show significant lipid vacuole accumulation in 3D cultures with no such accumulation taking place in control 3D cultures. Flow cytometric quantification of Nile Red stains of 2D and 3D cells induced along the adipogenic lineage for different time periods also suggest that cells grown in 3D scaffolds differentiate to a greater extent than those grown in 2D cultures. Hence, hMSC in 3D culture are able to form classical tissue-like three-dimensional constructs during proliferations stages while retaining their potential for induction into particular differentiated tissue types.

3.5 CONCLUSIONS

Three-dimensional scaffold design and development play an important role in tissue engineering. Fundamental cellular events such as cell adhesion, proliferation, differentiation and ECM secretion are intricately linked to the cells' spatial organization in 3D systems (Yamada and Clark, 2002). The results demonstrated that non-woven 3D PET matrices facilitate the distribution of cells via a depth-filtration process. The structure of the 3D PET scaffold dictates the growth and development patterns of the cells and influences the expression and organization

of ECM protein fibers. Directing the formation of the ECM network in 3D scaffolds is a critical aspect of the engineering of functional tissue constructs. These results suggest that tissue development patterns may be guided along predetermined pathways by configuring the various parameters of the 3D scaffold. HMSC cultured in 3D PET scaffolds display lowered proliferation potentials but actively create and remodel their ECM microenvironments resulting in tissue constructs that closely resemble native tissue architecture and maintain the potential to be differentiated along distinct lineages.

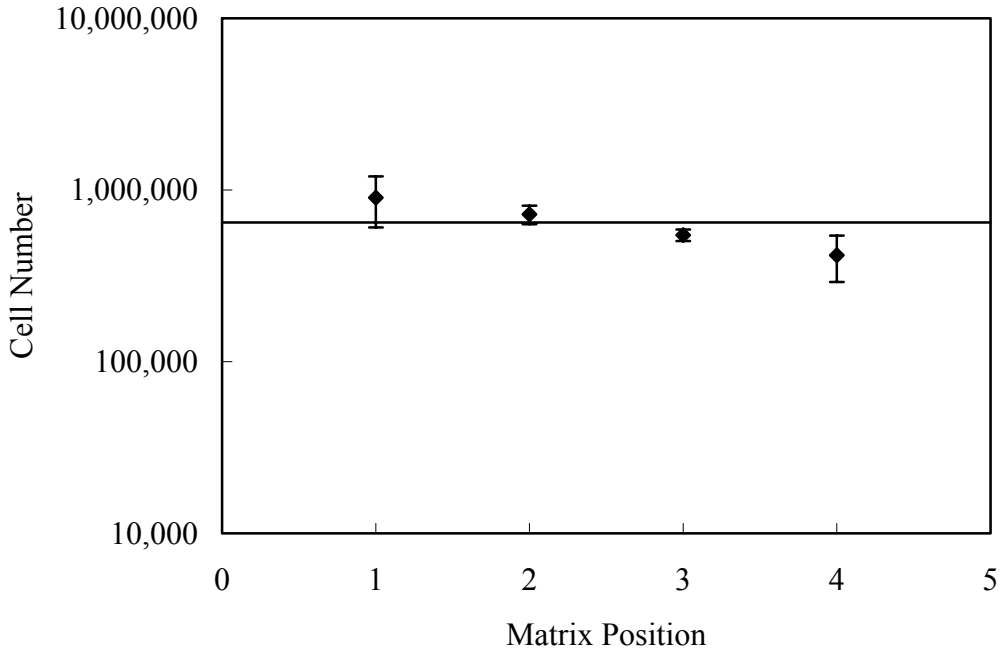


Figure 3.1. Cell Number vs. Matrix Position.
The number of cells seeded in each matrix was correlated to the position of the matrix in the stack (n=3). The higher the matrix was in the stack, the more cells it retained. The matrix at the top (#1) retained the largest number of cells and that at the bottom (#4) retained the least cells.

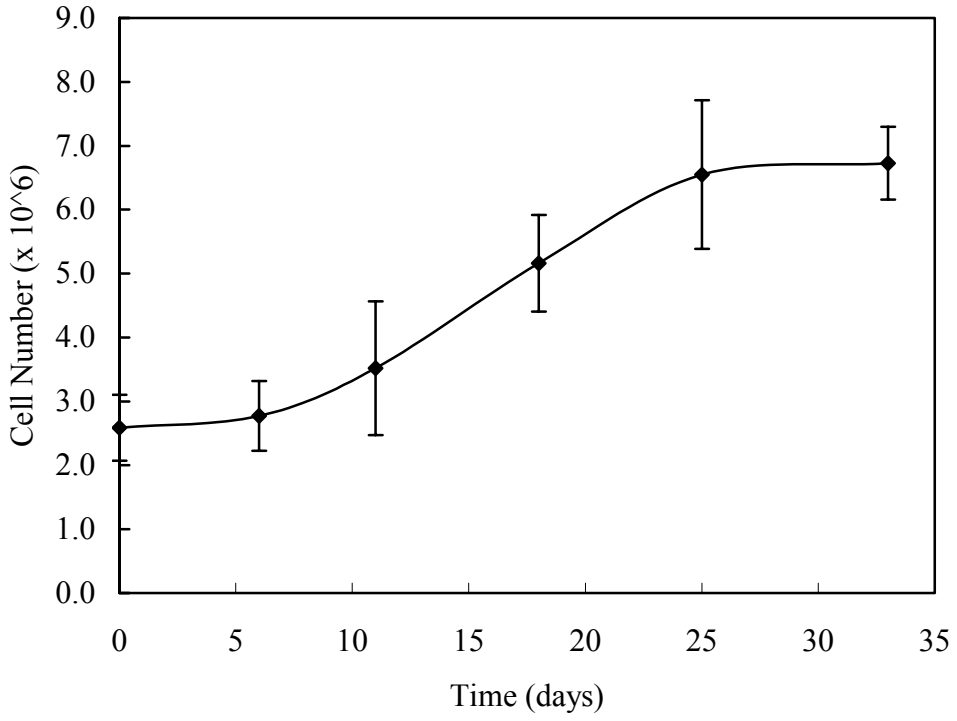


Figure 3.2a. Cell growth kinetics.

Each data point represents an average of the total number of cells in the four matrices that were seeded simultaneously. Data points were taken as duplicates. The curve shows three distinct regions: lag, log and plateau phases. Total cell number increased 260% over growth period of 33 days.

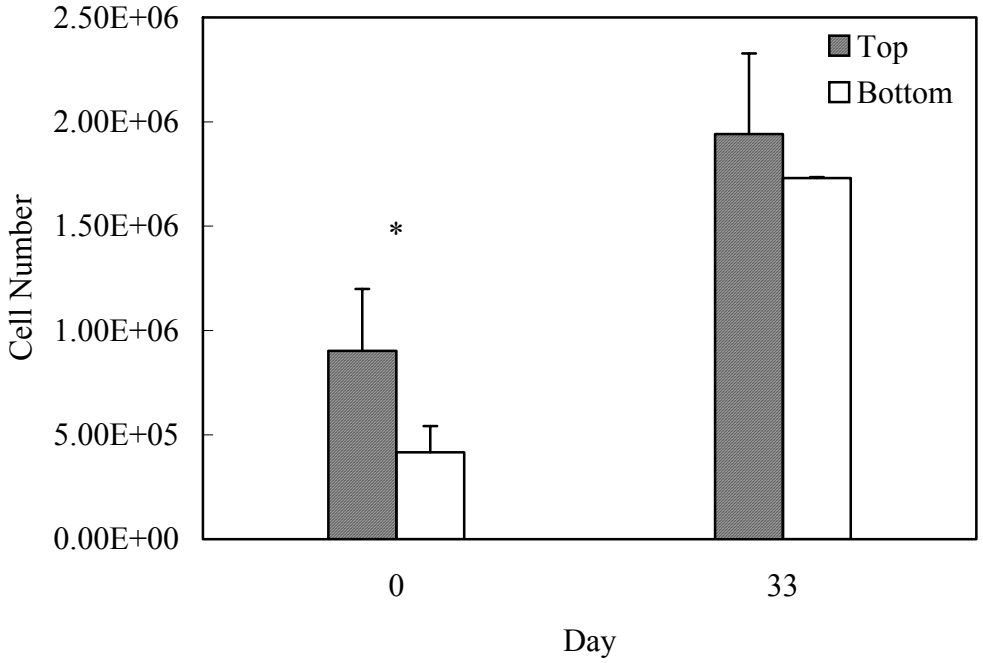


Figure 3.2b. Effect of Seeding Density on Growth.
The top matrix retained twice the number of cells as the bottom matrix (n=3, p= 0.04). After culturing for 33 days the number of cells in the top matrix was not significantly different from those in the bottom matrix (n=2, p= 0.29).

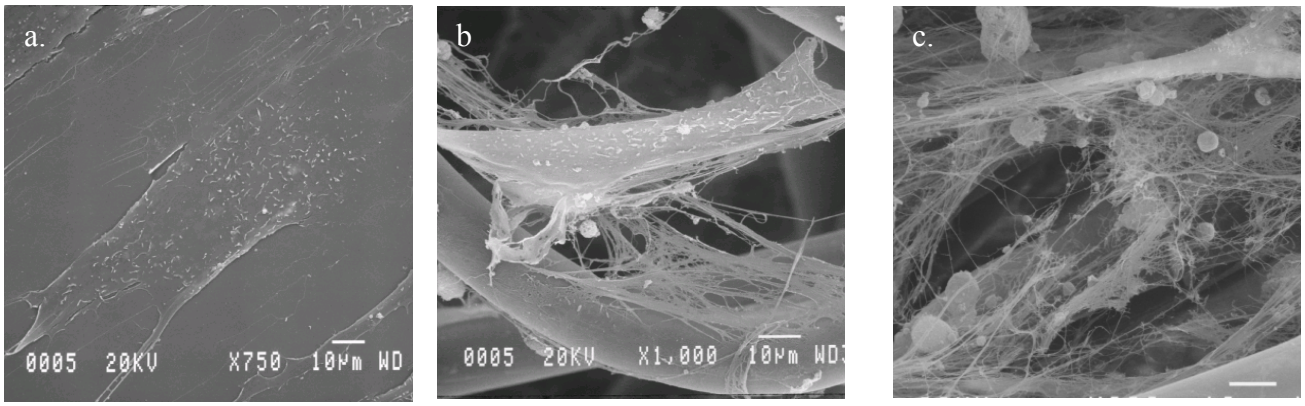
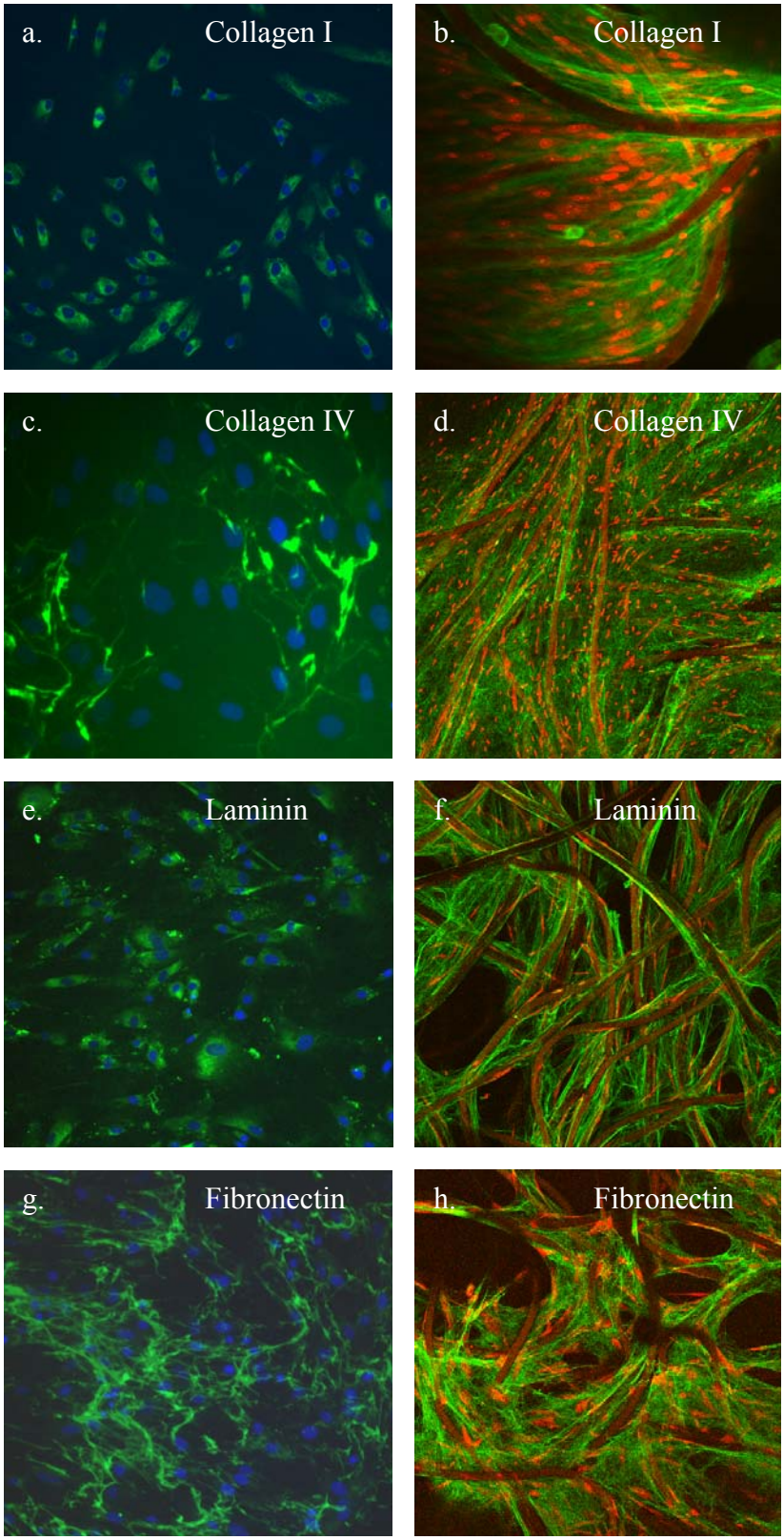


Figure 3.3 SEM micrographs of hMSC grown in 2D and 3D.
(a) SEM of hMSC grown on 2D coverslip. Shows a minimal production of ECM
(b) SEM of hMSC grown in 3D PET matrices. (c) Cells adopt various morphologies within matrix and secrete an extensive ECM network

Figure 3.4 Comparison of Immunofluorescent Staining of ECM Proteins on 2D and 3D. (a) Collagen I on 2D was confined to the cytoplasm of the cells but (b) showed distinctive fibrils on 3D. (c) Collagen IV was expressed sparingly on 2D. (d) Collagen IV showed extensive and structured expression in 3D. (e) Laminin was located predominantly around the nuclei of cells in 2D, with little or no localization in the extra-cellular spaces. (f) In 3D the laminin fibrils were organized mainly along the PET fibers but also in the pore spaces. (g) Fibronectin showed similar patterns in 2D and (h) 3D cell culture.


a.
Collagen I
b.
Collagen I
c.
Collagen IV
d.
Collagen IV
e.
Laminin
f.
Laminin
g.
Fibronectin
h.
Fibronectin

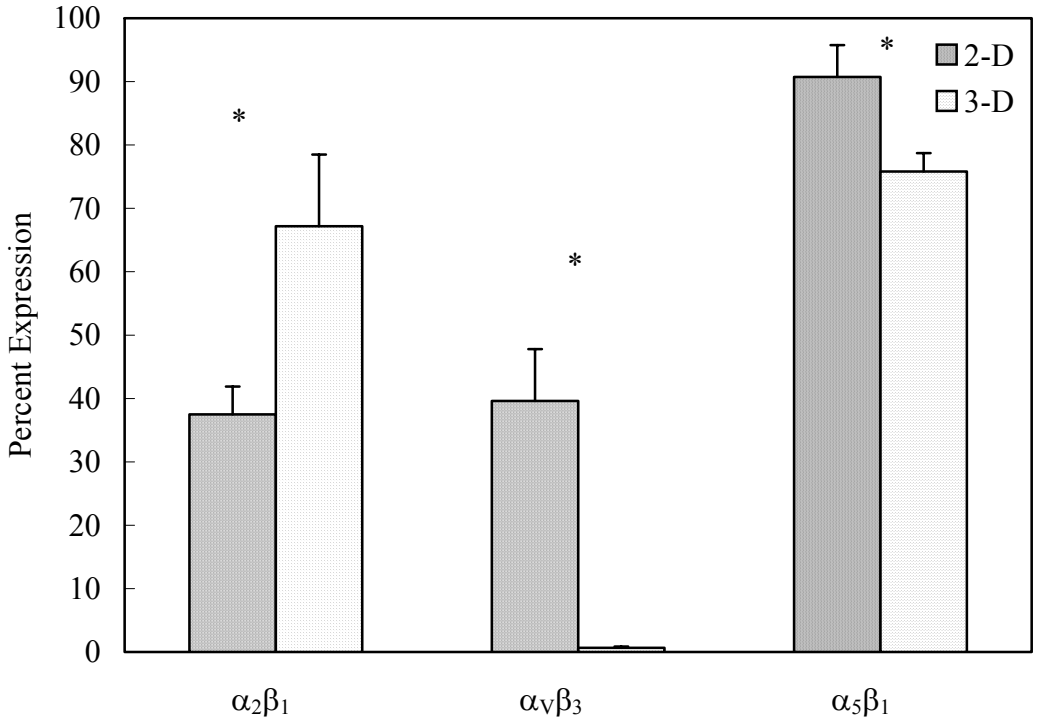


Figure 3.5 Comparison of integrin expression on 2D and 3D.

3D culture shows significantly higher proportion of cells expressing the $\alpha_2\beta_1$ integrin than the cells grown in 2D (n=3). $\alpha_V\beta_3$ is expressed weakly in 2D but not at all in 3D (n=3). There is a small but statistically significant drop in $\alpha_5\beta_1$ expression in 3D scaffolds compared to 2D culture (n=5).

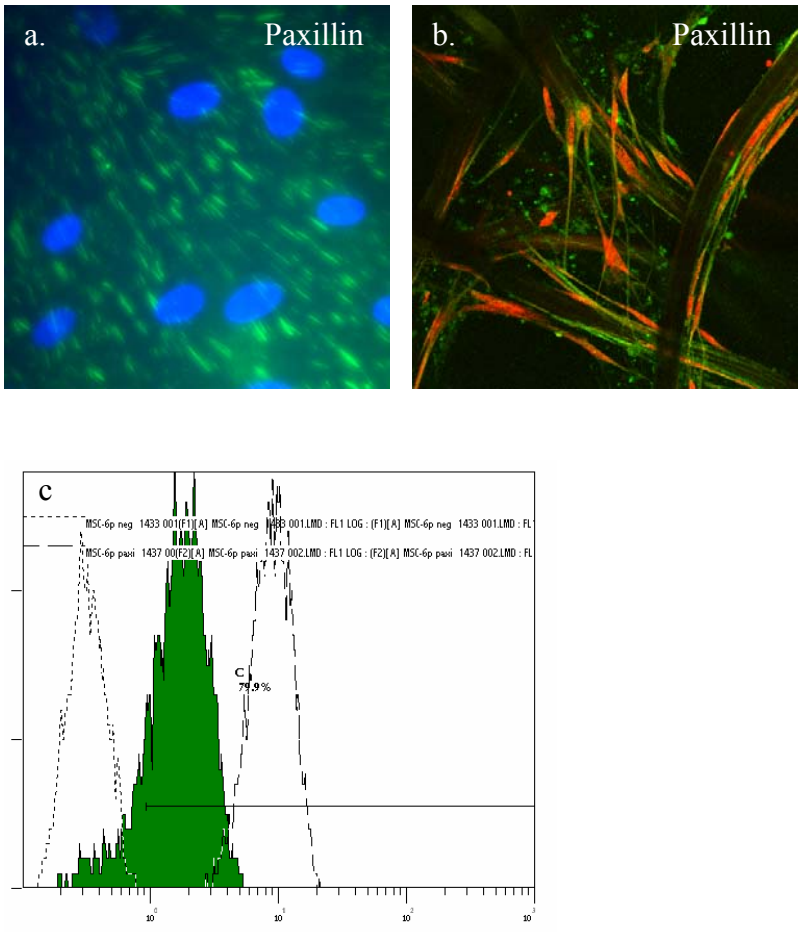


Figure 3.6 Expression of paxillin in 2D and 3D.
(a) Immunocytochemistry stains show ubiquitous expression of paxillin in cells cultured in 2D. (b) The localization of paxillin changes when cells are grown on 3D PET matrices. (c) Flow cytometry studies show a decreased expression of paxillin for cells grown on 3D as opposed to 2D. Green peak represents expression in cells cultured in 3D scaffolds. Transparent peak with solid line represents paxillin expression in cells cultured on 2D surfaces. Dotted peak is negative control.

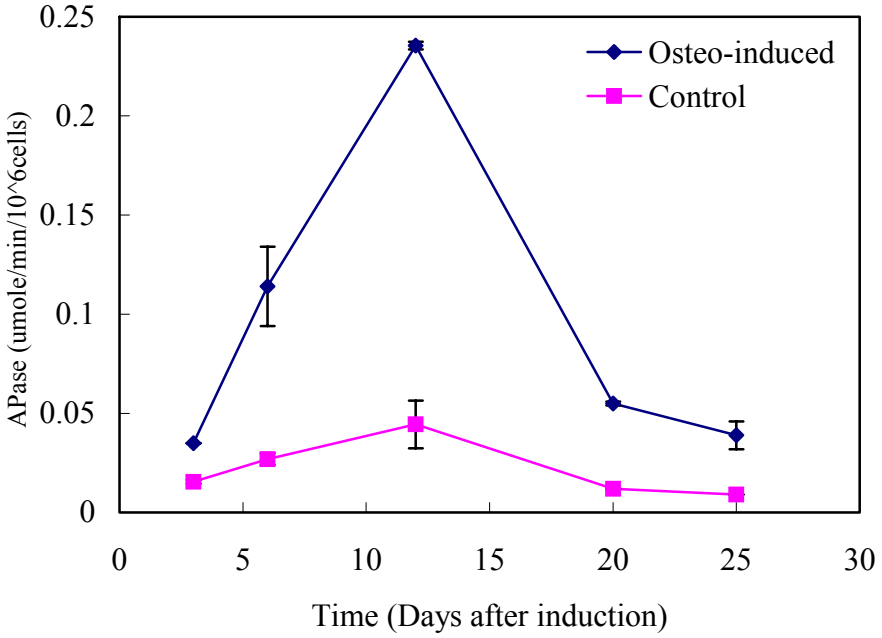


Figure 3.7 Alkaline Phosphatase activity of Osteo-Induced hMSC.
Cells induced to become osteoblasts have significantly higher AP activity than that of control (uninduced) cells. The AP activity of induced cells steadily increases to day 12 and then drops. At day 25 the activity is still twice as high as uninduced cells. Sample points were done in duplicate.

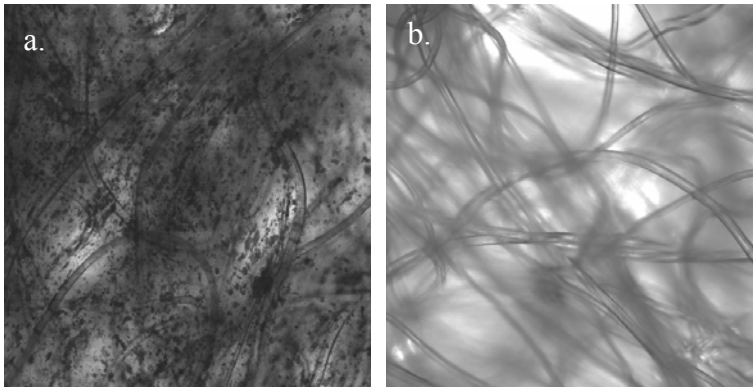


Figure 3.8 Von Kossa Staining.
Extensive mineralization of the ECM secreted by osteo-induced cells. Confocal microscopy shows calcium deposition along the fibers of the PET matrix. Control (uninduced) hMSC show no mineralization.

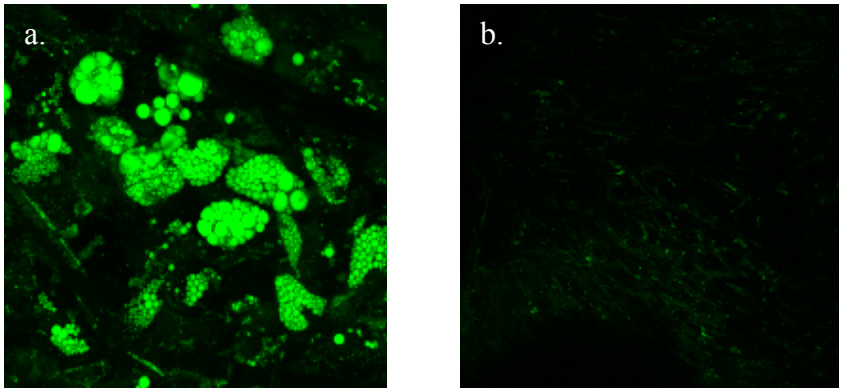


Figure 3.9 Nile red Staining.
Lipid vacuoles detected at FITC wavelength. (a) Cells induced to become adipocytes show extensive formation of lipid vacuoles. (b) Uninduced cells grown in matrix as control show no lipid vacuole formation after extended culture periods.

CHAPTER 4

PERFUSION BIOREACTOR AFFECTS PHENOTYPE OF HUMAN MESENCHYMAL STEM CELLS IN 3D SCAFFOLDS: CHANGES IN NUCLEAR SHAPE AND ECM STRUCTURE

4.1 INTRODUCTION

Despite the potential of hMSC to be used in multiple clinical applications (Prockop, 1997), major challenges exist during their *in vitro* expansion. HMSC are highly sensitive to their culture conditions, and the particular expansion method utilized impacts upon their functionality. Changes in seeding density (Sekiya *et al*, 2002a), growth-factors (Lennon *et al*, 1996; Stute *et al*, 2004), extracellular matrix (ECM) proteins (Meinel *et al*, 2004), substrate topography (Grayson *et al*, 2004), and medium flow (Li *et al*, 2004) affect their cell-cycle, differentiation, ECM expression, and onset of senescence. The plasticity of adult stem cells suggests that they are constantly in-transit while awaiting cues that direct cell fate (Zipori, 2004). The signals provided by the culture microenvironment provide several stimuli for determining their phenotype (Wagers & Weissman, 2004), and therefore it has become critical to understand how cell-preparation and culture conditions impact upon their characteristics.

One common tissue-engineering approach to tissue regeneration combines cells with three-dimensional (3D) scaffolds to form constructs that can be directly implanted into defect sites (Sharma and Elisseeff, 2004). However, developing 3D cellular constructs poses the twin challenges of achieving construct uniformity and mitigating oxygen/nutrient transport limitations (Martin *et al*, 2003). As a result, various bioreactor designs have been developed to alleviate these difficulties (Freed *et al*, 1994; Bancroft *et al*, 2003; Saini and Wick, 2003). Prior studies have investigated the effects of media flow on the seeding efficiency (Freed *et al*, 1997; Vunjak-Novakovic *et al*, 1998; Carrier *et al*, 1999; Holy *et al*, 2000; Wendt *et al*, 2003), nutrient

transport (Peng and Palsson, 1996), or functionality of end-stage differentiated cells (Ma *et al*, 1999b; Pei *et al*, 2002; Bancroft *et al*, 2002) in cell-based tissue constructs. In fact, using bioreactors to apply hydrostatic loading, or even shear stress, enhanced the differentiated phenotype of osteoblasts (Bancroft *et al*, 2002; Sikavitsas *et al*, 2003; Li *et al*, 2004) and chondrocytes (Saini and Wick, 2003) grown in reactor systems. The mechanical environment inherent in these systems is compatible with the native load-bearing functions of bone and cartilage, however, far less intuitive is the effect that bioreactor microenvironments will have on the phenotype or tissue-developmental processes of adult stem cells.

Growing hMSC in 3D polymer matrices significantly slows their growth, facilitates superior ECM expression and organization, and enhances subsequent multi-lineage differentiation (Grayson *et al*, 2004). When similar constructs were cultured under perfusion conditions, the improved nutrient and oxygen supplies led to greatly increased metabolic rates, oxygen consumption (Zhao *et al*, under review), and cell-proliferation (Zhao and Ma, 2005). Construct functionality though, is characterized by three distinct, yet inter-related parameters: cell density, ECM structure, and functional differentiation. Therefore, understanding the influences of media flow on the latter processes is critical for optimizing the mechanical and functional properties of tissue-constructs. In this study, we investigated and compared the effects that the mechanical and biochemical cues inherent in static and perfusion systems have on the phenotype and tissue-morphogenesis patterns of hMSC grown in 3D poly(ethylene terephthalate) (PET) matrices. We investigated the ECM structure and expression, and observed how this was related to the cell nuclear morphology, as well as correlated nuclear morphology with cellular traits, such as proliferation, differentiation, and progenicity. Our results demonstrate that while perfusion facilitates high-cellularity in 3D constructs, the phenotype of hMSC are significantly affected by the differences in culture conditions. This in turn significantly impacts upon their tissue development characteristics. These results highlight the need for careful consideration in the design of culture systems for stem cells since chosen parameters wield considerable influence over the properties of the tissue-construct.

4.2 MATERIALS AND METHODS

4.2.1 Cell - Culture

HMSC were obtained following a method outlined in one of our prior publications (Grayson *et al.*, 2004). These cells were expanded using α-MEM (Life technologies) with 10% FBS and 1% Penicillin/Streptomycin (Life technologies, MD) and grown at 37 °C and 5% CO_2. Cells at the 5^{th} passage were used for subsequent static or perfusion cultures. PET matrices were obtained and treated as previously described (Li *et al.*, 2001). Briefly, commercial non-woven PET fabrics were washed, hydrolyzed with NaOH and thermally compressed. The resulting matrices were about 1.2 mm thick and had a porosity of approximately 89%. After treatment, the matrices were cut into disks of diameter of 1.6 cm for perfusion culture, or 1.2 cm for static culture.

4.2.2 Perfusion Culture

A perfusion bioreactor system with modular perfusion chambers and integrated seeding loop was setup as described previously (Zhao and Ma, 2005). Four chambers were utilized in parallel for ease of growing multiple constructs that could be harvested at different time points. Using the seeding loop, 8.0×10^5 cells seeded in each at a flow rate of 0.1 mL/min. Media was fed to the upper and lower portions of each chamber continuously at a flow rate of 0.1 ml/min for up to 40 days. The media in the reservoir was changed every 5-8 days. The chambers were taken out at different time points, and the constructs aseptically removed and used for various assays.

4.2.3 Static Culture

Scaffolds seeded with hMSC were grown in a humidified incubator under static conditions to compare with the constructs grown in the perfusion system. Cells were seeded onto the PET matrices using the depth filtration method detailed in a prior publication (Grayson *et al.*, 2004). Approximately 3×10^6 cells were seeded unto four matrices. After seeding, the PET matrices were transferred to 12-well plates, with one matrix per well in 3 ml of media at 37 °C and 5% CO_2. The media was changed every other day and the cultures were maintained for up to 40 days.

4.2.4 DNA Assay

Cell numbers in the PET matrices were determined using DNA assays following a method reported in a prior publication (Ma *et al.*, 2000). Briefly, a DNA standard was prepared by dissolving salmon testes DNA (Sigma, MO) in TEX (10 mM Tris, 1mM EDTA, 0.1% Triton X-100) and its concentration determined using a spectrophotometer. A serial dilution was prepared and used to give a standard curve for each assay. Constructs were lysed using TEX with 0.1mg/ml proteinase K (Sigma, MO) at 50 °C overnight. Sample aliquots (100 μL) were placed in triplicate into a 96-well plate and 100 μL of Picogreen (Molecular Probes, OR) was added to each well. The plate was then incubated at 37 °C for 10 min in the dark and read on a fluorescent plate reader (Fluoro Count, Packard).

4.2.5 Protein Assays and Western Blot Analysis

Total protein was extracted from the PET matrices in a lysing buffer (120mM NaCl, 25mM HEPES (pH 7.4), 1mM EDTA) containing 1% Triton X-100 and protease inhibitors (phenylmethylsulfonylfluoride, leupeptin, antipain and aprotinin). A stock protein solution was prepared by dissolving bovine serum albumin (BSA) (Fisher Scientific, PA) in DI H_2O from which a serial dilution was prepared and used to give a standard curve for each assay. Samples of 10 μL crude protein extracts were placed in triplicate into a 96-well plate and 200 μL of protein dye solution (Biorad, CA) was added to each well. The plate was read on a microplate reader (Biorad, CA). The specific protein content was calculated by dividing the total protein by the cell number in each matrix, and the results for each set of three matrices were averaged.

For Western Blotting, protein samples were separated by SDS gel polyacrylamide electrophoresis and transferred electrophoretically onto nitrocellulose membranes (0.2 μm). The membranes were then blocked using a Tris buffer containing 0.1% Tween-20 and 5% dry milk. Membranes were then incubated with primary antibody overnight, washed with blocking buffer and incubated with an alkaline phosphatase-conjugated secondary antibody for 1 hour at room temperature. Protein bands were determined using a chemiluminescence assay and densitometer readings were used to determine the intensity of the resulting bands. After probing the antibodies, membranes were blocked and re-probed with antibodies to β-actin. Target protein

bands were normalized to β-actin content.

4.2.6 Immunocytochemistry Staining

The expression of various extracellular matrix (ECM) proteins was examined by immunocytochemistry staining following the method described previously (Grayson *et al*., 2004). Briefly, the constructs were washed with PBS and fixed with 4% paraformaldehyde for 30 min at room temperature, blocked using PBS with 1% BSA, and then incubated with the primary antibody overnight at 4 °C. Constructs were washed with blocking buffer again and incubated with FITC -conjugated secondary antibody (Jackson Laboratories, PA) for 1 hour at room temperature. Constructs were stored in TBS and counterstained with PI in order to view the nuclear morphology. Samples were viewed using a Zeiss 510 confocal microscope.

4.2.7 Colony-Forming Unit – Fibroblasts (CFU-F) Assay

The CFU-F protocol was adapted from Di Girolamo *et al*. (1999). Cells from the constructs were trypsinized with 0.5 % trypsin/0.25% collagenase/ 1mM EDTA in PBS. Cells were drawn up and down in a pipette several times to ensure cell separation and then carefully counted. The suspension was then diluted in complete media and 800 cells were plated into a 10 cm Petri dish. Three dishes were plated for each construct and two constructs were used to obtain a CFU-F number for each data point. The cells were grown for 14 days then washed with PBS and stained with 0.25% crystal violet solution. The visible colonies were counted manually.

4.2.8 Osteoblastic Induction

HMSC were grown in either perfusion or static culture and harvested at different time points. Cells were then induced into osteoblasts as described into an earlier assay (Grayson *et al*, 2004) for an additional 10 days. Briefly, cells were cultured in basal media containing 100 nM dexamethasone, 10 mM sodium-β-glycerophosphate, and 0.05 mM ascorbic acid-2-phophate. After 10 days, the alkaline phosphatase (AP) activity of the cells was determined. Cells were digested in 500 μL lysing buffer (1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 0.1 mg/ml phenylmethylsulfonylfluoride, 3% aprotinin in PBS). 50 μL of cell lysate were added to 50 μL alkaline buffer and 50 μL *para*-nitrophenolphosphate substrate at 37 ^{o}C for 15 min. *Para*-nitrophenol was used to develop a standard curve. The amount of p-nitrophenol liberated was

determined by reading the absorbance at 405 nm.

4.2.9 Statistics/Data Analysis

Experimental results were expressed as means ± standard deviation (SD) of the means of samples. All the collected data for seeding and cell culture were analyzed by ANOVA for multiple comparisons, and statistical significance was accepted at $p < 0.05$.

4.3 RESULTS

4.3.1 ECM Protein Organization

HMSC grown in 3D PET under static conditions develop very structured collagen I fibrillar networks (Figure 4.1a), and demonstrate a striking contrast to the collagen I organization observed for hMSC grown in perfusion cultures (Figure 4.1b). Cells grown in perfusion express collagen I, which remains perinuclear as cells appear unable to secrete collagen into the extra-cellular spaces and organize them into fibrils. A contrast is also observed for the laminin expression and structure. Copious amounts of laminin are expressed in static culture concomitant with considerable alignment of the fibrils (Figure 4.1c). HMSC in perfusion cultures are able to organize laminin into fibrils, however, there is no discernable orientation or alignment of adjacent fibrils (Figure 4.1d). No differences were observed for the organization of fibronectin. Fibronectin fibrils were formed but appear disorganized in both static and perfusion conditions (Figure 4.1 e, f).

4.3.2 Nuclear Shape

The cell nuclei, as seen by PI staining, exhibited significant morphological variations depending on the length of the culture periods, positions within the matrices and culture conditions. After one week in culture, cells cultured under both conditions exhibited uniform nuclear morphologies: small and slightly elongated (Figure 4.2a, b). However, the morphologies of the nuclei of cells from long-term culture under the two culture conditions were sharply contrasting. After 35 days, nuclei of cells at the upper surface of the static constructs appear smaller than those at Day 7 and remain oval-shaped (Figure 4.2c). In contrast, the cells growing at the lower surface displayed extremely elongated nuclei (Figure 4.2e). At this time point, cells grown in the

bioreactor were tightly compacted together, uniformly distributed throughout the matrices, and displayed spherical nuclei on both the upper and lower surfaces that were larger than the nuclei of the original cells (Figure 4.2d, f).

4.3.3 Protein Expression

The total protein expression was compared in the two culture conditions and normalized to cell numbers. This normalized protein content was 6 – 7 folds higher for the cells grown under static condition compared to those grown in the bioreactor at the end of the 35-day culture period (Figure 4.3). In fact, the specific protein content in perfusion culture remained relatively constant throughout the first three weeks, dropping only slightly toward the end of the culture period. In contrast, the cells cultured without media flow had steady increases in the normalized total protein content indicating significant ECM secretion, but reaching a plateau after roughly 30 days of culture.

4.3.4 CFU-F and Differentiation

The proportion of primitive cells in the constructs was determined by observing the colony-forming ability of the cells. The original population of cells seeded into the static and perfusion cultures gave rise to 80 colony forming units from 800 seeded cells (Figure 4.4a). Both culture-conditions resulted in a progressive reduction in the percentage of progenitors in the population with increasing culture time. Cells cultured in the bioreactor maintain statistically lower percentages of colony-forming progenitor cells than static-culture hMSC at Days 14, 28 and 35 (Figure 4.4a). However, analysis of the absolute numbers of colony-forming cells in the constructs indicates that the number of progenitor cells increase in perfusion constructs during the log growth phase (Figure 4.4b).

The osteogenic potential of the cells were also assayed in a time-dependent manner. HMSC were harvested from the constructs each week, induced along the osteoblastic lineage, and the AP activity of the cells determined after 10 days of induction. Both cultures exhibited similar trends, peaking in osteogenic potential after 2 weeks of culture followed by a gradual decline (Figure 4.5). Perfusion culture constructs exhibited higher AP activity at each time point, but

differed significantly from static counterparts at Weeks 2 and 3 before falling to comparable levels at later time points.

4.3.5 Western Blots

Western blot analysis confirmed that there were differences in the amount of fibronectin secreted, but also demonstrated that there were similar patterns of expression in static and perfusion cultures (Figure 4.6). Perfusion cultures had higher fibronectin levels at weeks 1 and 3, but the levels of fibronectin in both cultures decreased considerably by Week 5.

Western blots were also used to investigate relative expressions of p53, a cell-cycle regulator and indicator of apoptosis. Cells in static culture express minimal levels of p53 for the first 3 weeks of culture, but the p53 expression levels become detectable by Week 5 (Figure 4.6). This coincides with the stagnation of cell numbers within the scaffolds. Meanwhile, cells in perfusion culture upregulated p53 during the first 3 weeks, when the cell density in perfusion constructs is low, reaching a maximum at Week 3. P53 levels then fell to minimal levels at week 5 (Figure 4.6) when cells were proliferating exponentially.

4.4 DISCUSSION

4.4.1 Perfusion Affects Tissue Organization

Bioreactors facilitate adequate nutrient supply, and enhance uniform development of cells growing within 3D constructs, but it is their ability to provide controlled, reproducible environments that make them a useful tool for understanding tissue-development in 3D matrices and enhancing the quality of tissue-engineered constructs (Martin *et al*, 2003). Tissue-constructs cultured under static media conditions exhibit two major limitations: Inhomogeneities result in non-uniform tissue-developmental patterns throughout the constructs, and secondly, as a result of poor nutrient transfer to the construct core, there is an artificial limit to the maximum cell densities that can be achieved within the constructs (Freed *et al*, 1994). Our earlier reports demonstrate that constructs grown in the bioreactor system exhibit an order of magnitude higher cell density within the scaffolds compared to static-culture constructs after 5 weeks of growth

(Zhao and Ma, 2005). This enhanced proliferation is well supported by the continuous influx of nutrients and oxygen, which does not become limiting in the perfusion system. Additionally, convectional flow systems improve nutrient transfer to the core of tissue constructs and produce uniform spatial patterning of cells and ECM proteins. This improves cellular development in the center of the scaffolds, and in the case of chondrocytes or osteoblasts, results in considerably improved differentiated phenotypes (Martin *et al*, 1999; Ishaug *et al*, 1997). While previous research has demonstrated the ability of our system to improve cellularity and uniformity, the effect on hMSC physiology is unclear. The focus of this study therefore was to investigate how perfusion conditions affected tissue-development properties and physiology of stem cells.

The structure and composition of ECM proteins determine the mechanical and physiological properties of tissues, guide organ development (De Arcangelis and Georges-Labouesse, 2000), and regulate the nature of interactions between the ECM and cells (Katz *et al*, 2000). The inability of the cells in bioreactor constructs to organize collagen I, or to align their laminin fibrils after extended culture indicates that the physical, as well as biochemical stimuli, are different in static and perfusion cultures. Theoretically, the high resistance to convective flow through the scaffolds dictates that nutrient transfer to the interior of the scaffold would take place primarily by diffusion, and that the mechanical environment should remain similar to the static condition. However, one possibility suggested by the lack of collagen I structure in perfusion cultures, is that there may be creeping flow around the cells, and that this added mechanical stimuli may disturb essential mechanisms for fibril formation and/or organization. Consequently, the resulting lack of ECM protein structure in the constructs impacts considerably on the capacity of the ECM to exert a reciprocal effect on hMSC physiological characteristics (Boudreau & Bissell, 1998). Observations of nuclear morphology via PI staining demonstrated a clear relationship between ECM organization and nuclear shape: Cells that are unable to organize collagen I have spherical nuclei, while the nuclei of hMSC grown under static conditions are elongated, parallel to ECM fibers. The model of cellular tensegrity suggests that cell-matrix adhesions mediate reciprocal interactions between the ECM and cytoskeleton such that ECM scaffolds can regulate cell-shape by resisting tensile forces generated by the cells (Ingber, 1997, 2003a, b). Such a model may help to explain why hMSC have spherical nuclei in the perfusion system, where there are no resistive collagen fibrils, and suggest that cells at the

lower surface of static culture constructs exhibit highly elongated nuclei as a result of the synergistic influence of the 2D culture surface and PET fibers. Following seeding, all hMSC extend along PET fibers, which provide the initial growth template. However, hMSC at the lower surface are also in contact with a 2D plastic surface, which - being very rigid - provides high resistance to tensile stresses. This induces hMSC to elongate, while still being guided by the PET fibers. Thus, rather than become flattened, they adopt an exaggerated spindle morphology. Cells at the upper surface of the static constructs, while also aligning with ECM molecules, do not exhibit such extensive nuclear elongation (Figure 4.2d), neither do cells cultured exclusively on 2D surfaces (Grayson *et al*, 2004).

4.4.2 Role of Nuclear Shape in Determining Cell-Fate

Results from our studies add a new dimension to the ongoing debate of the effect of cell and nuclear morphology on cell-fate. Cell and nuclear shape play a role in determining whether cells proliferate (Folkman and Moscona, 1978; Ingber, 1990), undergo apoptosis (Chen *et al*, 1997) or differentiate (Huang and Ingber, 2000). Previous studies suggest that the phenomena of cell-cycle arrest and apoptosis in unattached, non-transformed, adhesion-dependent cells is directly related to the rounding of their nuclei (Folkman and Moscona, 1978; Chen *et al*, 1997). HMSC, which are traditionally isolated by their strong adherence to tissue-culture plastics, typically exhibit an elongated, fibroblastic morphology. However, it was recently shown that hMSC were successfully cultured in suspension cultures, and in spite of their spherical morphology, demonstrated the ability to survive, proliferate, form colonies when subsequently plated, and differentiate (Baksh *et al*, 2003). This report gives the first indication that the requirements of cell attachment and spreading for proliferation of non-transformed, anchorage-dependent cells may possibly be an acquired characteristic. If hMSC were first plated unto tissue-culture flasks and then resuspended, they did not maintain their original anchorage independence properties, suggesting that merely culturing hMSC *in vitro* can itself affect cell phenotype.

Our correlations of cell-shape with their growth and differentiation characteristics also differ from earlier reports in the literature. It should be noted that all studies investigating the effect of cells' shape on their fate have used non-adhesive coating of a tissue-culture surface to limit adhesion (Folkman and Moscona, 1978), or combined this with micro-patterning of adhesive

proteins to control cell shape (Singhvi *et al*, 1994), and the number of adhesions that can be formed by individual cells with a substrate (Chen *et al*, 1997). Hence, the effect of cell-shape is always coupled to the cell-attachment to 2D surfaces, resulting in polarized cells, and adding another dimension to the interpretation of the cell-shape effect, which is usually ignored. However, this aspect may contribute, at least in part, to the atypical regulatory effects that were observed in the perfusion system. Cells in the reactor exhibit spherical nuclei, but in spite of this, continue to proliferate and do not display signs of apoptosis. The cells in the constructs by contrast are not polarized, since they are surrounded on all sides by other cells and disorganized ECM protein. Furthermore, our findings also differ from reports that cell spreading is essential for hMSC to be induced into osteoblasts, or that a spherical shape is a pre-requisite for adipogenic differentiation (McBeath *et al*, 2004): Spherical reactor cells increase AP activity and mineralize their ECM when induced into osteoblasts (Zhao and Ma, 2005), while elongated static cells exhibit significant lipid vacuole formation upon adipocytic induction (Grayson *et al*, 2004). It appears possible that cells within a tissue-construct utilize alternative regulatory controls, or that the combination of input signals to the cells in 3D constructs is integrated differentially from those reported for cells on 2D cultures. Hence, some of the conclusions from 2D studies on the effects of cell-shape may not be valid in the 3D system.

4.4.3 Perfusion affects cellular phenotype

The concomitant changes in nuclear architecture with variations in cell shape induce changes in gene expression (Thomas *et al*, 2002; Vergani *et al*, 2004). The contrasting nuclear morphologies observed in cells cultured under flow and static conditions are accompanied by changes in growth kinetics, proliferation rates, metabolic profiles, protein expression, ECM structure and differentiation potential, indicating that cells in the bioreactor are phenotypically different from those in static conditions. These cell/construct parameters are all inter-related, and more work is required to determine whether they give rise to, or result from the nuclear morphologies. However, Thomas and coworkers (2002) showed that changing the nuclear shape of osteogenic cells in culture affected their expression levels of collagen I. Their results demonstrated that collagen synthesis increased with nuclear elongation, up to an optimal distention of the nucleus. Meanwhile, Vergani *et al* (2004) found that as the nuclei of CHO fibroblasts became more spherical, their collagen mRNA levels were upregulated. These two

contradictory reports suggest that there may be no direct relationship between nuclear shape and collagen synthesis, but that the influence of cell shape is integrated together with other input signals to determine the cellular response. In our experiments, immunofluorescent detection of collagen I and laminin indicates that their expression is significantly upregulated in static cultures, and may be a significant contributor to the increased total protein levels of hMSC in static culture. However, the range of nuclear elongation displayed by cells at different depths within the static constructs makes it unfeasible to correlate an actual nuclear distention value with the protein expression.

Another interesting observation is that many of the changes displayed by cells in perfusion cultures are characteristic of transformed cells; high proliferation and metabolic rates, apparent loss of anchorage-dependence and shape-responsive controls (Wittelsberger *et al*, 1981), and reduced fibronectin expression (Brenner *et al*, 2000) at the latter stages of culture. However p53 expression, another indicator of oncogenic transformation, is downregulated after Week 3 in perfusion culture. P53 is the most commonly mutated gene in human tumors, and its expression increases when cells are transformed (Santini *et al*, 2000). Hence, decreased p53 levels at latter stages of reactor culture contrast the other data and suggest that cells in the bioreactor may not be tumorigenic. In fact, p53 also plays an integral role as a cell-cycle inhibitor. Its expression patterns in both static and perfusion cultures correlate with the growth-kinetics in these cultures suggesting that its primary role in these cultures is to act as a check point in the cell-cycle. Cell-transformation though is a complex biological process, and more in-depth characterization, including chromosomal analysis, is needed to make a definitive conclusion on the nature of the transformed properties of cells grown in the perfusion bioreactor.

4.4.4 Colony-Forming Assays and Differentiation Potential

Despite the uncertainty of the phenotype of cells under different culture conditions, we have shown previously that constructs from both static and perfusion cultures maintain the ability to differentiate along both the osteoblastic and adipogenic lineages (Grayson *et al*, 2004; Zhao and Ma, 2005). Multi-lineage differentiation is used to test the functional characteristics of cells cultured within the constructs. In these experiments, constructs were again induced to differentiate into osteoblasts, and their AP activity measured to compare their osteogenic

potential. Surprisingly, cells in the reactor, despite their low collagen I expression prior to induction, rounded shape, and disorganized ECM, consistently maintained higher AP activity than those in static cultures at day 10 after induction. Recent studies of stem cell plasticity have demonstrated fully differentiated phenotypes being induced to display gene-expression characteristics of other cell types in culture. Hence, apparent phenotypic drift from the stem-cell phenotype during culture does not affect multi-lineage differentiation abilities in a predictable fashion, if at all. Other studies investigating the osteogenic potential of hMSC constructs with oscillating fluid–flow, showed that even when the gene expression of osteocalcin, osteonectin increased, AP activity was reduced relative to control cultures. This indicates that there are limitations to using solely AP activity as an absolute measure of osteogenic potential (Li *et al*, 2004).

4.5 CONCLUSIONS

Cells undergo different tissue-development processes in 3-D culture (Cukierman *et al*, 2001). Cell-survival, proliferation, differentiation and ECM secretion are all influenced by the spatial organization of cells (Yamada and Clark, 2002; Zahir and Weaver, 2004) in 3D constructs. However, the advantages of 3D scaffolds in tissue-engineering applications is checked by challenges, such as the uniformity and functionality of the construct. Bioreactor systems have succeeded in alleviating these concerns by enhancing nutrient transport and providing controlled microenvironments. Yet, as this and other studies demonstrate, the unique microenvironments created in the perfusion system impacts upon a broad range of cellular events. Thus, obtaining tissue constructs with desired functional characteristics may require a fundamental understanding of 3D cell biology, as well as exquisite control of the culture microenvironment. The effects of the culture parameters may be more pronounced for stem cells that have high proliferation potential and plasticity, are acutely sensitive to environmental cues, and whose development depends to a large extent on the orchestrated cellular events of growth, ECM secretion, and differentiation. This study demonstrates that perfusion conditions significantly impact on cellular gene expression and the physiological characteristics of the cells in the tissue-construct. Thus, as the field of cell-based, tissue-engineered constructs develops, so too must our understanding of the culture microenvironment on the functional properties of the end-product.

Figure 4.1 Immunofluorescent staining of ECM proteins in static and perfusion cultures. ECM protein expression of hMSC constructs cultured under static (a,c,e) and perfusion (b,d,f) conditions after 35 days culture. (a) HMSC grown under static conditions express large amounts of collagen I that is highly structured, whereas (b) collagen I expression in perfusion is restricted to cytoplasmic regions. (c) Laminin fibrils are also organized into fibrous networks in static culture. (d) Cells in perfusion can form laminin fibrils but they lack any particular orientation. (e,f) Fibronectin expression is similar in both culture conditions.

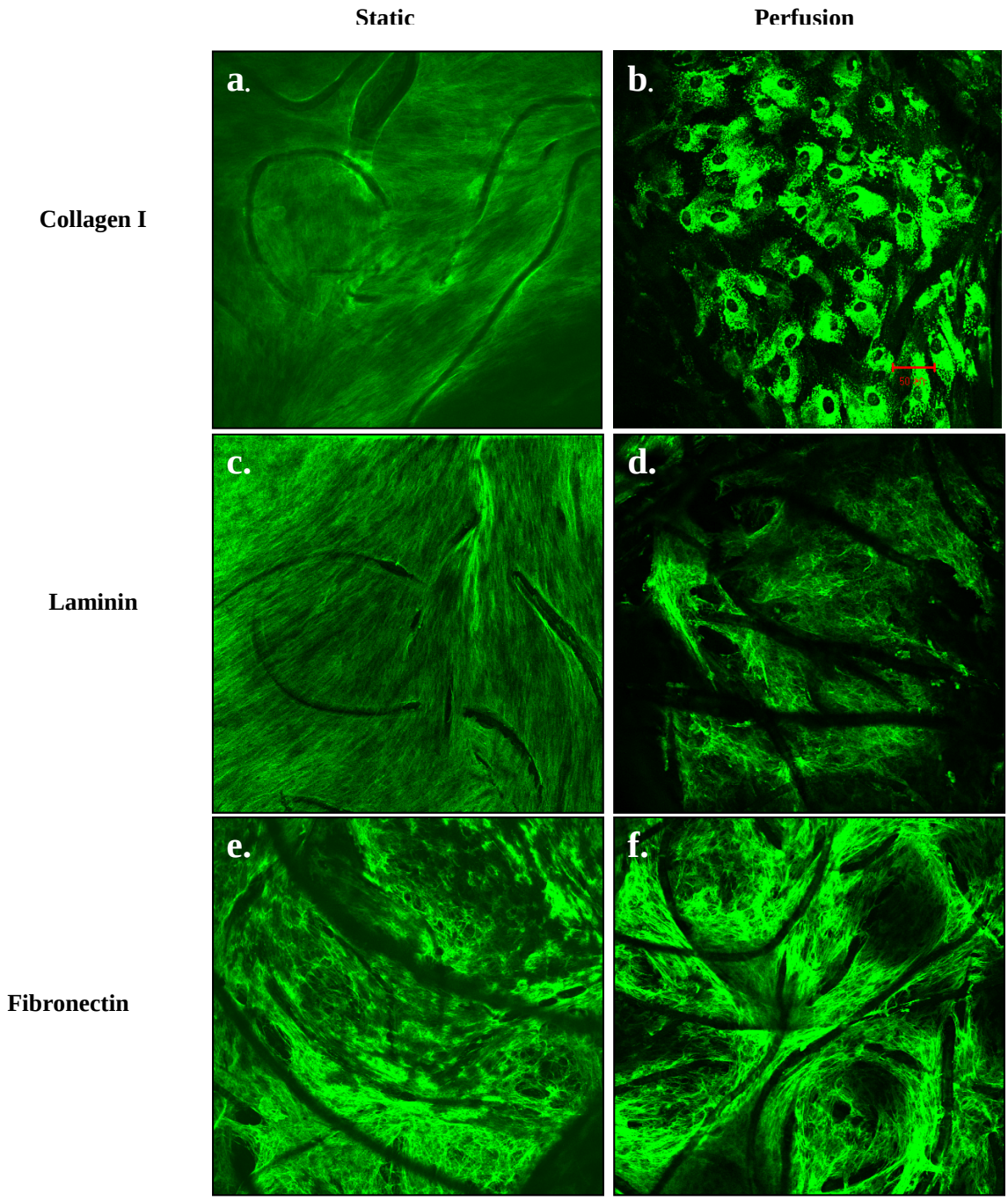
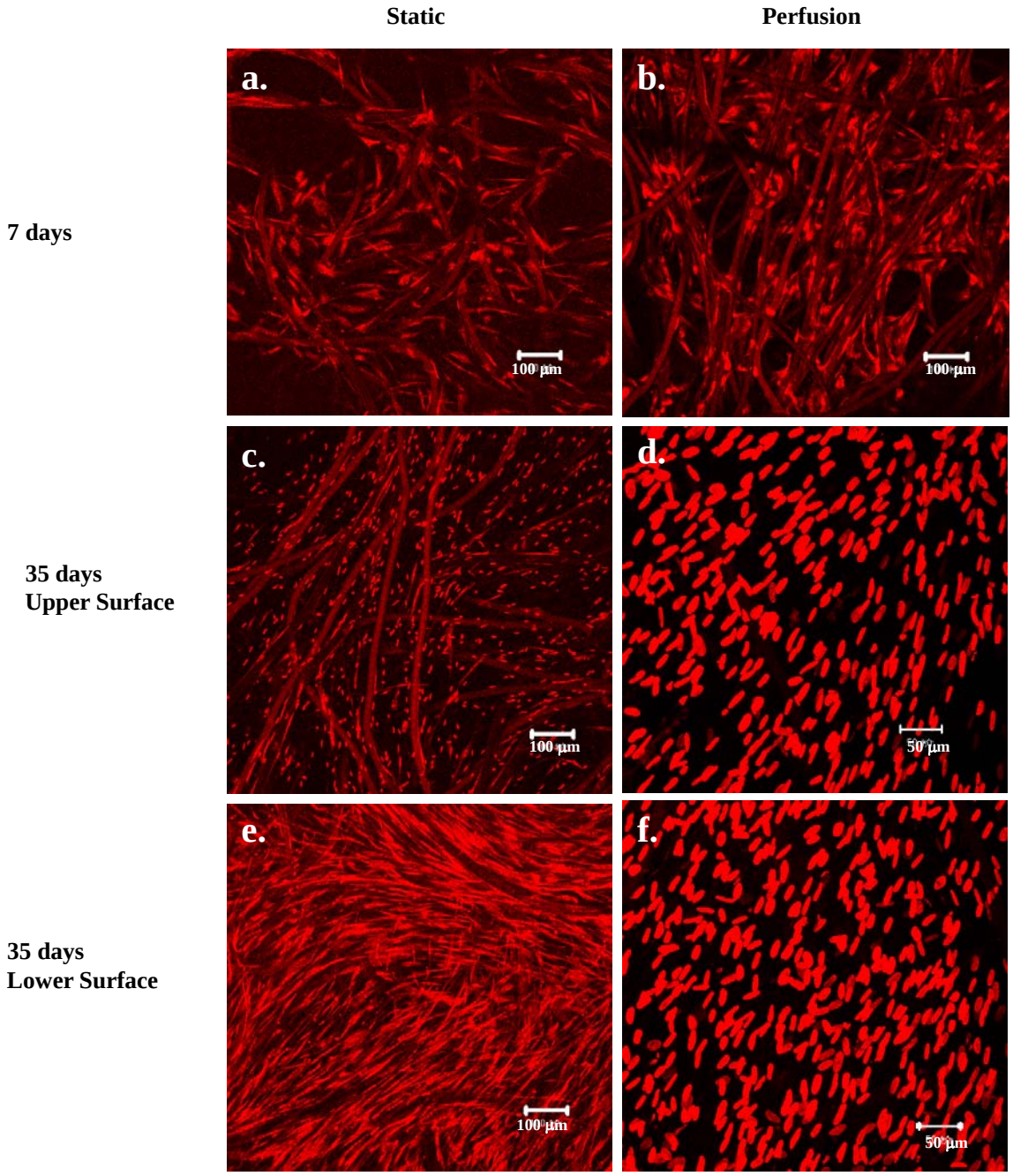

Static
Perfusion
Collagen I
Laminin
Fibronectin
a.
b.
c.
d.
e.
f.

Figure 4.2 PI staining showing morphology of cell nuclei.

Morphology of cell nuclei of the hMSC cultured under static (a,c,e) and perfusion (b,d,f) conditions. (a,b) After 7 days in culture cells cultured under both conditions exhibited uniform nuclear morphologies. After 35 days in culture, (c) cells at upper surface of static culture exhibit small, oval-shaped nuclei while (d) nuclei of cells from perfusion culture are large and spherical. (e) Cells at lower surface of static constructs have highly elongated nuclei but (f) no difference is observed in nuclear morphology at lower surface of perfusion constructs.


Static
Perfusion
7 days
35 days
Upper Surface
35 days
Lower Surface
a.
b.
c.
d.
e.
f.
100 µm
100 µm
100 µm
50 µm
100 µm
50 µm

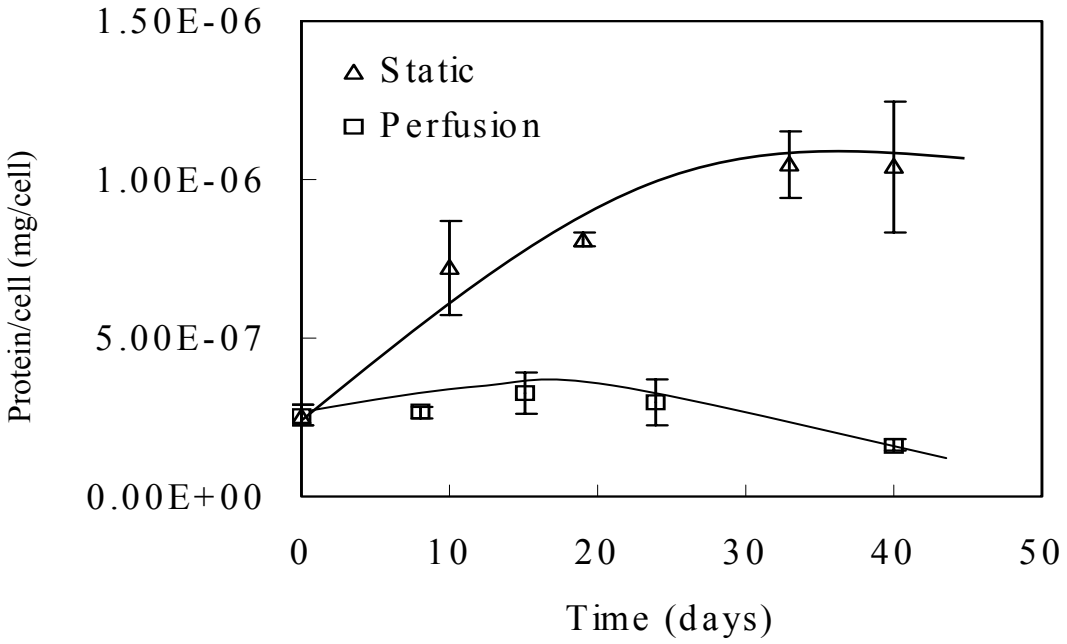


Figure 4.3 Total proteins content normalized to hMSC number.

(□) Perfusion and (△) Static culture conditions. Protein levels in static increased to approximately seven times the original content and are significantly higher than protein levels in perfusion cultures throughout the culture period. Protein levels in perfusion constructs remain relatively constant throughout the culture period.

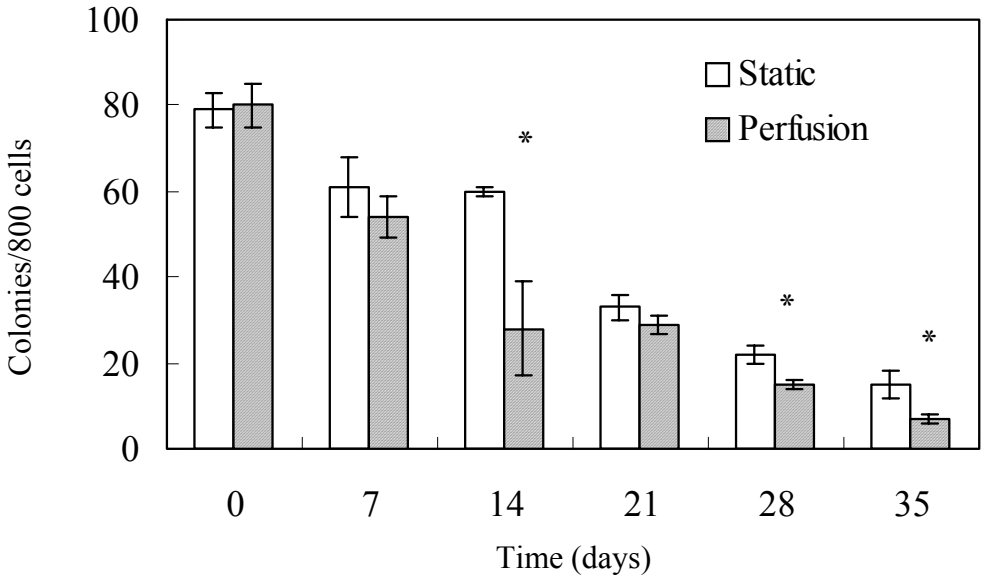


Figure 4.4a Variation of hMSC CFU-F potential with time in cultures.

Percentage of CFU-F present in the constructs under static and perfusion cultures. Static cultures maintain higher CFU-F percentages towards the end of the culture period (* $p < 0.05$).

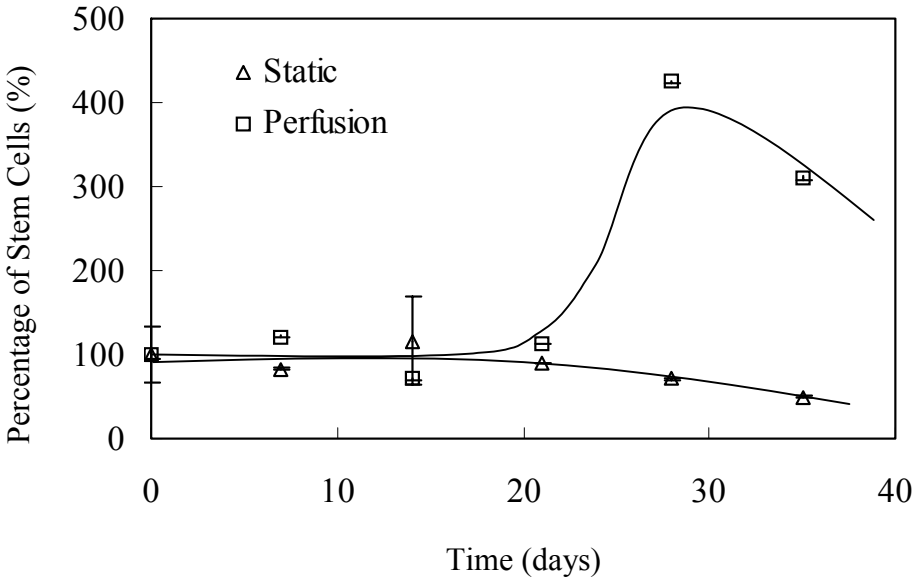


Figure 4.4b CFU-F numbers normalized to original CFU-F population.

Despite lower CFU-F percentages, significantly higher cell numbers in perfusion after day 21, indicate that the number of colony-forming progenitors actually increases in perfusion with time.

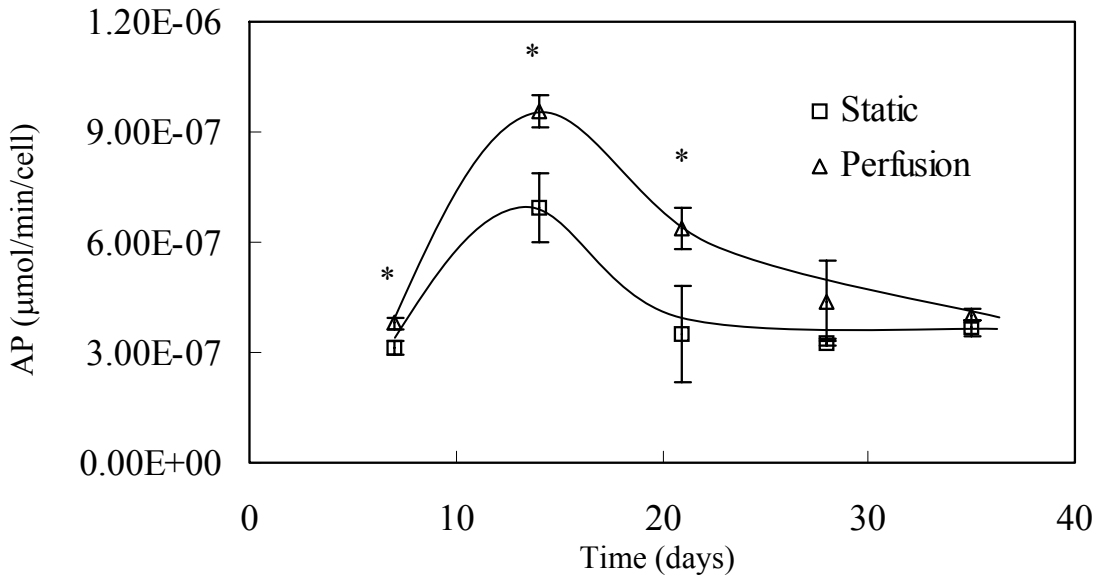


Figure 4.5 Variation of osteo-inductive potential with time in cultures.

AP level of the hMSC constructs after predetermined time (x-axis) of (△) perfusion or (□) static cultures followed by a 10-day period of osteogenic differentiation. AP levels are higher for perfusion constructs at all time points (* $p < 0.05$).

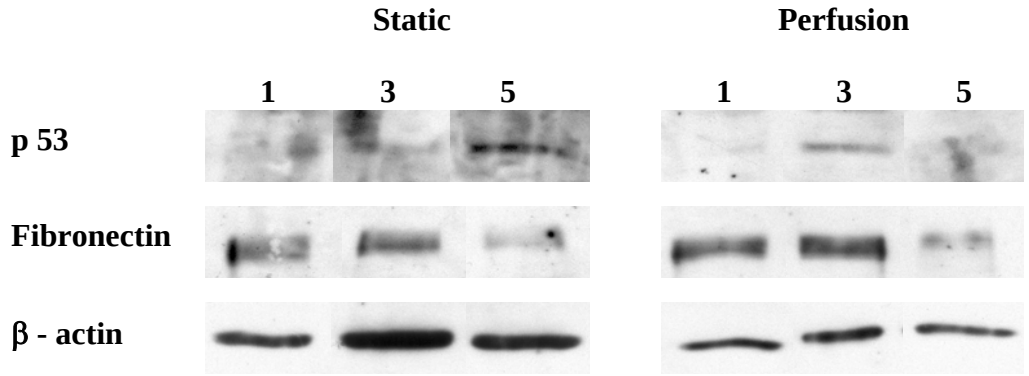


Figure 4.6 Western blots of fibronectin and p53 expression in hMSC constructs. Fibronectin levels are higher in perfusion constructs (relative to beta-actin immunoblots) but show the same trend in both static and perfusion cultures. P53 patterns differ under static and perfusion conditions. Perfusion cultures increase p53 expression after week 1 to a maximum expression occurring at week 3. Expression decreases thereafter. Static cultures demonstrate increasing expression of p53 with time in culture.

CHAPTER 5

EFFECT OF HYPOXIA ON THE TISSUE-DEVELOPMENT OF HUMAN MESENCHYMAL STEM CELLS

5.1 INTRODUCTION

A major limitation in the use of hMSC to develop functional tissue-constructs is the identification of appropriate *in vitro* conditions for the developing tissue, which retain the cells' native characteristics, prevent senescence and optimize their ability to treat damaged, diseased or degenerated tissues (Derubeis and Cancedda, 2004). Three-dimensional (3D) cell-biology methods possess fundamental advantages over traditional culture techniques for providing insight into the native characteristics of cells: They allow cells to adopt their native morphology, facilitate cell-cell contact as well as contact with the extra-cellular matrix (ECM), and significantly alter signaling processes (Abbott, 2003; Cukierman *et al*, 2001). Stem cells are particularly responsive to their microenvironments and hMSC grown within 3D scaffolds exhibit considerably altered growth characteristics, ECM patterning and differentiation capabilities (Grayson *et al*, 2004). Furthermore, 3D scaffolds are critical to tissue-engineering applications as they allow the *in vitro* development of living tissue-constructs that can be implanted into defect sites for clinical repair. Apart from altering cellular characteristics, synthetic 3D scaffolds offer a tangible level of control over tissue morphogenesis as they may guide cell development into complex structures (Levenberg *et al*, 2003). Yet, combining the physical influence of 3D scaffolds with biochemical cues further mimics the *in vivo* tissue-development environment, and allows even greater influence over these *in vitro* development processes. Oxygen is a potent biochemical signaling molecule. It regulates a broad range of cellular events critical to the development of multi-cellular organisms and plays important roles in normal and pathological physiological states. In this report, we investigate oxygen's role as a process parameter in developing engineered tissue constructs from hMSC.

Oxygen is a major gene-regulator during an organism's development, and also exerts significant effects on the *in vitro* growth and development of mammalian cells. The particular response to *in vitro* hypoxia is cell-type dependent, but oxygen affects critical cellular processes such as adhesion, (Lash *et al*, 2001), proliferation (Packer and Fuehr, 1977), metabolism (Loike *et al*, 1992), apoptosis (Carmeliet *et al*, 1998), growth factor expression (Minchenko *et al*, 1994), ECM secretion (Horino *et al*, 2002) and differentiation patterns (Lennon *et al*, 2001). Experimental evidence suggests that hematopoietic stem cells (HSC) and their progeny are spatially oriented within the bone marrow with respect to oxygen concentrations. HSC reside in regions having the lowest oxygen tension (Cipolleschi *et al*, 1993). Therefore, the cell-cycle patterns and differentiation characteristics of HSC are affected by changes in the oxygen concentrations during culture (Ivanovic *et al*, 2002). Several reports demonstrate that the *in vitro* characteristics of MSC may also be affected by the oxygen tension: Primary, nucleated cells from rat bone-marrow aspirates, cultured at 5% O_2 exhibited higher colony formation and increased their proliferation rates, as well as their *in vivo* and *in vitro* differentiation potential relative to control cultures (Lennon *et al*, 2001). Annabi *et al* (2003) grew late-passage murine MSC in Matrigel at 1% O_2 for 48 hours to examine their angiogenic properties. Hypoxia induced upregulation of bFGF, VEGF and MT-MMP1 compared to their normoxic counterparts, and increased migration rates and tube-formation were observed among the oxygen-deprived cells. This phenomenon indicates a role for MSC in tumor angiogenesis, but may also explain why MSC enhance myocardial neovascularization after infarction (Kocher *et al*, 2001). Hence, oxygen affects cellular characteristics and tissue-remodeling processes and may have the potential to direct stem cell fate in the development of 3D tissue constructs from hMSC.

In this study, we investigated the effects of reduced oxygen tension on the development of hMSC grown in 3D PET constructs. Key aspects of tissue-morphogenesis, including cell-growth characteristics, protein expression, ECM secretion, and differentiation were investigated under conditions of 2% and 20% oxygen tension. The results from our experiments indicate that hMSC tissue-constructs cultured at 2% oxygen demonstrated a switch in the metabolic pathways and exhibited increased proliferation potential compared to those cultured at normal oxygen tensions. Changes in total protein levels and ECM expression suggest that hypoxia modifies

hMSC tissue development processes. Furthermore, hypoxic conditions better maintain the stem cell properties of undifferentiated hMSC, and enhance their differentiation ability. These findings indicate that oxygen is an important component of the *in vivo* hMSC microenvironment, and regulating the oxygen concentration can impact upon the developmental patterns of hMSC.

5.2 MATERIALS AND METHODS

5.2.1 Reagents

Cell culture media, FBS and Penicillin/Streptomycin were obtained from Life Technologies (Rockville, MD). Antibodies to ECM proteins were obtained from ICN Biomedical (Costa Mesa, CA). All other reagents were obtained from Sigma (St. Louis, MO) unless otherwise stated.

5.2.2 Cell-Culture

Bone marrow derived hMSC were isolated as previously described (Sekiya *et al*, 2002a; Di Girolamo *et al*, 1999). Briefly, bone marrow aspirates of about 2 ml were drawn from healthy donors ranging in age from 19 to 49 years under an Institutional Review Board-approved protocol. Plastic adherent nucleated cells were separated from the aspirate and cultured using alpha MEM with 10% FBS and 1% penicillin/streptomycin at 37 ^{o}C and 5% CO_2. Approximately 3 x 10^6 cells were seeded onto four PET matrices via a depth filtration that was described previously (Grayson *et al*, 2004). The four PET matrices from each filtration were then transferred to a common chamber with 25 ml media where they were exposed to 95% Air - 5% CO_2 (normoxic) or 2% O_2 - 5% CO_2 - 93% N_2 (hypoxic) gas mixtures.

5.2.3 Metabolic Activity

Media samples were collected through sampling ports in the hypoxic chambers or by simple aspiration in normoxic cultures. Glucose and lactate concentrations were determined using a 2500 Biochemistry Select Analyzer (YSI Incorporated, OH). These values were used to determine glucose consumption (G) and lactate production (L) in cultures and generate a graph of the L/G molar ratios.

5.2.4 DNA Assays

A DNA standard was prepared by dissolving salmon testes DNA in TEX (10 mM Tris, 1mM EDTA, 0.1% Triton X-100) and its concentration confirmed using light at 260 nm from a μQuant UV spectrophotometer (Biotek Instruments, VT). Serial dilutions were prepared and used to construct a standard curve for each assay. Cells in the PET matrices were lysed by placing the entire scaffolds in TEX with 0.1mg/ml proteinase K (Fisher Scientific, PA) at 50^{o}C overnight. Samples of cell lysate (100 μL) were placed in duplicate or triplicate in to a 96-well plate and 100 μL of Picogreen (Molecular Probes, OR) added to each well. The plate was incubated at 37^{o}C for 10 min in the dark and then read on a Packard Fluoro Count fluorescent plate reader (GMI, MN).

5.2.5 BrdU Staining

BrdU was injected into the media of the cultures through a port to give a final concentration of 0.5 μg/ml. Since cells in the scaffolds proliferate slowly, a relatively long brdU incorporation time was used (20 hrs). Upon harvesting, cell-constructs were washed with PBS and fixed with 70% ice-cold ethanol for 30 min. Cells were denatured using 1N HCl - 0.1% Triton X-100, blocked and incubated with mouse monoclonal anti-BrdU conjugated to FITC overnight at 4 ^{o}C. Constructs were then washed and stored in TBS and counterstained with propidium iodide (PI) prior to viewing with a Zeiss 510 confocal microscope. In order to obtain statistical data, arbitrary view fields were chosen and the number of BrdU positive cells was recorded as a percentage of the total number of PI-stained cells.

5.2.6 Protein Assay and Western Blot Analysis

Total protein was extracted from the PET matrices in a lysing buffer (120mM NaCl, 25mM HEPES (pH 7.4), 1mM EDTA) containing 1% Triton X-100 and protease inhibitors (phenylmethylsulfonylfluoride, leupeptin, antipain and aprotinin). A small volume of the lysate was used to perform protein assays. A stock protein solution was prepared from bovine serum albumin (BSA) (Fisher Scientific, PA), and a serial dilution was generated to use as a standard curve. A colorimetric assay using a protein dye solution (Biorad, CA) was used to determine protein concentrations in the cell lysates. The specific protein content was calculated by dividing the total protein by the cell number in each matrix, and the results for each set of three matrices

were averaged.

For western blotting, cell lysate samples were separated by SDS gel polyacrylamide electrophoresis and transferred electrophoretically onto nitrocellulose membranes (0.2 μm). The membranes were then blocked using a Tris buffer containing 0.1% Tween-20 and 5% dry milk. Membranes were then incubated with primary antibody overnight, washed with blocking buffer and incubated with an alkaline phosphatase-conjugated secondary antibody for 1 hour at room temperature. A detection kit from Biorad (Hercules, CA) was used to detect the specified proteins using chemiluminescence. Membranes were then washed in blocking buffer and re-probed with antibodies to β-actin. Blots were read on a densitometer and normalized to β-actin contents to quantify relative amounts of proteins.

5.2.7 Colony Forming Unit – Fibroblast

The CFU-F protocol was adapted from (Di Girolamo *et al*, 1999). Cells from the construct were removed using a solution containing 0.5 % trypsin/ 0.25 % collagenase/ 1mM EDTA in PBS. Cells were drawn up and down in a pipette several times to ensure cell separation and then carefully counted. The suspension was then diluted in complete media and 800 cells were plated into a 10 cm Petri dish (14 cells/cm^2). Samples for each condition were done in duplicate. The cells were grown for 12 – 14 days at 37 ^oC and 5% CO_2 in a humidified incubator. Upon harvesting, cells were washed with PBS and stained with 0.5% crystal violet solution for 10 – 15 minutes at room temperature. Cells were washed twice with PBS and the visible colonies were counted.

5.2.8 Immunocytochemistry Staining

The expression of various extracellular matrix (ECM) proteins was examined by immunocytochemistry staining following the method described previously (Grayson *et al*., 2004). Briefly, the constructs were washed with PBS and fixed with 4% paraformaldehyde for 30 min at room temperature, blocked using PBS with 1% BSA, and then incubated with the primary antibody overnight at 4 °C. Constructs were washed with blocking buffer again and incubated with FITC -conjugated secondary antibody (Jackson Laboratories, PA) for 1 hour at

room temperature. Constructs were stored in TBS and counterstained with PI in order to view the nuclear morphology. Samples were viewed using a Zeiss 510 confocal microscope.

5.2.9 Osteoblast Differentiation

Differentiation of hMSC along osteoblastic lineages was carried out as previously described (Grayson *et al*, 2004). Firstly, cells were cultured in complete media for 28 days under hypoxia or normoxia, then transferred to induction media (αMEM, 10% FBS, 100 nM dexamethasone, 10 mM sodium-β-glycerophosphate and 0.05 mM ascorbic acid-2-phosphate)- at 20% O_2. Cell-constructs were harvested at days 5, 10, 14, and 19 after induction and cut into quarters. These were used to determine DNA content, ECM mineralization, alkaline phosphate activities and the osteonectin mRNA expression levels. The von Kossa staining technique was used to detect any mineralization of the ECM in the tissue-constructs. Matrices were removed from the wells and washed. The cells were fixed in 10% formalin solution for 1 hour then washed in silver nitrate under a bright light for approximately 15 minutes or until the calcium nodules turned black. The cells were differentiated in 5% sodium thiosulfate, washed and photographed with a digital camera. Cell-constructs were digested with 500 μL lysing buffer (PBS with 1% Triton X-100, 0.5% sodium deoxycholate, 0.1 % SDS, 0.1 mg/ml phenylmethylsulfonylfluoride, 3% aprotinin), 50 μL of cell lysate was then added to 50 μL of substrate and 50 μL alkaline buffer. The mixture was placed in 37 ^{o}C water bath for 15 minutes after which the reaction was stopped using 100 μL of 0.5 N NaOH solution. P-nitrophenol was used to construct a standard curve. The amount of p-nitrophenol liberated was determined using an absorbance reader with a 415 nm filter.

5.2.10 Adipocyte Differentiation

HMSC constructs were first cultured for 3 weeks in hypoxic or normoxic conditions before being harvested and placed in differentiation media. Cells were induced by treating with adipogenic induction (AI) medium (high glucose DMEM supplemented with 10% FBS, 0.2 mM indomethacin, 0.5 mM isobutyl-1-methyl xanthine, 1 μM dexamethasone and 5 μg/ml insulin). After 2 days, the AI media was removed and replaced with adipogenic maintenance (AM) medium (high glucose DMEM supplemented with 10% FBS and 10 μg/ml insulin). This treatment was continued in a cyclic fashion. Differentiation into adipocytes was detected using

the Nile Red assays, which stained lipid vacuoles. Stock Nile Red solution was prepared in acetone at a concentration of 1mg/ml following the protocol described by Greenspan *et al* (1985). The cells were fixed in 1.5% glutaraldehyde in PBS and then washed with PBS. Stock Nile Red solution was diluted in PBS at a ratio of 1:100 and the cells were incubated in this diluted solution for thirty minutes. The cells were viewed using a Zeiss 510 confocal microscope.

5.2.11 Scanning Electron Microscopy (SEM)

PET matrices containing the cells were removed from the wells, washed in PBS and fixed in 2.5% glutaraldehyde in 0.1M sodium cacodylate buffer (Electron Microscopy Sciences, Fort Washington, PA) at pH 7.4 for 2 - 4 hours. The cells were then rinsed in buffer and post-fixed in 2% osmium tetraoxide in 0.1 M cacodylate buffer (Electron Microscopy Sciences, Fort Washington, PA) for 2 hours. After rinsing, the cells were dried using a series of graded ethanol washings followed by critical point drying in CO_2. The dried samples were sputter coated using palladium with gold plating and then observed using a JSM 840 scanning electron microscope.

5.2.12 mRNA Extraction

Messenger RNA was extracted directly from the hMSC constructs using a kit obtained from Qiagen (Valencia, CA). Samples were then stored frozen at –80 ^{o}C and shipped in dry-ice overnight to Tulane University to be analyzed by RT-PCR.

5.2.13 Statistical Analysis

Experimental results were expressed as means ± standard deviation (SD) of the means of samples. All the collected data were analyzed by student's t-test for comparisons between normoxic and hypoxic cultures, and statistical significance was accepted at $p < 0.05$.

5.3 RESULTS

5.3.1 Metabolism Profile

Cell-constructs cultured under hypoxic conditions exhibited higher glucose consumption and lactate production relative to normoxic cultures throughout the culture period. The L/G ratio was also higher for hypoxic samples, but the difference only became significant after 12 days of culture. The L/G values of both cultures were similarly low at the initial stages of culture. However, within 10 days, the ratio in normoxic cultures stabilized at 1.6 – 1.7. L/G ratio for hypoxic cell-constructs increased steadily over the first 15 days then stabilized at approximately 2.5 (Figure 5.1). The huge differences in L/G ratios indicate alternative metabolic pathways may be utilized under the two conditions

5.3.2 Growth Kinetics

The growth kinetics of hMSC in the PET scaffolds under normal oxygen conditions follow a previously described pattern (Grayson *et al*, 2004). There is a lag-phase that lasts approximately 5 days, followed by a linear growth phase, and then cell numbers remain constant after 24 – 25 days when the number of cells in the matrix reaches 2×10^6. Reducing oxygen tension alters the growth kinetics of hMSC in 3D scaffolds. Cells cultured in hypoxic conditions exhibit a prolonged lag-phase, with essentially no growth taking place during the first nine days of culture (Figure 5.2). Between Days 10 and 23, cells in both constructs appeared to have similar growth rates. However, the major differences in growth occurred subsequent to day 23. While normoxic cultures entered a plateau phase, cells cultured under hypoxia continued proliferating. The cell numbers were similar after 30 days of culture, but cells in the two conditions appeared to be at different stages in the growth - cycle.

This growth patterns obtained from DNA assays were corroborated by doing a brdU analysis. BrdU positive cells were stained, counted and compared to the total number of cells in each view-field (Figure 5.3a). The percentage of cells incorporating brdU into their DNA was higher in normoxic samples at the early time points (days 9 and 16). However, there was a sharp

reversal at day 24 where brdU incorporation in the hypoxic samples was significantly higher than that of normoxic cultures (Figure 5.3b).

Further validation for the kinetic pattern was obtained from western blot analysis of cell-cycle proteins (Figure 5.4). Analysis of cyclin B1, an indicator of mitosis, revealed that the expression of cyclin B1 increased continually throughout the culture period in hypoxic samples, but did not change much in the normoxic samples where expression levels remained minimal. In contrast, p21 and p53 levels increased continuously from day 16 to day 30 in normoxic samples, correlating with decreased proliferation in these constructs. There was a slight increase in p21 levels for hypoxic cultures, but p53 levels remained constant throughout culture.

5.3.3 Protein Content and ECM expression

Hypoxic cells had statistically higher protein:DNA ratios than cells grown under 20% oxygen after just 5 days of culture (Table 5.1). As the cells proliferated, the protein:DNA ratios decreased significantly, but the hypoxic samples still retained significantly higher protein:DNA content. Immunofluorescent staining of collagen I, laminin and fibronectin at day 5 Discernable differences in fluorescence intensities were observed for fibronectin and laminin, where normoxic cultures stained much more intensely than the control constructs (Figure 5.5). Western blot analysis of total protein extracts confirmed higher fibronectin expression at the earlier time points, but also indicated temporal variation in fibronectin levels. Normoxic samples expressed high levels of fibronectin early in the culture period followed by a decrease in fibronectin expression at the latter stages. Conversely, there was a steady increase in the fibronectin expression of hypoxic cultures throughout culture time (Figure 5.6).

5.3.4 CFU-F and Differentiation

The colony-forming potential differed significantly for cells cultured under the two oxygen conditions (Figure 5.7a). Cells seeded at day 0 had CFU-F values of ~ 80 (out of 800 cells seeded into the Petri dish). However, after culture in 2% O_2, the CFU-F numbers of hypoxic hMSC averaged approximately 110 (Figure 5.7b), suggesting that low oxygen conditions may facilitate the survival of more primitive cells selectively over more mature cells. Opposite trends

were observed for cells grown at 20% O_2. CFU-F numbers decreased within the first 9 days of culture and remained in the range of 40 - 45 throughout one month of culture (Figure 5.7b).

Three assays were used to observe osteoblastic differentiation of hMSC. Von Kossa methods demonstrate darker staining of constructs cultured under hypoxia up to day 14 of induction. There was no observable difference at day 19 (Figure 5.8a). The AP activity increased for both sets of constructs (Figure 5.8b). Constructs cultured under 20% oxygen showed the characteristic AP peak at day 14. Hypoxic constructs maintained slightly higher AP values at Days 5 and 10, but while normoxic cultures peaked at day 14, the AP activity of the hypoxic samples continued to increase steadily during this period. A drastic increase in AP activity of hypoxic samples was seen at day 19, indicating that there is a temporal variation in the AP expression patterns after hMSC are cultured in hypoxia. The numerical values of AP activity were statistically similar at all time points except day 19, where it was higher for hypoxic samples. Osteonectin mRNA is strongly expressed by cells in both normoxic and hypoxic constructs (Figure 5.8c). The osteonectin levels remained roughly constant at different time points, particularly for the normoxic cultures. Both osteonectin and GAPDH mRNA levels appear lower in the hypoxic samples at day 19, however, if osteonectin levels are normalized to those of GAPDH, then osteonectin expression appears to be elevated at this time point.

Constructs from both conditions also exhibit adipogenic differentiation capabilities as evidenced by Nile Red staining (Figure 5.9a). Additionally, the cells adopt a spherical morphology characteristic of adipocytic differentiation (Figure 5.9b) and upregulate their LPL expression compared to un-induced controls (Figure 5.9c). Hypoxic samples also exhibit increased LPL expression relative to that of normoxic constructs.

5.3 DISCUSSION

5.4.1 HMSC Adaptation to Hypoxic Environments

Mammalian cells typically respond to hypoxic conditions by increasing their carbohydrate consumption and switching to anaerobic respiration in order to remain viable (Guillemin and Krasnow, 1997; Lin *et al*, 2000; Malhorta *et al*, 1999; Plas and Thompson, 2002; Downard,

2003). However, different cell types vary in their ability to cope with reduced oxygen, and many elicit cell-type specific responses to decreases in oxygen tensions e.g. HSC are partially characterized by their ability to thrive under hypoxic conditions (Krishnamurthy *et al*, 2004). Yet despite their close association with hMSC, little is known about the hypoxic response of hMSC. In our experiments hMSC exhibit increased glucose consumption and lactate production when incubated in 2% oxygen environments, and reveal an initial acclimatization phase where they also down-regulate non-essential functions i.e. cells did not proliferate and there was little ECM secretion during the first 9 days of culture. Their increasing L/G molar ratios during this period to a final value of 2.5 (compared to 1.7 for normoxic cultures) suggest that hypoxic hMSC gradually switch to a glycolytic pathway in order to maintain ATP levels. During the acclimatization period however, hypoxic hMSC expressed significantly higher protein levels. This is consistent with findings that *de novo* protein synthesis is a critical element of the cellular hypoxic response (Guillemin and Krasnow, 1997) as cells express a number of proteins required for increased glucose consumption, and the switch to glycolytic metabolism (Loike *et al*, 1992; Bashan *et al*, 1992, Firth *et al*, 1994). The lower ECM protein expression observed in hypoxic cultures at this time point indicates that the higher protein content is a result of increased intracellular proteins, most likely related to upregulation of protein components required for glycolysis.

Hypoxia induces cell-cycle arrest in many cell types. Gardner *et al* (2001), showed that culturing primary and transformed mouse embryonic fibroblasts (MEF) under hypoxia inhibited their progression through the G_1 phase of the cell-cycle. There was no concomitant increase in cell-cycle inhibitors p21, such as reported for endothelial cells (Iida *et al*, 2002), or p53 expression. Despite remaining quiescent, hMSC also show little variation in their p21 and p53 expression at the initial stages of hypoxic culture, indicating that hypoxia does not induce expression of these proteins in hMSC. P53 and p21 also have a role in regulating apoptotic events, therefore their lack of upregulation is also suggestive of continued hMSC viability. During the extended culture period afforded in 3D scaffolds, hMSC actually express lower levels of p21 and p53, and display higher cyclin B1 expression under hypoxia relative to normoxic controls. Thus it appears that hMSC *in vitro* proliferation is actually enhanced by chronic hypoxia. It has been demonstrated that hMSC exhibit three growth phases (Bruder *et al*, 1997),

which are all prolonged in 3D scaffolds (Grayson *et al*, 2004). The first two phases are further extended under hypoxia, which induces both a longer lag phase and a longer growth phase, and the plateau phase is not reached within the 30 days of hypoxic culture. Together, the growth kinetics curve, increased brdU incorporation, and higher cyclin B1 expression suggest that hypoxic hMSC can continue proliferating subsequent to day 30, and these enhanced self-renewal properties under hypoxia may indicate the maintenance of a more primitive stem cell phenotype.

5.4.2 Maintenance of Stem-Cell Characteristics

The average oxygen tension within bone marrow has been measured at ~5% (Mostafa *et al*, 2000). However, oxygen gradients exist within the marrow compartment as cells surrounding the sinuses compete for limited oxygen and nutrients leaving cells further away from the sinuses severely oxygen depleted. It is in this extremely hypoxic environment that HSC exist and are able to survive in a quiescent state using glycolytic pathways (Cipolleschi *et al*, 1993). Culturing hematopoietic stem or progenitor cells at different oxygen concentrations reveal that lower oxygen tensions (~ 1% O_2) maintain the earliest progenitor cells (Cipolleschi *et al*, 1993; Ivanovic *et al*, 2000b), while increasing the oxygen tension leads to differentiation into more mature hematopoietic phenotypes (Mostafa *et al*, 2000; Hevehan *et al*, 2000). HMSC are an integral component of the hematopoietic stem cell niche, suggesting that these two cellular phenotypes reside within similar compartments within the bone marrow. It is thus plausible to hypothesize that, as a result of this intimate relationship, hMSC survival and differentiation will be governed by similar mechanisms as those observed for HSC.

Hypoxia not only facilitated hMSC expansion as described above, but also allowed them to retain their stem cell characteristics. The hMSC population is heterogenous (Shur *et al*, 2002) and various culture conditions may selects for either more primitive or more mature sub-populations (Sekiya *et al*, 2002b). HMSC cultured at 2% O_2 maintain much higher colony forming numbers than cells cultured at 20% O_2. CFU-F numbers in hypoxic cultures were also higher than those of the original cell population seeded into the matrices indicating that the more primitive cells are being selected by oxygen deprivation. Thus hMSC, like HSC, appear significantly more resistant to hypoxia than their progeny. HSC express the breast cancer resistance protein (BCRP), which is thought to confer hypoxic resistance upon stem cells as the

interaction of BCRP with heme-containing porphyrins under hypoxic conditions reduces their toxic effects (Krishnamurthy *et al*, 2004). BCRP is conserved in many stem cell types (Bunting, 2002; Zhou *et al*, 2001), and is likely to be playing a similar role in maintaining hMSC in a viable state under hypoxia.

High CFU-F potential has been correlated with high *in vitro* lifespan (Di Girolamo *et al*, 1999), hence this data further corroborates the extended proliferation observed in hypoxic hMSC constructs. A correlation has also been established between cells having extended *in vitro* proliferation and those with higher differentiation potential (Bunting and Hawley, 2003). In light of the striking contrast in CFU potential and growth-kinetics between normoxic and hypoxic constructs then, the differences in osteoblastic and adipocytic differentiation appear uncharacteristically small. Still, following osteoblastic and adipocytic differentiation, von Kossa staining and AP activity, as well as osteonectin and LPL mRNA levels all conclusively indicated greater differentiation potential of the hypoxic constructs. Following induction, AP levels typically exhibit a characteristic peak during early differentiation processes, followed by a decrease as bone-formation progresses (Holtorf *et al*, 2005). Hypoxic constructs differed in levels of AP activity, as well as the temporal onset of the AP peak. Previous results indicate that AP values peak between day 12 and day 15 post-induction (Grayson *et al*, 2004). Normoxic cultures showed their highest AP values at Day 14 (although it is not known if the peak occurred between Day 10 and Day 14). Meanwhile, the AP levels for the hypoxic constructs increased consistently throughout the differentiation period indicating that the peak may have occurred after the sampling period.

5.4.3 Hypoxia and Tissue-Development

Oxygen concentrations significantly influence the way that cells remodel their environments. I*n vivo,* considerable vascular remodeling occurs in response to hypoxia, particularly in a wound model, and is largely directed by upregulated gene expression in endothelial cells (Faller, 1999). Cells in culture also exhibit remodeling capabilities in response to hypoxia. Human dermal fibroblasts regulated mRNA expression of VEGF, type IαI collagen and MMP-3 following exposure to hypoxia, mimicking gene expression patterns that occur *in vivo*, which are important to tissue-remodeling in wound-repair mechanisms (Steinbrech *et al*, 1999). Lung fibroblasts

from fetal rats upregulated collagen I synthesis when grown under 5% oxygen (Horino *et al*, 2002), while chondrocytes increase their collagen II expression and maintained their differentiated phenotypes better at lower oxygen tensions (Pfander *et al*, 2003; Kurz *et al*, 2004). Circulating stem and progenitor cells *in vivo* are also recruited to sites of tissue-damage by hypoxic environments through expression of stem-cell derived factor-1 (Ceradini *et al*, 2004).

Hypoxic environments during wound healing events persist for a relatively short period. Consequently, most studies of hypoxia were conducted within 2 days, and only transient responses in gene expression are observed in some cases (Steinbrech *et al*, 1999). It is not known if any transient changes occurred during the first 5 days of hypoxic culture. HMSC collagen synthesis after 5 days of hypoxia appeared unchanged, but major differences in hMSC ECM expression were observed for fibronectin. Fibronectin is a major ECM component, and is involved primarily in cell adhesion and migration. Earlier reports demonstrate that under hypoxia, trophoblasts and carcinoma cells decreased their ability to adhere to fibronectin, and downregulated their expression of α_5 integrin fibronectin receptor (Lash *et al*, 2001). HMSC cultured *in vitro* typically express fibrillar fibronectin (Grayson *et al*, 2004). The early decrease in fibronectin expression in hMSC in hypoxic constructs is thought to be associated with the acclimatization phase. However, hypoxic hMSC increase fibronectin expression throughout the culture period, whereas hMSC cultured at normal oxygen tension decrease their fibronectin expression after two weeks. Loss of fibronectin expression by hMSC has been correlated with differentiation (Munoz-Elias *et al*, 2003), hence these results provide further evidence that hypoxia helps to maintain the undifferentiated state of hMSC, and may explain why fibronectin expression, like growth rate, continues to increase with time in culture.

5.5 CONCLUSIONS

Cellular responses to hypoxia are mediated by the hypoxia-inducible factor-1α (HIF-1α), which shows the greatest sensitivity in oxygen concentrations within the range of 0.5 – 2.0 % (Jiang *et al*, 1996). This range coincides with the hypothesized oxygen concentrations in the *in vivo* hMSC environment. Oxygen gradients exist within 3D cellularized scaffolds particularly under no media-flow conditions (Malda *et al*, 2004), hence, using 2% oxygen to culture hMSC results

in a physiologically relevant range of oxygen tensions within the constructs. Understanding the combined effects of oxygen and 3D topography on hMSC allow the manipulation of these parameters in order to expand these cells and develop functional tissue-constructs. HMSC thrive in prolonged conditions of extreme hypoxia suggesting that low-oxygen tensions may be an essential component of the hMSC niche. The majority of experiments reported in the literature were typically performed on two-dimensional substrates and within 48 hours of the onset of hypoxia in order to mimic the wound environment. However, sustained hypoxia is a parameter of the normal physiology of hMSC, and hypoxic constructs maintained hMSC proliferation and differentiation characteristics much better than normoxic samples. Further studies are required to determine how the higher cell proliferation and fibronectin expression will impact on the mechanical properties of the ECM fibrils under hypoxia. The findings from this study demonstrate that oxygen tension is a key parameter in the *in vitro* culture of hMSC and their development into tissues.

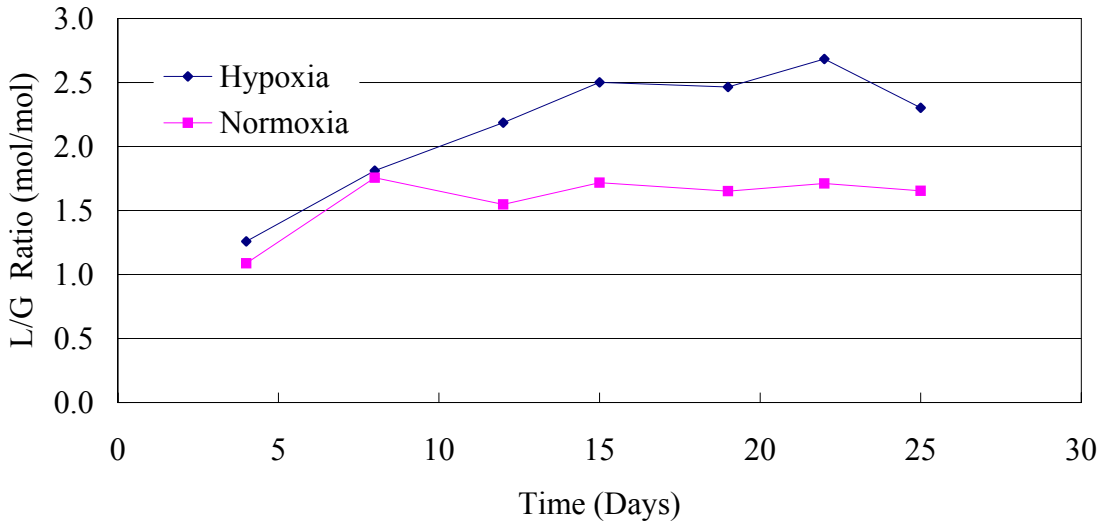


Figure 5.1. Molar Lactate:Glucose Ratios for HMSC in Culture
Ratio of lactate production (L) to glucose consumption (G) for constructs under both hypoxic and normoxic culture conditions over a 24-day time-period demonstrate higher ratios under hypoxic culture (2.5) compared to normoxic culture (~1.7)

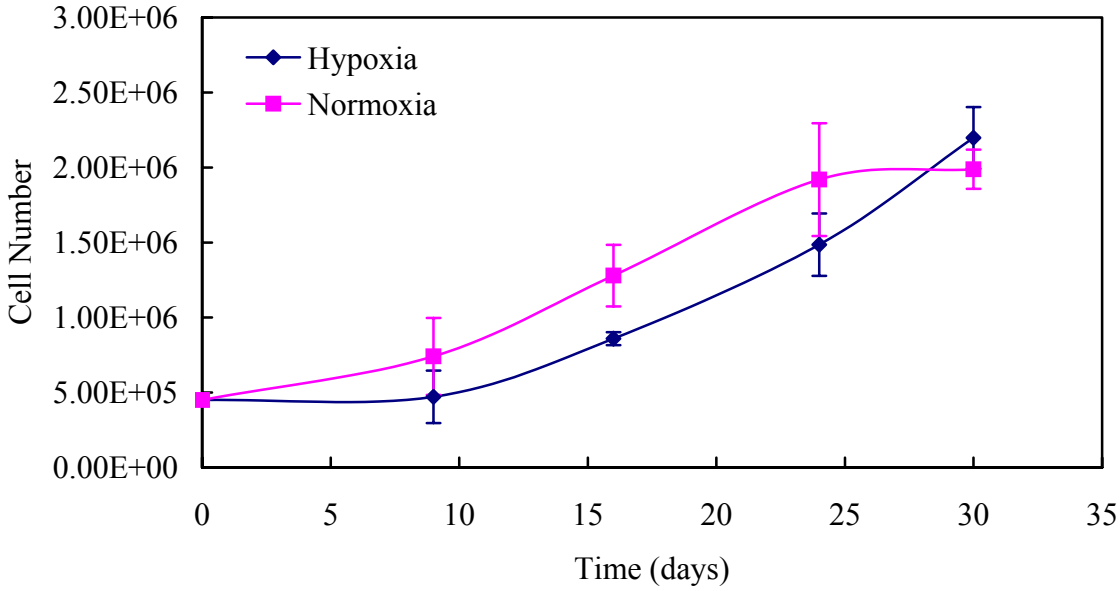


Figure 5.2. Growth kinetic curves.
HMSC grown within the 3D constructs under hypoxic and normoxic culture conditions.

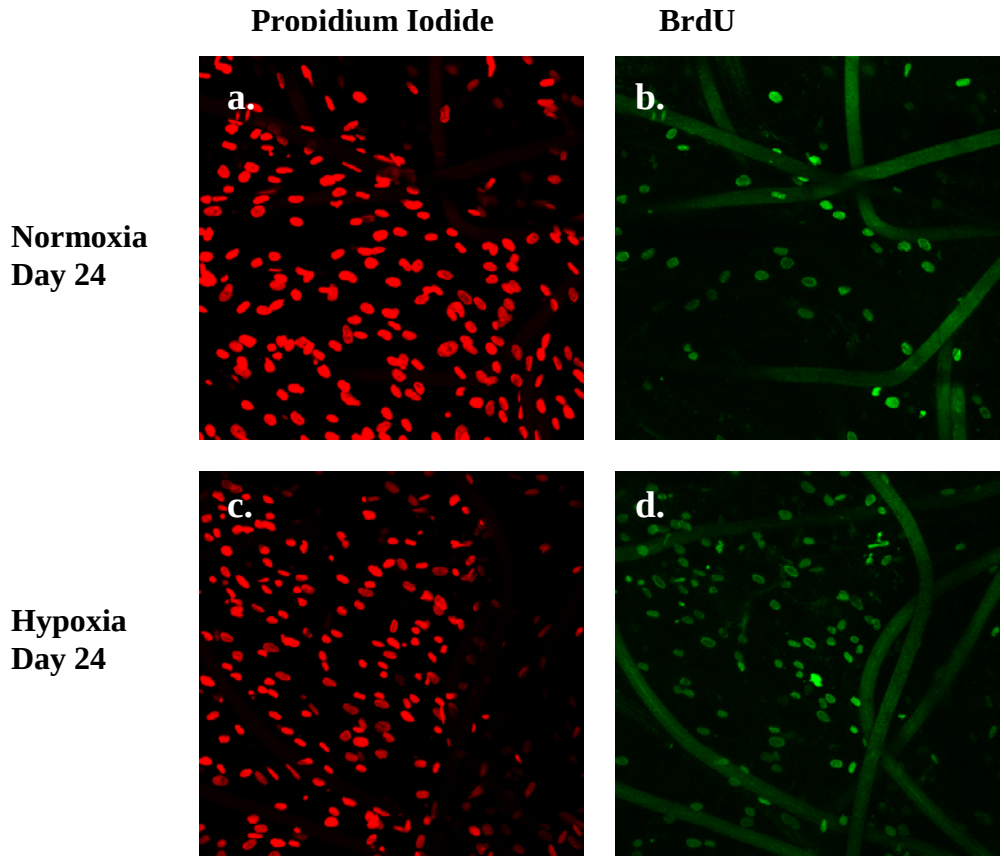


Figure 5.3a. Immunofluorescent Staining of BrdU and PI
Representative view fields from both conditions showing (**a**, **c**) PI stained cells (red) and (**b**, **d**) brdU positive cells (green) at Day 24 or culture.

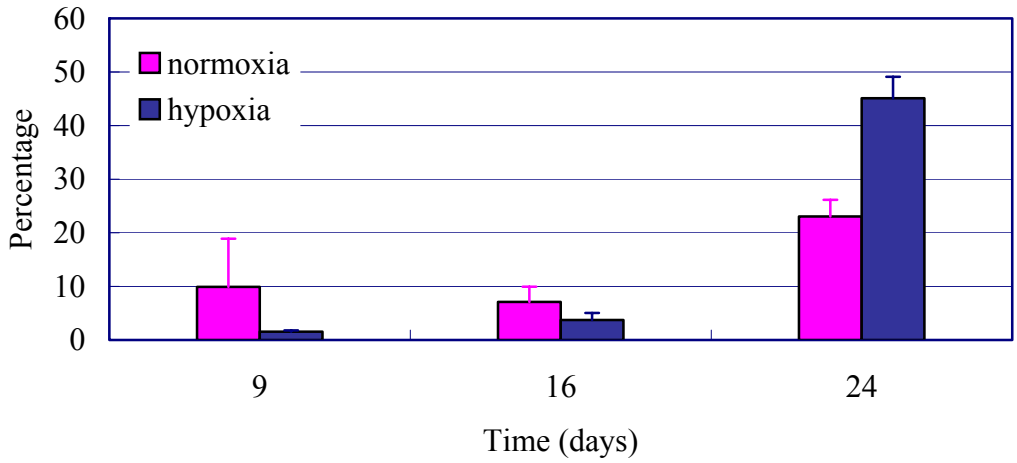


Figure 5.3b. Percentage of brdU-positive cells in culture.
Percentages were obtained from arbitrary view fields at different times in culture. Results from several view fields (n = 4) were collated and statistical analysis performed using student's t-test. (* $p < 0.05$)

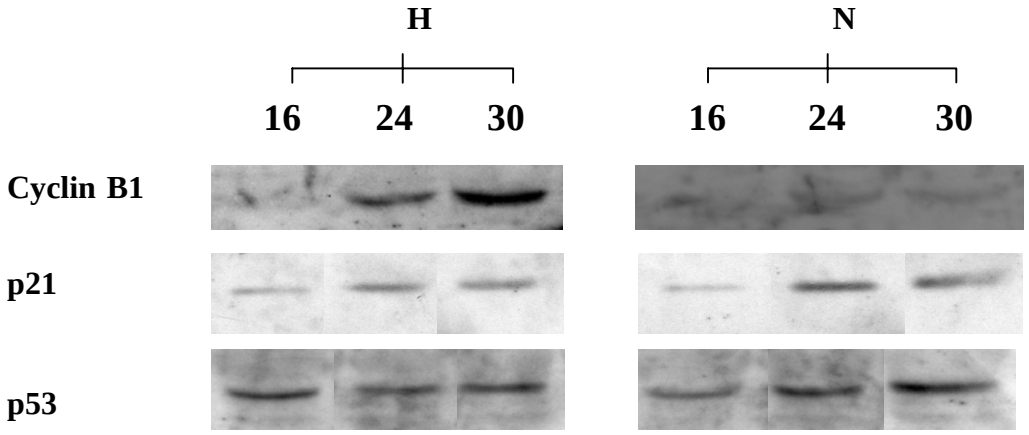


Figure 5.4. Western blots of cyclin B1, p21 and p53 expression.
Normoxic cultures maintain basal levels of cyclin B1 expression but a gradual increase in both p21 and p53 expression with time in culture. Hypoxic cultures exhibit gradual increase in cyclin B1 expression, with significantly higher expression at day 30. P21 levels increase slightly throughout culture but p53 expression remains relatively unchanged.

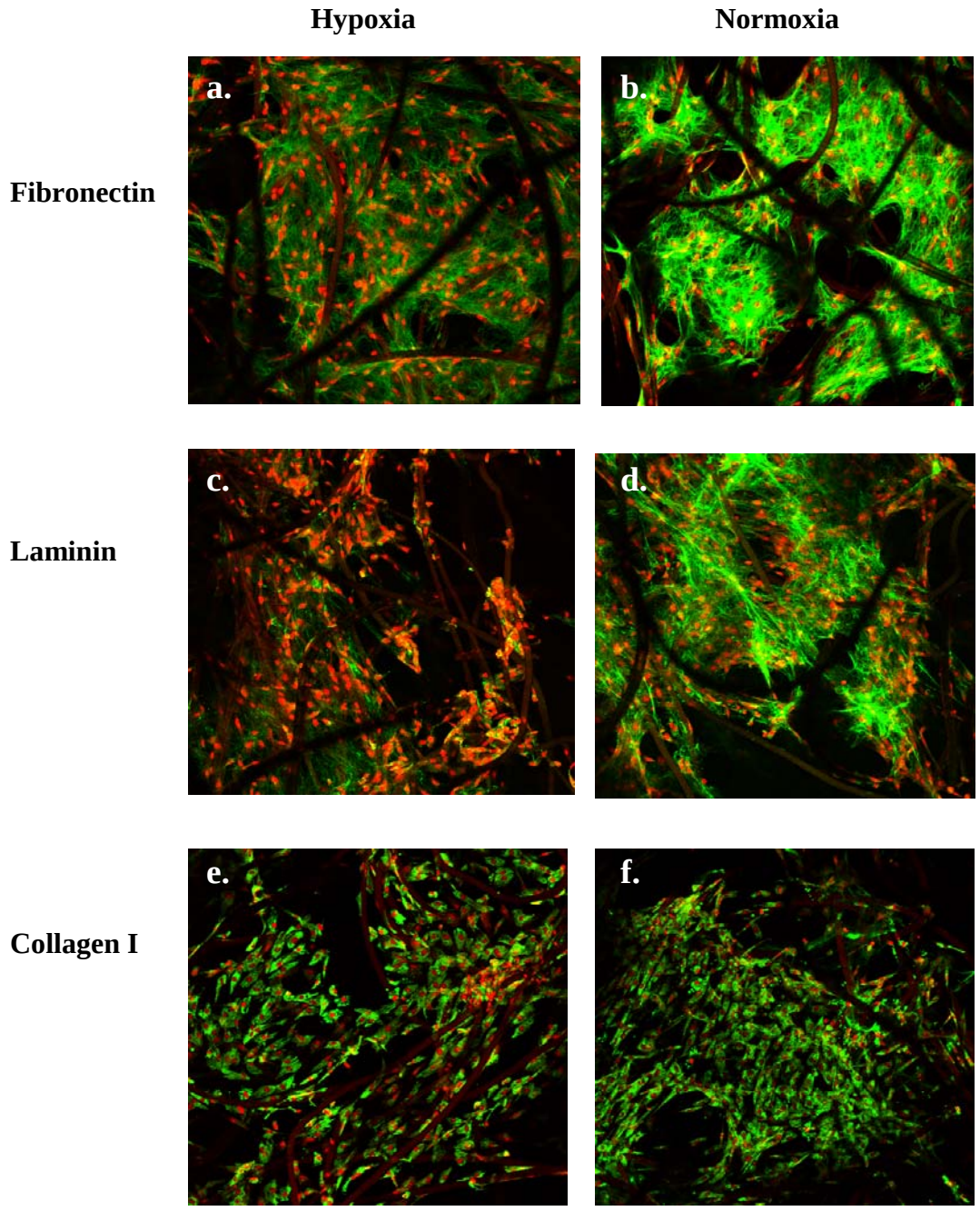


Figure 5.5 Immunofluorescence Staining of ECM Proteins
(a, b) fibronectin, (c, d) laminin and (e, f) collagen I on 3-D PET matrices after 5 days of hypoxic or normoxic culture. The intensity of the normoxic stains for fibronectin (b) and laminin (d) are significantly brighter than those of the hypoxic cultures (a, c). There is no discernable difference in intensity of Collagen I stains (e, f).

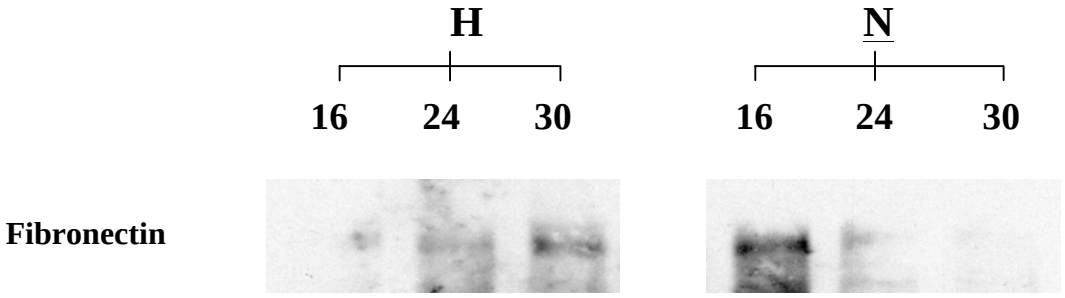


Figure 5.6 Western Blots of Fibronectin Expression.
Time course analysis at days 16, 24 and 30 of culture period showing significantly different expression patterns for hypoxic and normoxic cultures.

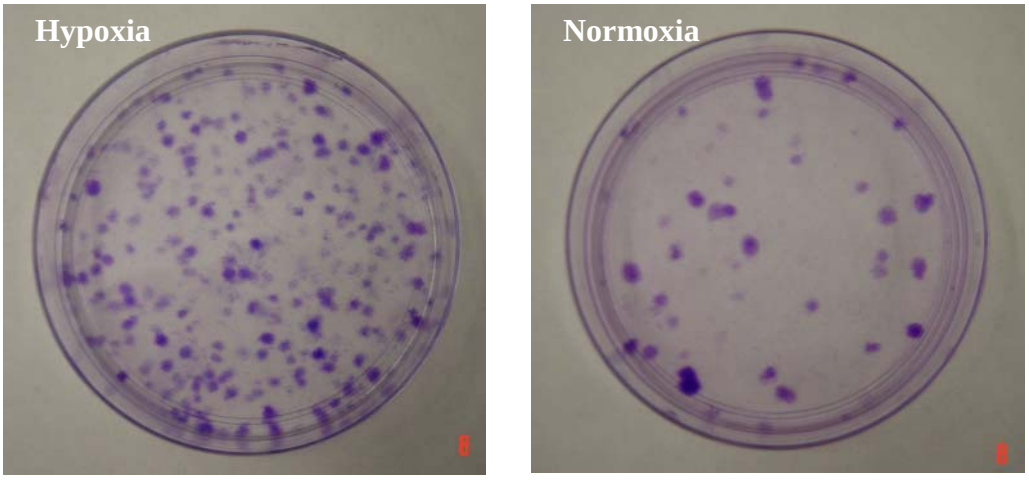


Figure 5.7a Crystal Violet Staining of hMSC Colonies.
Representative CFU-F assay results showing drastic differences in colony-forming abilities of normoxic and hypoxic cells.

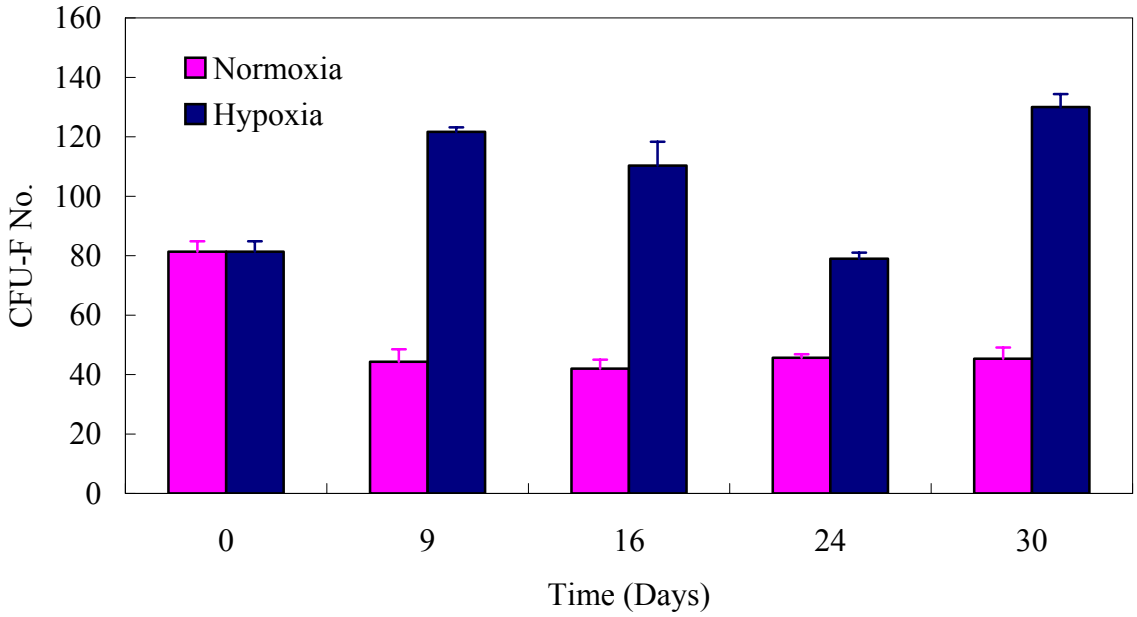


Figure 5.7b Statistical analysis of CFU-F Numbers at Different Time Points.
Statistical analysis was performed using student's t-test. ($n = 2$; * $p < 0.05$)

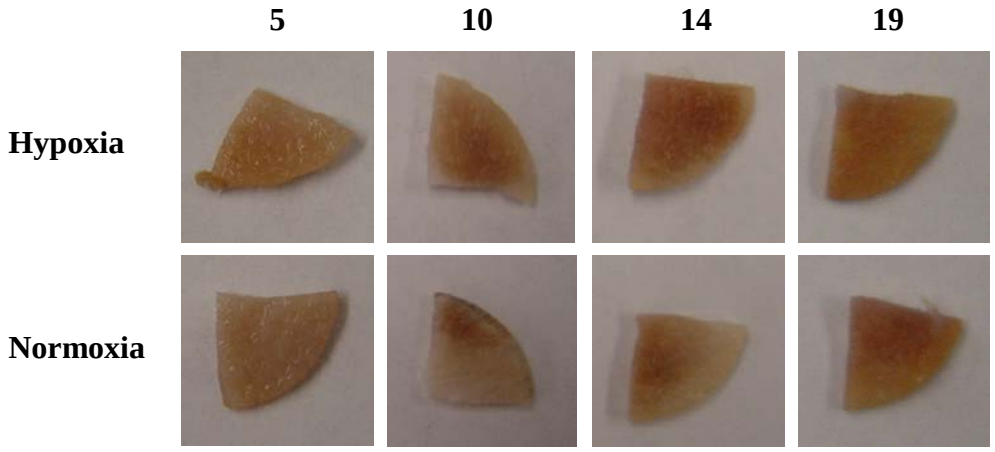


Figure 5.8a Von Kossa staining of Osteo-Induced hMSC Constructs.
Samples from hypoxic cultures stain more intensely than normoxic cultures at days 5, 10 and 14. There is no discernable difference at day 19.

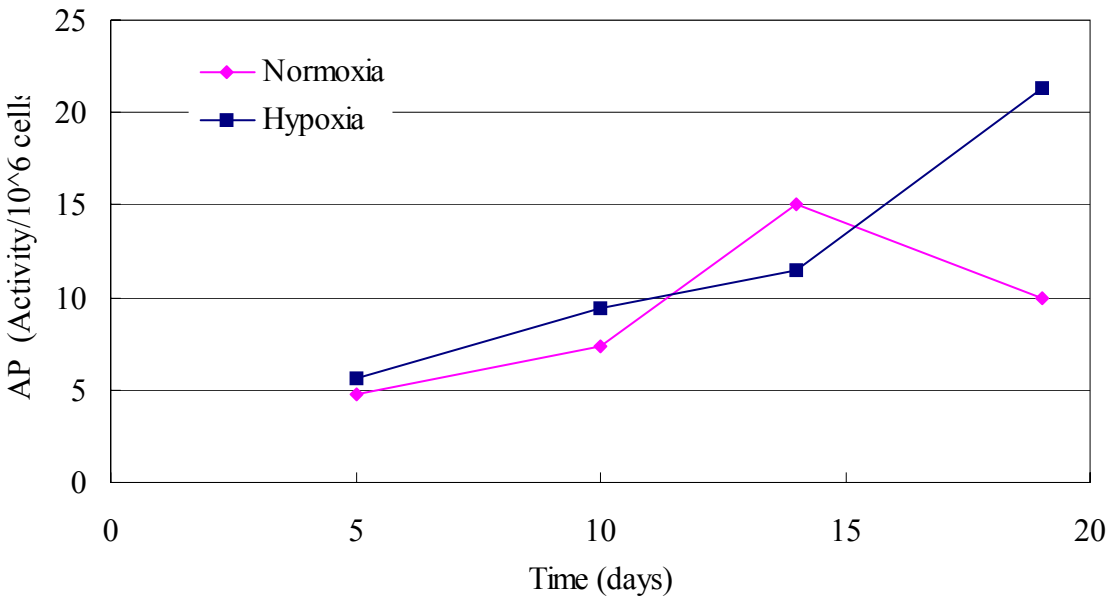


Figure 5.8b AP activity of Osteo-Induced hMSC constructs.
Normoxic samples show characteristic peak at day 14, unlike hypoxic samples, where AP values rise consistently and highest AP activity is observed at day 19. Peak values for hypoxia are significantly higher than those of normoxic samples.

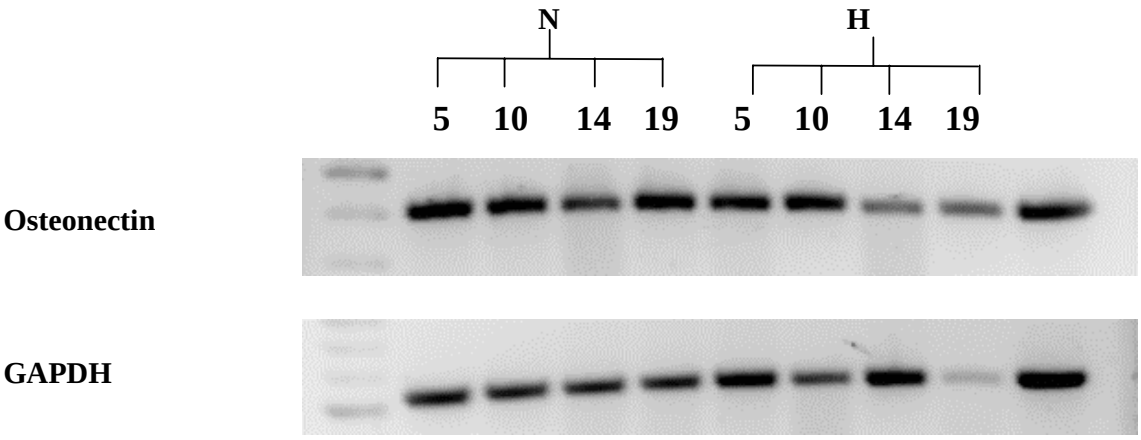


Figure 5.8c RT-PCR Analysis of Osteonectin Expression in Osteo-Induced Constructs
Time course of osteonectin expression after induction is shows fairly constant for expression levels for normoxic samples. Levels in hypoxia increase from day 5 to day 10 and then day 19 (when normalized to GAPDH expression). GAPDH mRNA is used as a loading control.

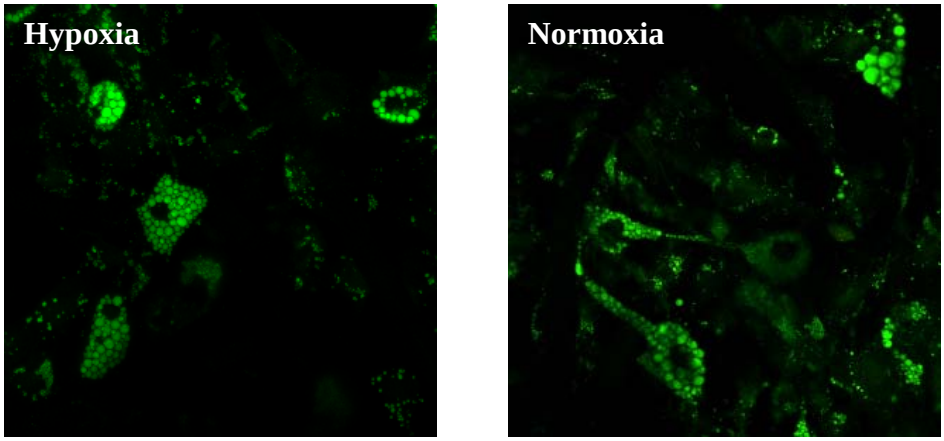


Figure 5.9a Nile red staining of Adipo-Induced hMSC Constructs.
Both hypoxic and normoxic samples maintained their adipogenic differentiation capabilities.

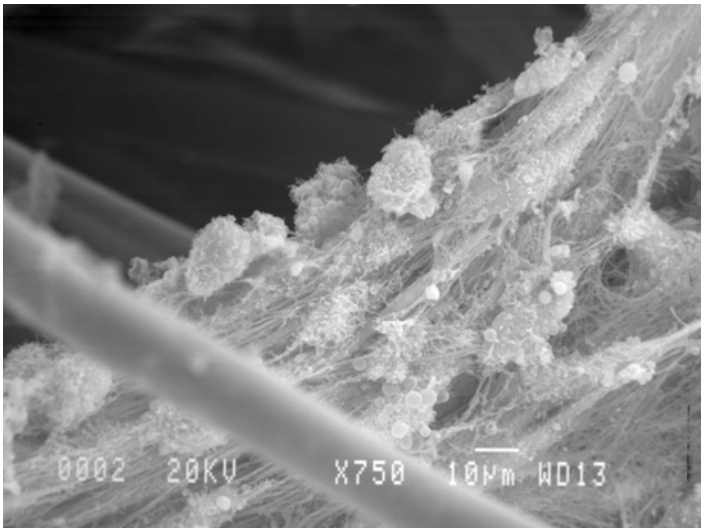


Figure 5.9b SEM Image of Adipo-Induced Cells showing Spherical Morphologies.

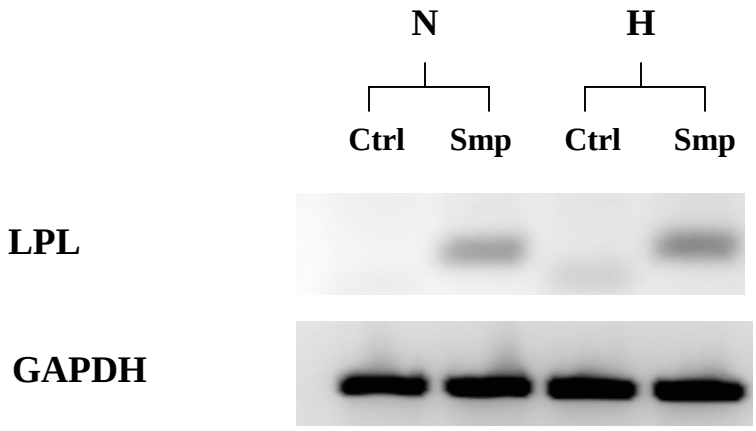


Figure 5.9c RT-PCR Analysis of LPL Expression in Adipo-Induced Constructs. Un-induced hMSC (ctrl) do not express LPL mRNA, but induced constructs (smp) upregulate LPL expression. Higher LPL expression is detected in hypoxic relative to normoxic samples.

Table 1. Protein:DNA ratios for hMSC constructs.

The ratio of protein:DNA was determined for hypoxic and normoxic constructs at Day 5 and Day 28 of culture. Ratio decreased with time in culture but much higher ratios are obtained for hypoxic cultures, at both culture stages.

Conditions	Ratio of Protein:DNA (mg/ng)	
	5 days	28 days
Hypoxia	1.3	0.12
Normoxia	0.4	0.06

CHAPTER 6

HYPOXIA AFFECTS THE IN VITRO LIFE-SPAN OF HUMAN MESENCHYMAL STEM CELLS

6.1 INTRODUCTION

Human mesenchymal stem cells (hMSC) are potential therapeutic cell sources for treating a plethora of diseases (Prockop *et al*, 2003). However, clinical applications of hMSC are hampered by an incomplete understanding and control of *in vitro* culture parameters. Traditional cell-culture techniques facilitate billion-fold expansion of hMSC (Pittenger *et al*, 1999), but result in a gradual loss of their primitive characteristics and unlimited self-renewal properties (Bruder *et al*, 1997). Current approaches toward overcoming this problem aim at replicating aspects of the hMSC *in vivo* environment. HMSC are obtained primarily from the bone marrow, which has an average oxygen tension of 5% (Mostafa *et al*, 2000). Oxygen tension is also potent gene-regulator, affecting cellular calcium concentrations (Berna *et al*, 2002), metabolic pathways (Guillermin and Krasnow, 1997), adhesion (Lash *et al*, 2001), migration (Annabi *et al*, 2003) and ECM remodeling (Pfander *et al*, 2003), and has been extensively studied as a process parameter for hematopoietic stem cell (HSC) *in vitro* expansion (Cipolleschi *et al*, 1993, Hevehan *et al*, 2000; Ivanovic *et al*, 2000a; b; 2002). However its role in the expansion of hMSC has not yet been studied.

HMSC and HSC co-exist in a symbiotic relationship in culture. HMSC secrete cytokines that support HSC survival, and have been used in co-culture methods to preserve the most primitive HSC progenitors (Kadareit *et al*, 2002). Further studies (Zandstra *et al*, 1994; Baksh *et al*, 2003) suggest that the presence of HSC may also enhance hMSC *in vitro* proliferation characteristics. The intimate interaction between these two stem cell types strongly indicate that they reside in similar niches within the bone marrow, hence some parameters used to optimize HSC expansion

may be extended to the hMSC phenotype. An abundance of experimental evidence suggests that HSC reside in severely hypoxic regions of the bone marrow, and in fact, high resistance to hypoxic conditions has been used as a defining characteristic of HSC (Krishnamurthy *et al*, 2004). Consequently, maintaining HSC under severely hypoxic conditions in culture significantly improves their proliferation properties: HSC at 1% oxygen are able to maintain higher percentages of stem and progenitor cells in their populations and exhibit enhanced potential for repopulating ablated murine marrow (Ivanovic *et al*, 2000a; b). Considerably less is known about culture conditions that optimize hMSC characteristics. Sekiya *et al*, (2002a) reported that the initial plating density significantly affects the quality of cells that are maintained in the population. In studies done with rat mesenchymal stem cells (rMSC), Lennon *et al* (2001) found that primary rMSC exhibited higher colony-forming potential, as well as increased proliferation rates, and enhanced osteoblast differentiation characteristics. Other studies have found that using hypoxia to isolate hMSC from adult bone marrow selected for a highly primitive cell-type that had significantly improved self-renewal abilities (D'Ippolito *et al*, 2004). However, hypoxia was not used as a continuous culture parameter in their experiments.

In this study, hMSC are cultured under 2% oxygen conditions in Petri dishes and compared to cells from the same donor cultured at the traditional 20% oxygen conditions. At 2% O_2, changes in HIF-1α localization in the cellular compartments are observed. Hypoxic hMSC display significantly improved morphological traits as well as exhibited an order of magnitude difference in expansion potential over six weeks of culture. Hypoxia also preserved the colony-forming potential of hMSC better than high oxygen conditions. These changes in hypoxia are concomitant with higher expression of cyclin B1, but little difference is seen in their p21 and p53 expression, thus the molecular mechanisms of their enhanced proliferation remain unknown.

6.2 MATERIALS AND METHODS

6.2.1 Reagents

Cell culture media, FBS and Penicillin/Streptomycin were obtained from Life Technologies (Rockville, MD). Antibodies to ECM proteins were obtained from ICN Biomedical (Costa

Mesa, CA). All other reagents were obtained from Sigma (St. Louis, MO) unless otherwise stated.

6.2.2 Cell Culture

Bone marrow derived hMSC were isolated as previously described (Sekiya *et al*, 2002a; Di Girolamo *et al*, 1999). Briefly, bone marrow aspirates of about 2 ml were drawn from healthy donors ranging in age from 19 to 49 years under an Institutional Review Board-approved protocol. Plastic adherent nucleated cells were separated from the aspirate and cultured using complete media (alpha MEM with 10% FBS and 1% penicillin/streptomycin) at 37 ^{o}C and 5% CO_2. For normoxic cultures, hMSC were cultured at 95% Air - 5% CO_2. For hypoxia studies, hMSC were cultured in hypoxic chambers that were flushed with gas mixtures of composition 2% O_2 - 5% CO_2 - 93% N_2 (hypoxic).

6.2.3 HIF-1α Staining

HMSC were plated unto glass cover-slips and placed in both culture conditions for one day. Samples were removed, washed in cold DPBS and fixed in methanol at –20 ^{o}C for 5 minutes. Samples were then washed with PBS, blocked and incubated with HIF-1α 1^{o} mouse monoclonal antibody for 1 hour at 37 ^{o}C. Samples were again washed in blocking buffer and incubated with 2^{o} antibody conjugated to Alexafluor 488 for 1 hour at 37 ^{o}C. Samples were then washed, counterstained with DAPI and allowed to dry. Samples were viewed on an Olympus IX70 microscope.

6.2.4 Growth Analysis

Analyses of growth potential, as well as, the growth kinetics of the cells were carried out. In order to assess growth potential, a vial of hMSC was thawed, cells were counted and plated into four 10 cm Petri dishes at 2000 cells/cm^2. Cell-culture media was changed every 3 – 4 days. During media changes and at harvest times cell-morphology was observed using phase-contrast filters in an Olympus IX70 microscope. At harvest, cells were washed in PBS, trypsinized and re-suspended in 10 ml complete media. Cells were passed through an 18-gauge needle to break up cell clumps and a 0.5 ml sample was aliquoted and used to carefully count the cell-concentration using a hemacytometer. Cells were then replated at the same seeding density and

the procedure repeated. For growth kinetics experiments, cells previously expanded in normoxic conditions were seeded into sixteen 10 cm Petri dishes; Eight were cultured at 20% O_2 and the others at 2% O_2. Cells were harvested at days 1, 3, 5 and 7 and counted.

6.2.5 Protein Assay and Western Blot Analysis

Total protein was extracted from the PET matrices in a lysing buffer (120mM NaCl, 25mM HEPES (pH 7.4), 1mM EDTA) containing 1% Triton X-100 and protease inhibitors (phenylmethylsulfonylfluoride, leupeptin, antipain and aprotinin). A small volume of the lysate was used to perform protein assays. A stock protein solution was prepared from bovine serum albumin (BSA) (Fisher Scientific, PA), and a serial dilution was generated to use as a standard curve. A colorimetric assay using a protein dye solution (Biorad, CA) was used to determine protein concentrations in the cell lysates. The specific protein content was calculated by dividing the total protein by the cell number in each matrix, and the results for each set of three matrices were averaged.

For Western Blotting, cell lysate samples were separated by SDS gel polyacrylamide electrophoresis and transferred electrophoretically onto nitrocellulose membranes (0.2 μm). The membranes were then blocked using a Tris buffer containing 0.1% Tween-20 and 5% dry milk. Membranes were then incubated with primary antibody overnight, washed with blocking buffer and incubated with an alkaline phosphatase-conjugated secondary antibody for 1 hour at room temperature. A detection kit from Biorad (Hercules, CA) was used to detect the specified proteins using chemiluminescence. Membranes were then washed in blocking buffer and re-probed with antibodies to β-actin. Blots were read on a densitometer and normalized to β-actin contents in order to quantify relative amounts of proteins.

6.2.6 Colony Forming Unit – Fibroblast

The CFU-F protocol was adapted from (Di Girolamo *et al*, 1999). After harvest and cell counting, the suspension was diluted in complete media and 800 cells were plated into a 10 cm Petri dish. Samples from each condition were done in duplicate. The cells were grown for 12 – 14 days at 37 ^{o}C and 5% CO_2 in a humidified incubator. Upon harvesting, cells were washed

with PBS and stained with 0.5% crystal violet solution for 10 – 15 minutes at room temperature. Cells were washed twice with PBS and the visible colonies were counted.

6.3 RESULTS

6.3.1 HIF-1α Staining

HMSC cultured under normoxic and hypoxic conditions express high levels of HIF-1α. In normoxic hMSC, HIF-1α is localized almost exclusively in the cytoplasmic regions, and distinct outlines of the unstained nuclear regions are apparent (Figure 6.1a). While HIF-1α is also strongly expressed in the cytoplasm of hypoxic cells, the nuclear regions are also stained, indicating both cytoplasmic and nuclear localization for hypoxic hMSC (Figure 6.1b).

6.3.2 Cell Morphology

Early stage, passage 2 hMSC exhibit small, spindle-shaped morphologies in both normoxic and hypoxic cultures (Figure 6.2a, b). However, there was a considerable change in the morphology of hMSC grown under normoxia with time. Passage 4 hMSC grown at 20% oxygen exhibit very broad, flattened morphologies and do not grow to confluence (Figure 6.2c). In contrast, passage 4 cells from hypoxic conditions, maintain their spindle morphologies and are able to achieve high cell-density (Figure 6.2d).

6.3.3 Growth Potential

HMSC were expanded at 2% and 20% oxygen tensions for eight passages. Cells under hypoxia exhibited higher growth than cells cultured under normoxic conditions at each passage. On average an approximate 8-fold expansion was observed at each passage for hypoxic cells, whereas cells cultured at 20% O_2 only expanded about 5 times during each passage period (Figure 6.3a). This corresponded to over 330000 fold expansion over 6-weeks of culture in hypoxia as opposed to a mere 16000 fold expansion for normoxic cells during the same period (Figure 6.3b).

6.3.4 Western Blots

Western blot analysis was done for samples harvested at day 7 of passages 4, 5 and 6. The results shown in Figure 6.4 indicate that there were no discernable differences in p53 expression from hMSC in the two culture conditions. P21 levels were slightly elevated in Passage 5 and 6 hypoxic samples, but levels were similar at passage 4. Likewise, there was a slight increase in cyclin B1 expression in hypoxic cultures, particularly at Passage 6. There was no consistent pattern in the fibronectin or HSP 47 expression between the two culture conditions, indicating that hypoxia may not play a distinct role in affecting their expression in these cultures.

6.3.5 Growth Kinetics

The growth kinetics of hMSC were observed for Passage 4 cells. Similar plating efficiencies were observed for hypoxic and normoxic cultures at day 1. Of the 96000 cells seeded, 62000 adhered to the plate after 24 hours. The growth rate for the cells remained similar for the first 5 days of culture, and there were no significant differences in the cell numbers during this period. However, there were significantly more cells in hypoxic cultures by day 7 (Figure 6.5a).

6.3.6 CFU-F Assays

Cells harvested at days 1, 3, 5 and 7 were re-plated for CFU-F assays. Hypoxic and normoxic cells exhibited similar colony-forming potential over the first three days of culture. However, there were significant decreases in the CFU-F potential of the cells grown under normoxia, relative to hypoxic samples at days 5 and 7 (Figure 6.5b).

6.4 DISCUSSION

6.4.1 HIF-1α and the Hypoxic Response

HIF-1α is the primary mediator of the hypoxic response in most cell types. Under hypoxic conditions, HIF-1α sub-unit is stabilized, allowing HIF-1α to move into the cell nucleus, bind the HIF-1β counterpart and act as a transcription factor (TF) for many of the genes associated with the hypoxic response (Semenza, 1999). While it appears that hMSC constitutively express HIF-1α even at 20% O_2, only cells cultured at 2% oxygen conditions demonstrate evidence of widespread HIF-1α translocation into the nucleus, suggesting that it is only under hypoxic

conditions that it can fulfill its role as a TF. Indeed hMSC consume more glucose when cultured at low oxygen conditions (Grayson *et al*, In preparation); this process is typically associated with higher expression of glucose transporters in the cell membrane (Loike *et al*, 1992; Bashan *et al*, 1992; Sivitz *et al*, 1992), as well as upregulated expression of other genes associated with the glycolytic pathway (Firth *et al*, 1994; Hellkamp *et al*, 1991). Although, a role for HIF-1 has been demonstrated in upregulating these genes, considerably more work is required to determine what role, if any, HIF-1 plays in affecting the cell-cycle and proliferation potential of hMSC.

6.4.2 Effects of Hypoxia on Cell Proliferation

Hypoxia significantly affected hMSC proliferation *in vitro*: HMSC exhibit more than 20-fold increase in expansion potential under hypoxia compared to normoxic conditions over a 6-week culture period. During each passage, hMSC numbers were higher at day 7 of culture, and in half the cases, the differences were statistically significant (Figure 6.3a). Even though the cultures were more confluent at the time of harvesting, hMSC cultured at 2% O_2 maintain elevated cyclin B1 levels relative to normoxic cultures, suggesting the possibility that there may be reduced sensitivity to contact inhibition signals under hypoxic conditions. However, cell morphology analysis may provide alternative explanations: The broadening and flattening of hMSC in normoxic culture could be indicative of differentiation mechanisms, hence, reduced proliferation. Or another contributing factor is that the smaller, spindle-shaped hypoxic cells can be packed more closely. Further analysis is required to elucidate the mechanisms that result in increased cellularity in hypoxic culture.

Higher cell numbers could be the result of either increased proliferation rates stimulated by hypoxia, or an extended growth phase. Interestingly, hypoxia does not seem to impact the growth-rate of the cells during the first five days of passage (Figure 6.5a). The higher cell numbers on day 7 of culture therefore suggests that hypoxia may be prolonging the growth phase, and validates the cyclin B1 data at the day 7 time point. A similar, unexplained trend was observed when the cells were cultured in three-dimensional polymer scaffolds (Grayson *et al*, In preparation). However, this phenomenon might be understood by studying cell-cycle activators and inhibitors. The role of cell-cycle regulators, particularly p21 and p27, in modulating expansion, differentiation and apoptotic mechanisms of the hematopoiesis process has been

studied (Steinman, 2002). While differences in growth were observed in these experiments, there was no observable difference in p21 expression. Hence, more studies are required to provide greater insight into hMSC growth kinetics, or the different parameters influencing the mesengenic lineage.

6.4.3 Effect of Hypoxia on Stem Cell Characteristics

HMSC cultures are comprised of heterogenous sub-populations (Shur *et al*, 2002; Colter *et al*, 2001; Sekiya *et al*, 2002a). A rudimentary colony-forming technique (CFU-F) provides an indication of the proliferation potential of the cells (DiGirolamo *et al*, 1999) by determining the relative number of primitive cells within the total population. Hypoxic hMSC maintain higher CFU-F numbers than hMSC cultured in normoxic conditions despite superior growth. The enhanced colony-forming potential of hMSC in low oxygen cultures indicate that hypoxic conditions may stimulate hMSC to give rise to more primitive stem cells upon dividing, rather than dividing to give one or two partially committed progenitor cells, thereby progressively diluting the stem cell pool. Alternatively, primitive stem cells may be more resistant to hypoxia than progenitor, or more mature cell types. Hence under low oxygen conditions, a higher percentage of stem cells is preserved. The similar p21 and p53 expression of hMSC under both conditions also indicate that hMSC are not being adversely affected by hypoxia and are not being directed down apoptotic pathways as a result of an inability to maintain required ATP levels (Malhorta *et al*, 2001). Prior reports demonstrate that as hMSC proliferate, they become broader, flatter and exhibit a reduction in their proliferation and differentiation abilities (Bruder *et al*, 1997; Sekiya *et al*, 2002a). However, this is not observed under hypoxia. Cellular morphology under hypoxia indicate that hMSC maintain their size and morphology, while normoxic cells exhibit vast changes in size and morphology by the end of the fourth passage.

6.5 CONCLUSIONS

Similarities in HSC and hMSC microenvironments and cellular characteristics suggest that many of the approaches previously used to unearth enigmas of the hematopoietic process, and improve HSC *in vitro* expansion, may be applied to hMSC. However, the analogy has its limitations; the hematopoietic lineage has been fully characterized, including the transit amplifying progenitor

cells for different pathways, but this has not been achieved for hMSC, which exhibit significant variation in surface protein expression under different culture conditions, while maintaining their pluripotency. Hence, even the hMSC phenotype has not been completely defined, necessitating the use of more generalized characteristics such as morphology and colony-forming potential. Yet, this study verifies that hMSC, like HSC, also appear resistant to extended hypoxia, and maintain their primitive characteristics during the expansion period at lowered oxygen tensions. Oxygen concentration may be a critical component of the stem cell compartment in bone-marrow, and thus may provide important cues to hMSC and HSC alike, that influence *in vivo* differentiation and affect *in vitro* characteristics as well.

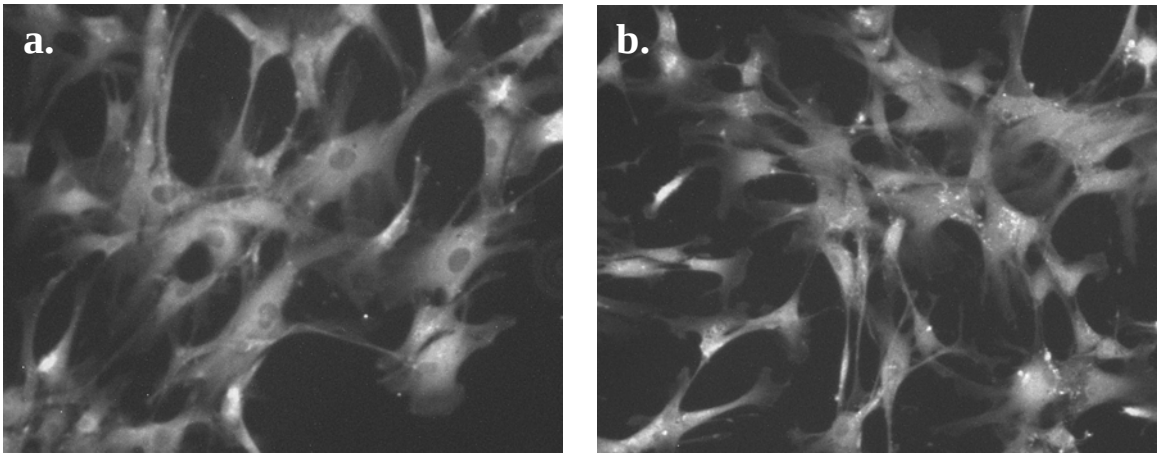


Figure 6.1 Immunofluorescent staining of HIF-1α.
HMSC cultured at (a) 20% oxygen show distinct HIF-1α staining that is localized in the cytoplasmic regions, leaving cell-nuclei unstained. (b) 2% oxygen also exhibit cytoplasmic staining, but nuclear regions are also densely stained.

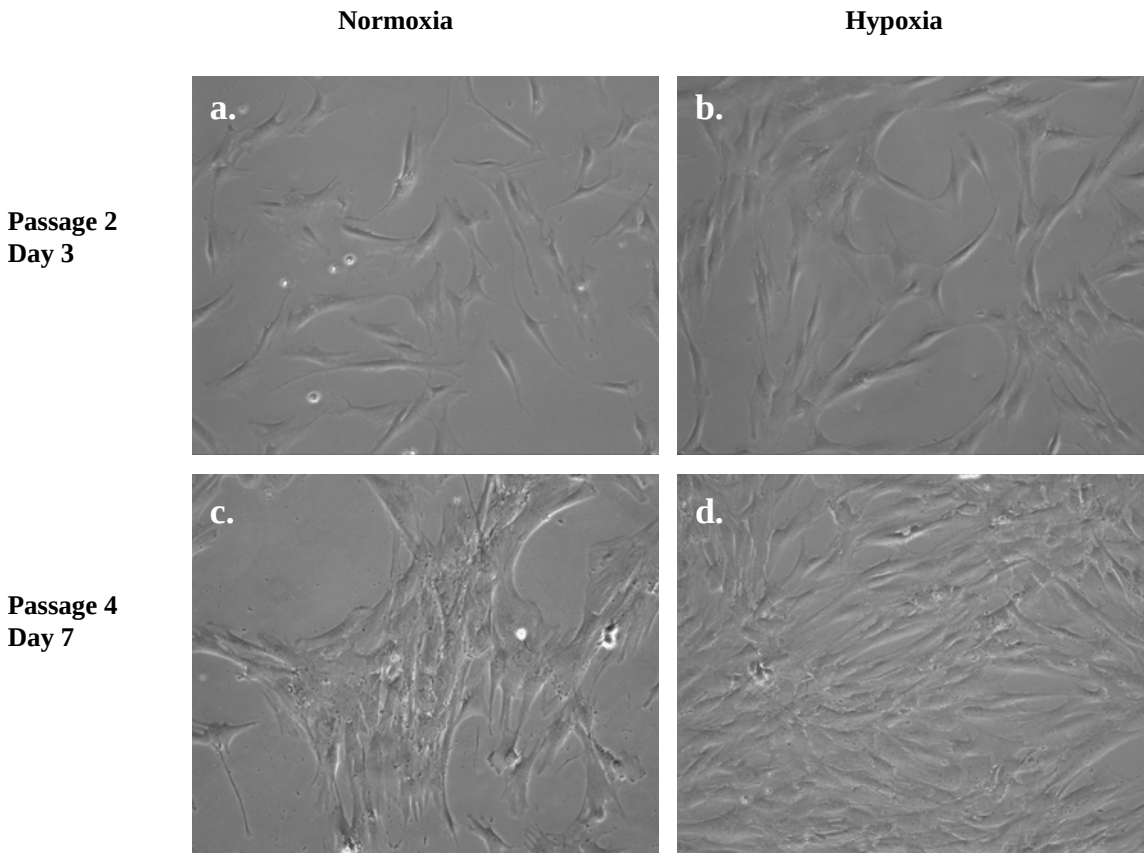


Figure 6.2 Morphology of hMSC at Different Stages.
(a, b) Passage 2 show similarities in original hMSC cultured at both (a) normoxic and (b) hypoxic conditions. (c, d) By Passage 4 cells distinct morphological variations are seen. (b) Normoxic hMSC become broad and flattened, whereas (d) hypoxic cells maintain spindle morphology.

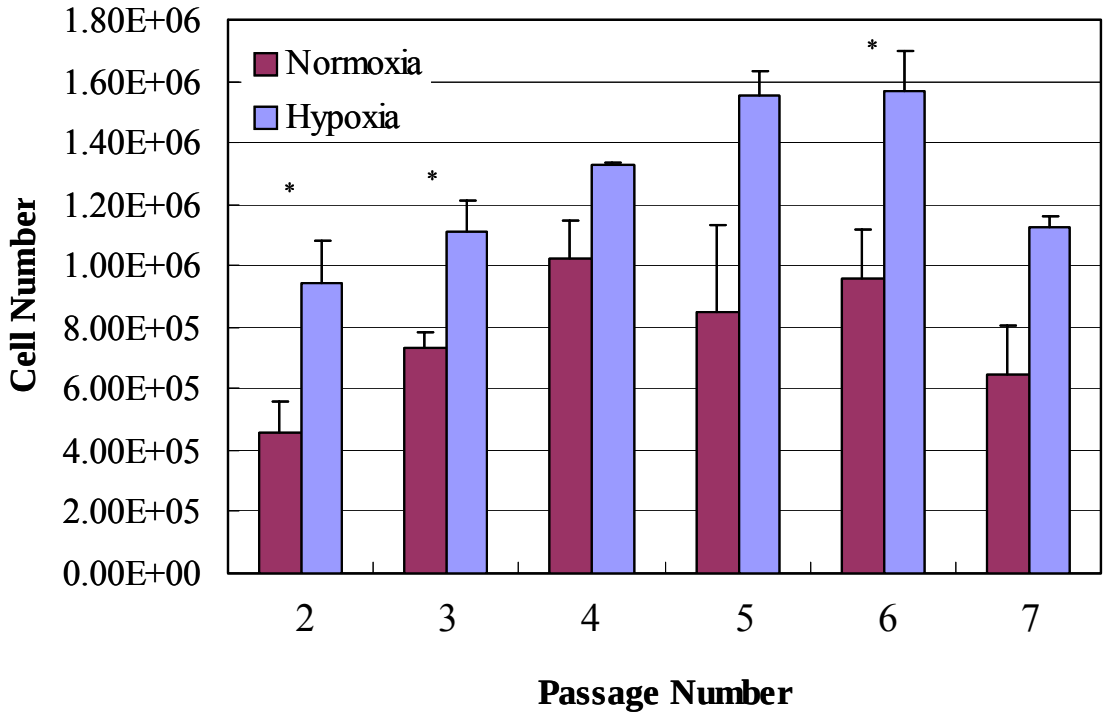


Figure 6.3a HMSC Numbers at Different Passages

HMSC were plated at 1.5 x 10^5 cells per 10 cm Petri dish and harvested at day 7 at each passage. Cells from 2 dishes were counted (* $p < 0.05$)

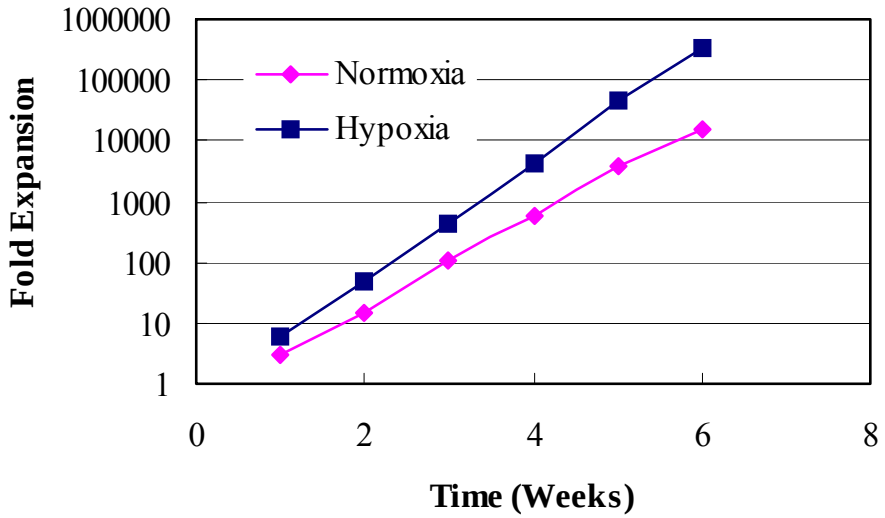


Figure 6.3b Expansion of hMSC as a function of time in culture.

A 20-fold difference in cell expansion was observed after 6 weeks (Passage 7) of culture.

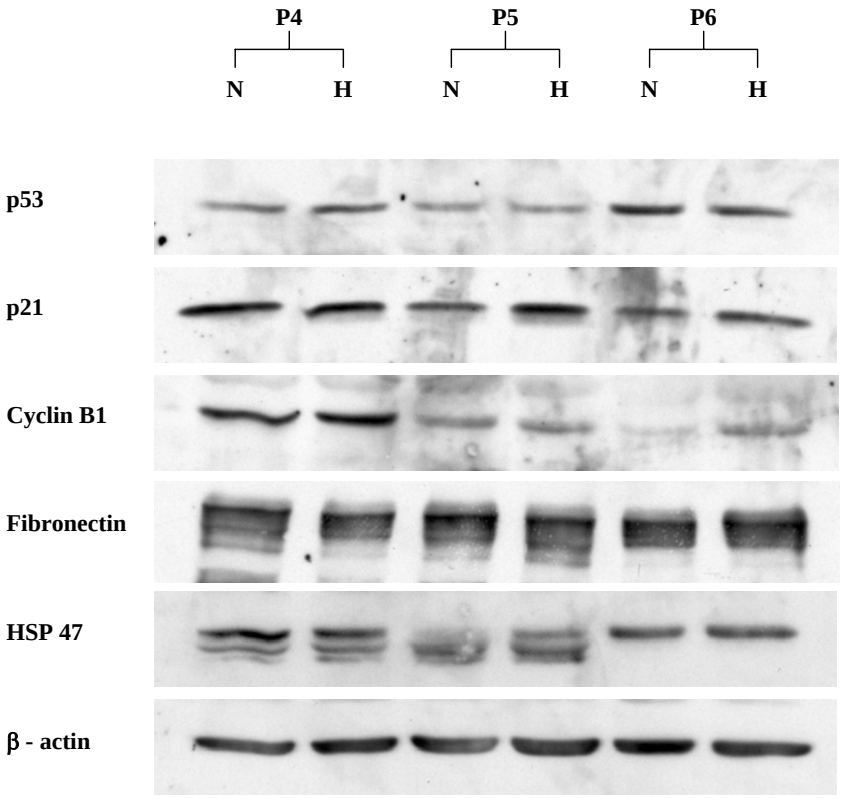


Figure 6.4 Western Blot of Various Markers in Hypoxia and Normoxia

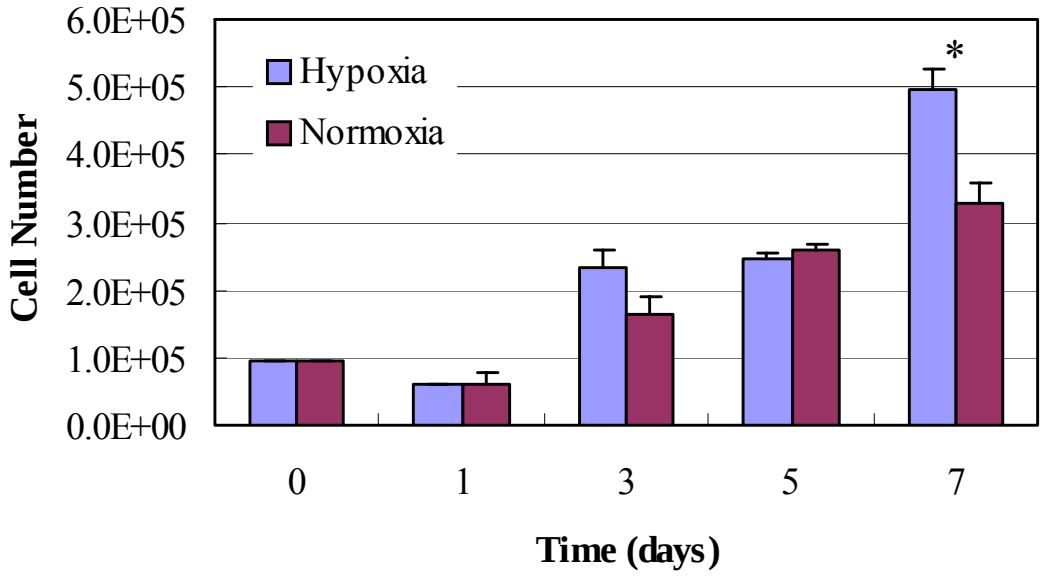


Figure 6.5a Growth kinetics of Passage 4 hMSC

No statistical differences in cell numbers are observed for the first five days of culture. By day 7 hypoxic cultures have significantly higher cell numbers ($p < 0.05$)

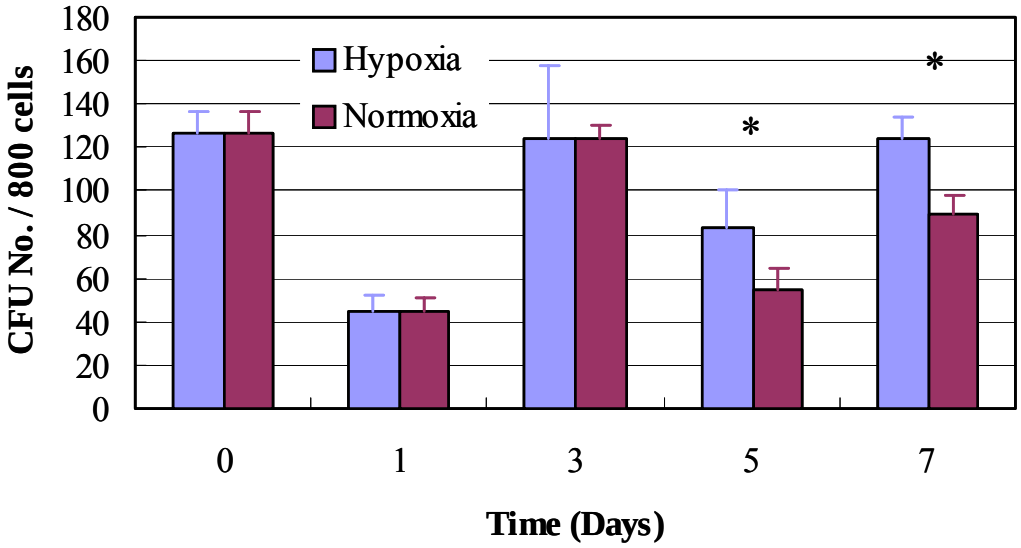


Figure 6.5b Kinetics of CFU-F Potential of Passage 4 hMSC

Statistical difference in CFU-F numbers were only observed after 5 days of culture ($p < 0.05$)

CHAPTER 7

CONCLUSIONS AND FUTURE WORK

7.1 A TISSUE-ENGINEERING PERSPECTIVE

Cells in the developing organism respond to spatial patterning, chemotactic gradients, autocrine and paracrine signaling events, and hormonal cues to elicit a coordinated response involving cell proliferation, migration, differentiation and apoptosis. The end result is a complex organism, comprised of multiple cell types with specialized functions, which are precisely oriented in space with respect to each other, and communicate via various mechanisms. Tissue-engineering is in its infancy stage however, and the immediate goal is to develop uniform constructs that reflect the cell-patterning and protein-organization of a single differentiated tissue such as bone or cartilage. The underlying paradigm of tissue engineering is to combine cells with scaffolds to generate new tissues, thus integrating principles of cell and developmental biology, materials engineering, and bioreactor design toward a common goal. To this end, extensive research has been done to investigate the effects of scaffold-type, as well as the biochemical and physical stimuli of the 3D culture environment on the growth and developmental patterns of cells. Several parameters play critical roles and must be considered for each application. These include (i) material architecture, biocompatibility, and bioactivity (ii) cell type (iii) spatial and temporal delivery of growth factors and (iv) the transport of nutrients into, and metabolic waste out of tissue-constructs. The overall complexity of the task is heightened when stem cells are utilized because of an incomplete understanding of their characteristics or responses to variations in environmental stimuli. Therefore current approaches for the *in vitro* development of tissues cannot realistically attempt to replicate too many of the parameters important in normal development of organisms, but instead present critical, controllable, reproducible cues that guide cellular development, and result in functional, differentiated tissue-engineered constructs (Lavik and Langer, 2004).

7.2 SCIENTIFIC CONTRIBUTION OF THIS STUDY

The results detailed in the previous chapters provide insight into several factors affecting key developmental characteristics of hMSC. Construct topography, efficiency of nutrient transfer, and oxygen tension significantly influence hMSC growth kinetics, metabolic profiles, ECM secretion and organization, total protein expression, CFU-F potential, and differentiation abilities. As a broad array of parameters were investigated, it is now possible to deduce specific aspects of the culture-environment that affect key developmental characteristics e.g. which conditions favor exponential growth, or maintain the primitive state relative to the differentiated state. Equally important were the effects that culture conditions had on the resulting tissue-architecture. The cell shape and packing density as well as the ECM structure all combine to confer specific properties to a tissue. Prior studies of 3D tissues have primarily used histological stains, which are able to determine spatial distribution of the ECM components, but do not give information about the structure of the protein fibers. The drastic difference in cell-shape and ECM structure observed in static and flow conditions indicate that media flow not only improves nutrient transfer efficiency and construct uniformity, but significantly affects other developmental properties and cellular characteristics.

While each parameter may have been studied separately, the results from each study cannot be viewed in isolation. The media flow in perfusion cultures will also enhance oxygen supply to the cells, so that the effects on nutrient transport properties and the mechanical environment are coupled with cellular responses to changes in biochemical cues within the scaffold. Also, it is not known whether the increased glucose consumption by hMSC under hypoxic conditions resulted in steeper nutrient gradients within the construct, and how this may have affected cellular characteristics. These raise further questions. For example, if the media in the perfusion system is equilibrated to 2% ambient oxygen, what effect will this have on CFU-F potential of hMSC, total protein levels, or fibronectin expression throughout the culture period. As such, the results were presented in distinct sections, but there is an overarching link between these sections as they all provide approximations to the in vivo hMSC environment, and it is necessary to view the results holistically to facilitate additional insights.

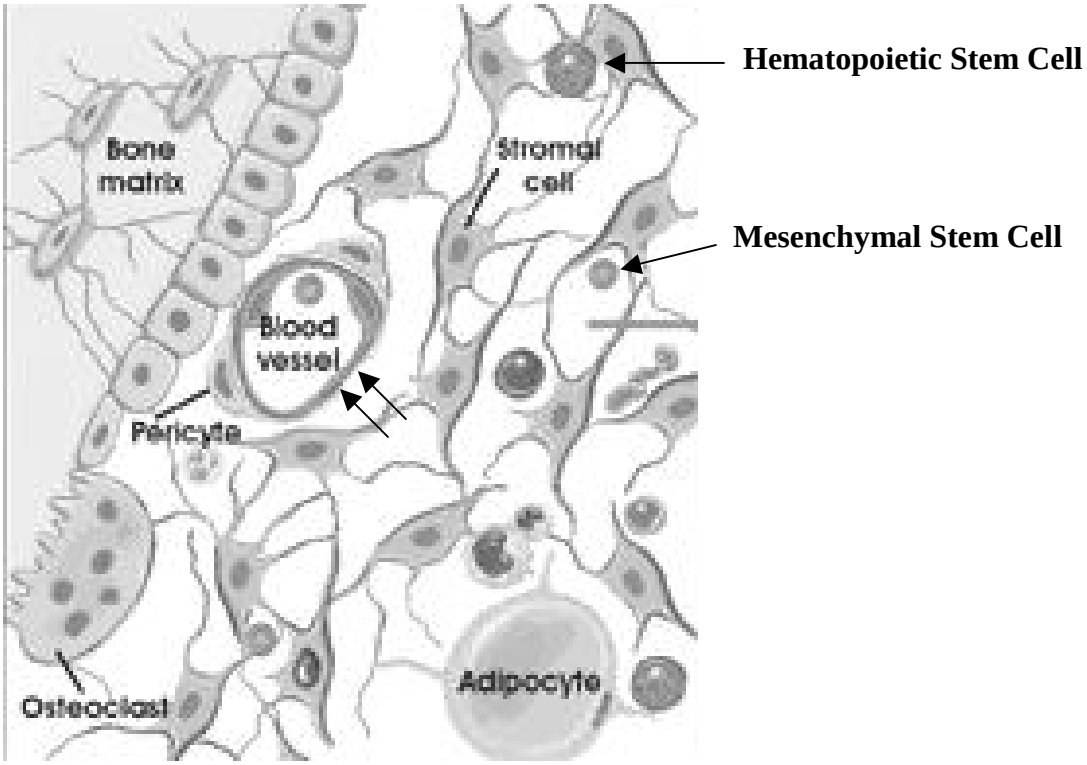


Figure 7.1 Reconstructing the hMSC In Vivo Microenvironment

Schematic diagram of the bone marrow environment. Each aspect of experiments approximate the *in vivo* hMSC environment. HMSC in the bone marrow associate with several cell types as well as ECM (not shown) in a 3D arrangement. Diffusion of nutrients from blood vessel (double arrow) to the hMSC is roughly approximated in the perfusion system. Oxygen from the blood is supplied to hMSC by diffusion through a number of other cell types leaving hMSC in a severely oxygen depleted environment.

7.3 FUTURE RESEARCH

Knowledge gained from these endeavors will provide the backdrop for future studies attempting to generate complex tissues comprised of different cell types, and importantly, a vascular system. The ability of stem cells, and in particular hMSC, to give rise to various cell phenotypes makes them primary candidates for these applications. Further studies are needed to better define hMSC, and identify their phenotype, genotype and molecular controls that regulate differentiation pathways. One critical aspect governing the use of hMSC for developing tissues is understanding the environmental stimuli and signaling pathways that support and maintain the undifferentiated stem cell in addition to those that induce differentiation along specific lineages (Tuan *et al*, 2003) as has been done with the hematopoietic system (Duncan et al, 2005). However, these are generalized projects that will be more aptly addressed by cell and molecular biologists. More specific research is detailed below.

Specifically, the changes in cell-growth patterns as a result of hypoxic conditions need to be further studied. Differences in cyclin B1 expression were observed particularly towards the end of culture periods. While cyclin B1 is required for cells to progress through mitosis, it is not typically considered a cell-cycle regulator. Meanwhile, the p21 regulator remained relatively unchanged, as did p53. Hence, it is necessary to study other cell-cycle regulators. Cyclin D1, cyclin E and p27 are good candidates for initial studies. Additionally, WB and RT-PCR techniques can be used to investigate the long-term effects of hypoxia on the expression of stem cell markers Oct-4, Rex-1 and Sox-2, as well as differences in the telomerase activity of hMSC.

Secondly, as mentioned in the background, hypoxia results in tube-formation in murine MSC, indicating a role for MSC in formation of new blood vessels. An interesting corollary of this would be to determine what effects does hypoxia have on the differentiation of hMSC into an endothelial phenotype. Initial studies should be done on 2D plates. Flow-cytometry and immunocytochemistry staining can be used to look at markers specific to endothelial cells. These results can be supplemented with quantitative studies of hMSC elongation at low cell densities (using staining techniques in conjunction with computer imaging programs), and hMSC migration rates.

APPENDIX A

Commonly Used Abbreviations

2D	-	two-dimensional
3D	-	three-dimensional
APC	-	adipose progenitor cells
ATP	-	adenosine tri-phosphate
BAEC	-	bovine aortic endothelial cells
bFGF	-	basic fibroblastic growth factor
BPAEC	-	bovine pulmonary artery endothelial cells
Cyt	-	cytochrome
DFO	-	desferriooxamine
DMD	-	Duchenne's muscular dystrophy
DPI	-	diphenylene iodonium
ECM	-	extracellular matrix
EPO	-	erythropoietin
ESC	-	embryonic stem cell
FA	-	focal adhesion
FAK	-	focal adhesion kinase
FBS	-	fetal bovine serum
GAG	-	glucosaminoglycans
GAPDH	-	glyceraldehydes phosphate dehydrogenase
GLUT	-	glucose transporter
GvHD	-	graft versus host disease
HDF	-	human dermal fibroblasts
HIF	-	hypoxia-inducible factor
hMSC	-	human mesenchymal stem cells
HRE	-	hypoxia response element
HSC	-	hematopoietic stem cell
MAPC	-	multipotent adult progenitor cell
MI	-	myocardial infarct
MIAMI	-	marrow-isolated adult multilineage inducible
MMP	-	metallo matrix proteinase
mMSC	-	murine mesenchymal stem cells
MSC	-	mesenchymal stem cell
PET	-	poly(ethylene terephthalate)
PG	-	proteoglycans
PGA	-	poly(glycolic acid)
PLA	-	poly(lactic acid)
PLA	-	processed liposuction aspirates
pVHL	-	protein von Hippel Lindau
rMSC	-	rat mesenchymal stem cells
ROI	-	reactive oxygen intermediates
RS-1	-	rapidly self-renewing
TEX	-	Tris-EDTA-Triton X-100
TF	-	transcription factor
VEGF	-	vascular endothelial growth factor

APPENDIX B

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REFERENCES

Abbott A. 2003. Cell culture: biology's new dimension, Nature 424: 870 – 872

Abdallah BM, Haack-Sorensen M, Burns JS, Elsnab B, Jakob F, Hokland P, Kassem M. 2005. Maintenance of differentiation potential of human bone marrow mesenchymal stem cells immortalized by human telemorase reverse transcriptase gene in despite of extensive proliferation, Biochem Bioph Res Co 326: 527–538

Acker H. 1994a. Cellular oxygen sensors, Ann. NY Acad Sci 718: 3- 10

Acker H.1994b. Mechanisms and meaning of cellular oxygen sensing in the organism Resp Phys 95: 1 – 10

Acker H. 1989. PO_2 chemoreception in arterial chemoreceptors, Ann. Rev Phys 51: 835 – 844

Adams J. 2001. Cell-matrix contact structures, Cell Mol Life Sci 58: 371 -392

Agani FH, Pichiule P, Chavez JC, LaManna JC. 2000. The role of mitochondria in the regulation of hypoxia-inducible factor 1 expression during hypoxia, J Biol Chem 275: 35863 – 35867

Aggarwal S, Pittenger MF. 2005. Human mesenchymal stem cells modulate allogeneic immune cell responses, Blood 105: 1815 – 1822

Annabi B, Lee Y, Turcotte S, Naud E, Desrosiers RR, Champagne M, Eliopoulos N, Galipeau J, Béliveau R. 2003. Hypoxia promotes murine bone marrow-derived stromal cell migration and tube formation, Stem Cells 21: 337 – 347

Aung T, Miyoshi H, Tun T, Ohshima N. 2002. Chondroinduction of mouse mesenchymal stem cells in three-dimensional highly porous matrix scaffolds, J Biomed Mat Res 61: 75-82

Awad HA, Bovin GP, Dressler MR, Smith FNL, Young RG, Butler DL. 2003. Repair of patellar tendon injuries using a cell-collagen composite, J Orthopaed Res 21: 420 – 431

Azizi SA, Stokes D, Augelli BJ, DiGirolamo C, Prockop DJ. 1998. Engraftment and migration of human bone marrow stromal cells implanted in the brains of albino rats, Proc Natl Acad Sci 95: 3908 –3913

Badylak S, Liang A, Record R, Tullius R, Hodde J. 1999. Endothelial cell adherence to small intestinal submucosa: an acellular scaffold, Biomaterials 20: 2257 - 2263

Baksh D, Davies JR, Zandstra PW. 2003. Adult human bone marrow-derived mesenchymal progenitor cells are capable of adhesion-independent survival and expansion, Exp Hematol 31: 723 –732

Bancroft GN, Sikavitsas VI, Mikos AG. 2003. Design of a flow perfusion bioreactor system for bone tissue-engineering applications. Tissue Eng 9: 549 - 554.

Bancroft GN, Sikavitsas VI, van den Dolder J, Sheffield TL, Ambrose CG, Jansen JA, Mikos AG. 2002. Fluid flow increases mineralized matrix deposition in 3D perfusion culture of marrow stromal osteoblasts in a dose-dependent manner. Proc Natl Acad Sci 99: 12600-12605.

Barry F, Murphy JM. 2004. Mesenchymal stem cells: clinical applications and biological characterization, Int J Biochem Cell Bio 36: 568 - 584

Bartholomew A, Sturgeon C, Siatskas M, Ferrer K, McIntosh K, Patil S, Hardy W, Devine S, Ucker D, Deans R, Moseley A, Hoffman R. 2002. Mesenchymal stem cells suppress lymphocyte proliferation in vitro ad prolong skin graft survival in vivo, Exp Hematol 30: 42 – 48

Bashan N, Burdett E, Hundal HS, Klip A. 1192. Regulation of glucose transport and GLUT 1 glucose transporter by O2 in muscle cells in culture, Am J Phys 262: C682 – 690

Behonick DJ, Werb Z. 2003. A bit of give and take: the relationship between the extracellular matrix and the developing chondrocyte, Mech Dev 120: 1327 -1336

Bissell MJ, Nelson WJ. 1999. Cell-to-cell contact and extracellular matrix. Integration of form and function: the central role of adhesion molecules, Curr Opin Cell Biol 11: 537 - 539

Bittner RE, Schöfer C, Weipoltshammer K, Ivanova S, Streubel B, Hauser E, Freilinger M, Höger H, Elbe-Bürger A, Wachtler F. 1999. Recruitment of bone-marrow-derived cells by skeletal and cardiac muscle in adult dystrophic mdx mice, Anat Embryol 199: 391 - 396

Bluow B, Song H, Tihan T, Bosze J, Ferrara N, Gerber H, Johnson RS, Bergers G. 2003. The Hypoxic response of tumors is dependent on their microenvironment, Cancer Cell 4: 133 – 146

Bonnet D. 2003. Biology of human bone marrow stem cells, Clin Exp Med 3: 140 – 149

Bonyadi M, Waldman SD, Liu D, Aubin JE, Grynpas MD, Stanford WL. 2003. Mesenchymal progenitor self-renewal deficiency leads to age-dependent osteoporosis in Sca-1/Ly-6A null mice, Proc Natl Acad Sci 100: 5840 – 5845

Boudreau N, Bissell M. 1998. Extracellular matrix signaling: integration of form and function in normal and malignant cells, Curr Opin Cell Biol 10: 640 - 648

Boudreau N, Werb Z, Bissell MJ. 1996. Suppression of apoptosis by basement membrane requires three-dimensional tissue organization and withdrawal from the cell cycle, Proc Natl Acad Sci 93: 3509 – 3513

Brenner KA, Corbett SA, Schwarzbauer JE. 2000. Regulation of fibronectin matrix assembly by activated Ras in transformed cells, Oncogene 19: 3156 - 3163

Bruder SP, Jaiswal N, Haynesworth SE. 1997. Growth Kinetics, Self-Renewal, and the Osteogenic Potential of Purified Human Mesenchymal Stem Cells During Extensive Subcultivation and Following Cryopreservation, J Cell Biochem 64: 278 – 294

Bruder SP, Kraus KH, Goldberg VM, Kadiyala S. 1998. The effect of implants loaded with autologous mesenchymal stem cells on the healing of canine segmental bone defects, J Bone Joint Surg 80: 985 – 996

Bruder SP, Kurth AA, Shea M, Hayes WC, Jaiswal N, Kadiyala S. 1998. Bone regeneration by implantation of purified, culture-expanded human mesenchymal stem cells, J Orthopaed Res 16: 155 – 162

Bunn HF, Poyton RO. 1996. Oxygen sensing and molecular adaptation to hypoxia, Phys Rev 76: 839 - 885

Bunting KD. 2002. ABC transporters as phenotypic markers and functional regulators of stem cells, Stem Cells 20: 11 – 20

Bunting KD, Hawley RG. 2003. Integrative molecular and developmental biology of adult stem cells, Biol Cell 95: 563 -578

Burridge K, Chrzanowska-Wodnicka M. 1996. Focal adhesions, contractility and signaling, Ann Rev Cell Dev Biol 12: 463 - 518

Cadenas E. 1989. Biochemistry of Oxygen Toxicity, Ann Rev Biochem 58: 79 – 110

Campoccia D, Doherty P, Radice M, Brun P, Abatangelo G, Williams DF. 1998. Semisynthetic resorbable materials from hyaluronan esterification, Biomaterials 19: 2101 - 2127

Caplan AI, Bruder SP. 2001. Mesenchymal stem cells: building blocks for molecular medicine in the 21st century, Trends Mol Med 7: 259 – 264

Caplan AI. 1991. Mesenchymal stem cells J Orthopaed Res 9: 641 – 650

Caplan AI. 1994.The mesengenic process, Clin Plastic Surg 21: 429 – 435

Carlberg AL, Pucci B, Rallapalli R, Tuan RS, Hall DJ. 2001. Efficient chondrogenic differentiation of mesenchymal cells in micromass culture by retroviral gene transfer of BMP-2, Differentiation 67: 128 - 138

Carmeliet P, Dor Y, Herbert J, Fukumura D, Brusselmans K, Dewerchin M, Neeman M, Bono F, Abramovitch R, Maxwell P, Koch CJ, Ratcliffe P, Moons L, Jain RK, Collen D, Keshet E. 1998. Role of HIF-1 in hypoxia-mediated apoptosis, cell proliferation and tumor angiogenesis, Nature 394: 485 – 490

Carrier RL, Papadaki M, Rupnick M, Schoen FJ, Bursac N, Langer R, Freed LE, Vunjac-Novakovic G. 1999. Cardiac tissue engineering: cell seeding, cultivation parameters, and tissue construct characterization, Biotechnol Bioeng 64: 580 - 589

Cartmell SH, Porter BD, Garcia AJ, Guldberg RE. 2003. Effects of medium perfusion rate on cell-seeded three-dimensional bone constructs in vitro. Tissue Eng 6:1197-1203.

Ceradini DJ, Kulkarni AR, Callaghan MJ, Tepper OM, Bastidas N, Kleinman ME, Capla JM, Galiano RD, Levine JP Gurtner GC. 2004. Progenitor cell trafficking is regulated by hypoxic gradients through HIF-1 induction of SDF-1, Nat Med 10: 858 - 864

Chandel NS, Maltepe E, Goldwasser E, Mathieu CE, Simon MC, Schumacker PT. 1998. Mitochondrial reactive oxygen species trigger hypoxia-induced transcription, Proc Natl Acad Sci 95: 11715 – 11720

Chen CS, Mrksich M, Huang S, Whitesides GM, Ingber DE. 1997. Geometric control of cell life and death, Science 276: 1425 – 1428

Cheng SL, Yang J, Rifas L, Zhang AF, Avioli LV. 1994. Differentiation of human bone marrow osteogenic stromal cells in vitro: induction of the osteoblast phenotype by dexamethasone, Endocrinology 134: 277 - 286

Cheng T, Rodrigues N, Shen H, Yang Y, Dombkowski D, Sykes M, Scadden D. 2000. Hematopoietic stem cell quiescence maintained by $p21^{cip1/waf1}$, Science 287: 1804 – 1808

Cipolleschi MG, Sbarba PD, Olivotto M. 1993. The role of hypoxia in the maintenance of hematopoietic stem cells, Blood 82: 2031 – 2037

Cockman ME, Masson N, Mole DR, Jaakkola P, Chang G, Clifford SC, Maher ER, Pugh CW, Ratcliffe PJ, Maxwell PH. 2000. Hypoxia-inducible factor-α Binding and Ubiquitylation by the von Hippel-Lindau Tumor Suppression Protein, J Biol Chem 275: 25733 – 25741

Colter DC, Sekiya I, Prockop DJ. 2001. Identification of a subpopulation of rapidly self-renewing and multipotential adult stem cells in colonies of human marrow stromal cells, Proc Natl Acad Sci 98: 7841 -7845

Compernolle V, Brusselmans K, Acker T, Hoet P, Tjwa M, Beck H, Plaisance S, Dor Y, Keshet E, Lupu F, Nemery B, Dewerchin M, Van Veldhoven P, Plate K, Moons L, Collen D, Carmeliet P. 2002. Loss of HIF-2α and inhibition of VEGF impair fetal lung maturation, whereas treatment with VEGF prevents fatal respiratory distress in premature mice, Nat Med 8: 702 – 710

Conget PA, Minguell JJ. 1999. Phenotypical and functional properties of human bone marrow mesenchymal progenitor cells, J Cell Phys 181: 67 – 73

Cukierman E, Pankov R, Yamada KM. 2002. Cell interaction with three-dimensional matrices, Curr Opin Cell Biol 14: 633 – 639

Cukierman E, Pankov R, Stevens DR, Yamada KM. 2001. Taking Cell-Matrix Adhesions to the Third Dimension, Science 294: 1708 – 1712

Damsky CH, Ilić D. 2002. Integrin signaling: it's where the action is, Curr Opin Cell Biol 14: 594 -602

D'Ipploito G, Diabira S, Howard GA, Meneri P, Roos BA, Schiller PC. 2004. Marrow-isolated adult multilineage inducible (MIAMI) cells, a unique population of postnatal young and old human cells with extensive expansion and differentiation potential, J Cell Sci 117: 2971 - 2981

De Arcangelis A, Georges-Labouesse E. 2000. Integrin and ECM functions role in vertebrate development, Trends Gen 16: 389 - 395

Deans RJ, Moseley AB. 2000. Mesenchymal stem cells: Biology and potential clinical uses, Exp Hematol 28: 875 – 884

Deb A, Wang SH, Skelding KA, Miller D, Simper D, Caplice NM. 2003. Bone-marrow derived cardiomyocytes are present in adult human heart – a study of gender-mismatched bone marrow transplantation patients, Circulation 107: 1247 – 1249

Dennis JE, Charbord P. 2002. Origin and differentiation of human and murine stroma, Stem Cells 20: 205 - 214

Derubeis AR, Cancedda R. 2004. Bone marrow stromal cells (BMSCs) in bone engineering: Limitations and Recent Advances, Ann Biomed Eng 32: 160 - 165

Desplat V, Faucher J, Mahon FX, Sbarba PD, Praloran V, Ivanovic Z. 2002. Hypoxia Modifies Proliferation and Differentiation of CD34+ CML Cells, Stem Cells 20: 347 – 354

DiGirolamo CM, Stokes D, Colter D, Phinney DG, Class R, Prockop DJ. 1999. Propagation and senescence of human marrow stromal cells in culture: a simple colony-forming assay identifies samples with the greatest potential to propagate and differentiate, Br J Hemat 107**:** 275–281

Dominici M, Hofmann TJ, Horwitz EM. 2001. Bone marrow mesenchymal cells: biological properties and clinical applications, J Biol Reg Homeo Agents 15: 28 -37

Downard J. 2003. Metabolism meets death, Nature 424: 896 – 897

Duncan AW, Rattis FM, Di Mascio LN, Congdon KL, Pazianos G, Zhao C, Yoon K, Cook JM, Willert K, Gaiano N, Reya T. 2005. Integration of Notch and Wnt signaling in hematopoietic stem cell maintenance, Nat Immunol *In Print*

Ebert BL, Firth JD, Ratcliffe PJ. 1995. Hypoxia and Mitochondrial Inhibitors Regulate Expression of Glucose Transporter-1 via Distinct Cis-acting Sequences, J Biol Chem 270: 29083 – 29089

Eckblom P, Lonai P, Talts JF. 2003. Expression and biological role of laminin-1, Matrix Biol 22: 35 - 47

Elisseeff J. 2004. Injectable cartilage tissue engineering, Expert Opin Biol Ther 4: 1849 – 1859

Faller DV. 1999. Endothelial cell responses to hypoxic stress, Clin Exp Pharm Phys 26: 74 - 84

Fandrey J, Frede S, Jelkmann W. 1994. Role of hydrogen peroxide in hypoxia-induced erythropoietin production, Biochem J 303: 507 – 510

Fandrey J, Seydel FP, Siegers CP, Jelkmann W. 1990. Role of Cytochrome P450 in the Control of the Production of Erythropoietin, Life Sci 47: 127 – 134

Fauza D. 2004. Amniotic fluid and placental stem cells, Best Prac Res Clin Ob Gyn 18: 877 – 891

Fedele AO, Whitelaw ML, Peet DJ. 2002. Regulation of gene expression by the hypoxia inducible factors, Mol Interven 2: 229 – 243

Ferrari G, Cusella-De Angelis G, Coletta M, Paolucci E, Stornaiuolo A, Cossu G, Mavilio F. 1998. Muscle regeneration by bone marrow-derived myogenic progenitors, Science 279: 1528 - 1530

Firth JD, Ebert BL, Pugh CW, Ratcliffe PJ. 1994. Oxygen-regulated control elements in the phosphoglycerate kinase 1 and Lactate Dehydrogenase A genes: Similarities with the Erythropoietin 3' Enhancer, Proc Natl Acad Sci 91: 6496 – 6500

Folkman J, Moscona A. 1978. Role of cell shape in growth control, Nature 273: 345 – 349

Francis Suh JK, Matthew HWT. 2000. Application of chitosan-based polysaccharaide biomaterials in cartilage tissue engineering: a review, Biomaterials 21: 2589 - 2598

Freed LE, Langer R, Martin I, Pellis NR, Vunjac-Novakovic G. 1997. Tissue engineering of cartilage in space, Proc Natl Acad Sci 94: 13885 - 13890

Freed LE, Hollander AP, Martin I, Barry JR, Langer R, Vunjac-Novakovic G. 1998. Chondrogenesis in a cell-polymer-bioreactor system, Exp Cell Res 240: 58 - 65

Freed LE, Vunjak-Novakovic G, Marquis JC, Langer R. 1994. Kinetics of chondrocytes growth in cell-polymer implants. Biotechnol Bioeng 43: 597-604.

Friedenstein AJ, Gorskaja UF, Kulagina NN. 1976. Fibroblast precursors in normal and irradiated mouse hematopoietic organs, Exp Hematol 4: 267 – 274

Gage FH. 2000. Mammalian Neural Stem Cells, Science 287: 1433 - 1438

Gamini A, Paoletti S, Toffanin R, Micali F, Michielin L, Bevilacqua C. 2002. Structural investigations of cross-linked hyaluronan, Biomaterials 23: 1161 -1167

Gang EJ, Hong SH, Jeong JA, Hwang SH, Kim SW, Yang IH, Ahn CY, Han H, Kim H. 2004. In vitro mesengenic potential of human umbilical cord blood-derived mesenchymal stem cells, Biochem Bioph Res Co, 321: 102 – 108

Gardner LB, Li Q, Park MS, Flangan WM, Semenza GL, Dang CV. 2001. Hypoxia inhibits G1/S transition through regulation of p27 expression , J Biol Chem 276: 7919 - 7926

Geiger B. 2001. Encounters in Space, Science 294: 1661-1662

Giancotti FG, Ruoslahti E. 1999. Integrin signaling, Science 285: 1028 - 1032

Gilding DK, Reed AM. 1979. Biodegradable polymers for use in surgery – polyglycolic/poly(lactic acid) homo- and copolymers: 1, Polymer 20: 1459 – 1464

Gleadle JM, Ebert BL, Firth JD, Ratcliffe PJ. 1995a. Regulation of angiogenic growth factor expression by hypoxia, transition metals and chelating agents, Am J Phys 268: C1362 – C1368

Gleadle JM, Ebert BL, Ratcliffe PJ. 1995b. Diphenylene iodonium inhibits the induction of erythropoietin and other mammalian genes by hypoxia, Eur J Biochem. 234: 92 – 99

Goldberg MA, Schneider TJ. 1994. Similarities between the oxygen-sensing mechanisms regulating the expression of vascular endothelial growth factor and erythropoietin, J Biol. Chem 269: 4355 – 4359

Goldberg MA, Dunning SP, Bunn HF. 1988. Regulation of the erythropoietin gene: evidence that the oxygen sensor is a heme protein, Science 242: 1412 – 1414

Görlach A, Fandrey J, Holtermann G, Acker H. 1994. Effects of cobalt on haem proteins of erythropoietin-producing HepG2 cells in multicellular spheroid culture, FEBS Letters 348: 216 – 218

Görlach A, Holtermann G, Jelkmann W, Hancock JT, Jones SA, Jones OTG, Acker H. 1993. Photometric characteristics of haem proteins in erythropoietin-producing hepatoma cells (HepG2), Biochem J 290: 771 – 776

Graven KK, Troxler RF, Kornfeld H, Panchenko MV, Farber HW. 1994. Regulation of endothelial-cell glyceraldehydes-3-phosphate dehydrogenase expression by hypoxia, J Biol Chem 269: 24446 - 24453

Grayson WL, Ma T, Bunnell B. 2004. Human mesenchymal stem cell tissue development in 3D PET matrices, Biotechnol Prog 20: 905 – 912

Grinell F. 2003. Fibroblast biology in three-dimensional collagen matrices, Trends Cell Biol 13: 264 - 269

Gronthos S, Franklin DM, Leddy HA, Robey PG, Storms RW, Gimble JM. 2001. Surface protein characterization of human adipose tissue-derived stromal cells, J Cell Phys 189: 54 – 63

Guilak F, Awad HA, Fermor B, Leddy HA, Gimble JM. 2004. Adipose-derived adult stem cells for cartilage tissue engineering. Biorheology 41: 389 – 399

Guillemin K, Krasnow MA, 1997. The hypoxic response: huffing and HIFing, Cell 89: 9 - 12

Gussoni E, Bennett R, Musckiewicz KR, Meyerrose T, Nolta JA, Gilgoff I, Stein J, Chan Y, Lidov HG, Bönnemann CG, von Moers A, Morris GE, den Dunnen JT, Chamberlain JS, Kunkel LM, Weinberg K. 2002. Long-term persistence of donor nuclei in a Duchenne muscular dystrophy patient receiving bone marrow transplantation, J Clin Invest 110: 807 –814

Gussoni E, Soneoka Y, Strickland CD, Buzney EA, Khan MK, Flint AF, Kunkel LM, Mulligan RC. 1999. Dystrophin expression in the mdx mouse restored by stem cell transplantation, Nature 401: 390-394

Hall PA, Watt FM. 1989. Stem cells: the generation and maintenance of cellular diversity, Development 106: 619 –633

Hawley RG, Sobieski DA. 2002. Somatic Stem Cell Plasticity: To Be or Not To Be, Stem Cells 20: 195 –197

Haynesworth SE, Baber MA, Caplan AI. 1992. Cell Surface Antigens on Human Marrow-Derived Mesenchymal Cells are detected by Monoclonal Antibodies, Bone 13: 69 – 80

Haynesworth SE, Goshima J, Goldberg VM, Caplan AI. 1992. Characterization of cells with osteogenic potential from human marrow, Bone 13: 81 - 88

Haynesworth SE, Baber MA, Caplan AI. 1996. Cytokine expression by human marrow-derived mesenchymal progenitor cells in vitro: effects of dexamethasone and IL-1α, J Cell Phys 166: 582-595

Haynesworth SE, Reuben D, Caplan AI. 1998. Cell-based tissue engineering therapies: the influence of whole body physiology, Adv Drug Del Rev 33: 3 – 14

Heath CA. 2000. Cells for Tissue Engineering, *TIBTECH* 18: 17 – 19

Hellkamp J, Christ B, Bastian H, Jungermann K. 1991. Modulation by oxygen of the glucagon-dependent activation of the phosphoenolpyruvate carboxylase gene in rat hepatocyte cultures, Eur J Biochem 198: 635 – 639

Hevehan DL, Papoutsakis T, Miller WM. 2000. Physiologically significant effects of pH and oxygen tension on granulopoiesis, Exp Hemat 28: 267 – 275

Ho VT, Bunn HF. 1996. Effects of transition metals on the expression of the erythropoietin gene: further evidence that the oxygen sensor is a heme protein, Biochem Bioph Res Co 223: 175 – 180

Holden C, Vogel G. 2002. Plasticity: time for a reappraisal, Science 296: 2126 – 2129

Holtorf H, Jansen JA, Mikos AG. 2005. Flow perfusion culture induces the osteoblastic differentiation of marrow stromal cell-scaffold constructs in the absence of dexamethasone, J Biomed Mat Res A 72A: 326 – 334

Holy CE, Shoichet MS, Davies JE. 2000. Engineering three-dimensional bone tissue in vitro using biodegradable scaffolds: investigating initial cell-seeding density and culture period, J Biomed Matl Res 51: 376 - 382

Horino Y, Takahashi S, Miura T, Takahashi Y. 2002. Prolonged hypoxia accelerates the posttranscriptional process of collagen synthesis in cultured fibroblasts, Life Sci 71: 3031 – 3045

Horwitz AF. 1997. Integrins and Health, Sci Am 276: 68 - 75

Horwitz EM, Gordon P, Koo WKK, Marx JC, Neel MD, McNall RY, Muul L, Hofmann T. 2002. Isolated allogeneic bone-marrow derived mesenchymal cells engraft and stimulate growth in children with osteogenesis imperfecta: Implications for cell therapy of bone, Proc Natl Acad Sci 99: 8932 – 8937

Horwitz EM, Prockop DJ, Fitzpartick LA, Koo WW, Gordon PL, Neel M, Sussman M, Orchard P, Marx JC, Pyeritz RE, Brenner MK. 1999. Transplantability and therapeutic effects of bone marrow-derived mesenchymal cells in children with osteogenesis imperfecta. Nat Med 5:309-313

Huang LE, Arany Z, Livingston DM, Bunn HF. 1996. Activation of hypoxia inducible transcription factor depends primarily upon redox-sensitive stabilization of its α subunit, J Biol Chem 271: 32253 – 32259

Huang LE, Gu J, Schau M, Bunn HF. 1998. Regulation of hypoxia inducible factor 1α is mediated by an O2-dependent degradation domain via the ubiquitin proteasome pathway, Proc Natl Acad Sci 95: 7987 – 7992

Huang S, Ingber DE. 2000. Shape-dependent control of cell-growth, differentiation, and apoptosis: switching between attractors in cell regulatory networks, Exp Cell Res 261: 91 - 103

Hung S, Chen N, Hsieh S, Li H, Ma H, Lo W. 2002. Isolation and characterization of size-sieved stem cells from human bone marrow, Stem Cells 20: 249 – 258

Hynes RO. 1999. Cell adhesion: old and new questions, Trends Cell Biol 9: M33 – M37

Hynes RO. 1992. Integrins: versatility, modulation and signaling in cell adhesion, Cell 69: 11 – 25

Igura K, Zhang X, Takahashi K, Mitsuru A, Yamaguru S, Takahashi TA. 2004. Isolation and characterization of mesenchymal progenitor cells from chorionic villi of human placenta, Cytotherapy 6: 543 – 553

Iida T, Mine S, Fujimoto H, Suzuki K, Minami Y, Tanaka Y. 2002. Hypoxia-inducible factor-1α induces cell cycle arrest of endothelial cells, Genes Cells 7: 143 – 149

Ingber DE. 2003a. Tensegrity I: Cell structure and hierarchical systems, J Cell Sci 116: 1157 – 1173

Ingber DE. 1997. Tensegrity: The architectural basis of cellular mechanotransduction, Ann Rev Phys 59: 575 – 599

Ingber DE. 2003b. Tensgrity II: How structural networks influence cellular information processing networks, J Cell Sci 116: 1397 – 1408

Ingber DE. 1990. Fibronectin controls capillary endothelial cell growth by modulating cell-shape, Proc Natl Acad Sci 87: 3579 – 3583

Ishaug SL, Crane GM, Miller MJ, Yasko AW, Yaszemski MJ, Mikos AG. 1997. Bone formation by three-dimensional stromal osteoblast culture in biodegradable polymer scaffolds, J Biomed Mat Res 36: 17 - 28

Ivan M, Kondo K, Yang H, Kim W, Valiando J, Ohh M, Salic A, Asara JM, Lane WS, Kaelin Jr WG. 2001. HIF-α targeted for VHL-mediated destruction by proline hydroxylation: Implications for O2 Sensing, Science 292: 464 – 468

Ivanovic Z, Bartolozzi B, Bernabei PA, Cipolleschi MG, Rovida E, Milenkovic P, Praloran V, Sbarba PD. 2000a. Incubation of murine bone marrow cells in hypoxia ensures the maintenance of marrow-repopulating ability together with the expansion of committed progenitors, Br J Hemat 108: 424 – 429

Ivanovic Z, Belloc F, Faucher J, Cipolleschi MG, Praloran V, Sbarba PD. 2002. Hypoxia maintains and interleukin-3 reduces the pre-colony forming cell potential of dividing CD34+ murine bone marrow cells, Exp Hematol 30: 67 – 73

Ivanovic Z, Sbarba PD, Trimoreau F, Faucher J, Praloran V. 2000b. Primitive human HPCs are better maintained and expanded in vitro at 1 percent oxygen than at 20 percent, Transfusion 40: 1482 – 1488

Iyer NV, Kotch LE, Agani F, Leung SW, Laughner E, Wenger RH, Gassmann M, Gearhart JD, Lawler AM, Yu AY, Semenza GL. 1998. Cellular and developmental control of O2 homeostasis by hypoxia-inducible factor 1α, Genes Dev 12: 149 – 162

Jaakkola P, Mole DR, Tian YM, Wilson M, Gielbert J, Gaskell SJ, von Kriegshem A, Hebestreit HF, Mukherji M, Schoffield CJ, Maxwell PH, Pugh CW, Ratcliffe PJ. 2001. Targeting of HIF-α to the von Hippel-Lindau Ubiquitylation Complex by O2-Regulated Prolyl Hydroxylation, Science 292: 468 – 472

Jaiswal N, Haynesworth SE, Caplan AI, Bruder SP. 1997. Osteogenic differentiation of purified, culture-expanded human mesenchymal stem cells in vitro, J Cell Biochem 64: 295 –312

Javazon EH, Beggs KJ, Flake AW. 2004. Mesenchymal stem cells: paradoxes of passaging, Exp Hematol 32: 414 –425

Jensen UB, Lowell S, Watt FM. 1999. The spatial relationship between stem cells and their progeny in the basal layer of human epidermis: a new view based on whole-mount labeling and lineage analysis, Development 126: 2409 – 2418

Jiang BH, Semenza GL, Bauer C, Marti HH. 1996. Hypoxia-inducible factor 1 levels vary exponentially over a physiologically relevant range of O2 tension, Am J Phys 271: C1172 – C1180

Jiang BH, Zheng ZH, Leung SW, Roe R, Semenza GL. 1997. Transactivation and inhibitory domains of hypoxia-inducible factor 1 alpha. Modulation of transcriptional activity by oxygen tension. J Biol Chem 272: 19253 - 19260

Jiang YH, Jahagirdar BN, Reinhardt RL, Schwartz RE, Keene CD, Ortiz-Gonzalez XR, Reyes M, Lenvik T, Lund T, Blackstad M, Du JB, Aldrich S, Lisberg A, Low WC, Largaespada DA, Verfaillie CM. 2002. Pluripotency of mesenchymal stem cells derived from adult bone marrow, Nature 418: 41 –49

Jones PH, Harper S, Watt FM. 1995. Stem cell patterning and fate in human epidermis, Cell 80: 83 –93

Kadareit S, Deeds L, Haynesworth S, Koc O, Kozic M, Szekely E, Daum-Woods K, Goetchius G, Fu P, Welniak L, Murphy W, Laughlin M. 2002. Expansion of LTC-ICs and maintenance of p21 and BCL-2 expression in cord blood CD34+/CD38- early progenitors cultured over human MSCs as a feeder layer, Stem Cells 20: 573 – 582

Kallio PJ, Okamoto K, O'Brien S, Carrero P, Makin Y, Tanaka H, Poellinger L. 1998. Signal Transduction in Hypoxic Cells: Inducible Nuclear Translocation and Recruitment of the CBP/p300 Coactivator by the Hypoxia Inducible Factor-1α, EMBO J 17: 6573 –6586

Kan C, Abe M, Yamanaka M, Ishikawa O. 2003. Hypoxia-induced increase of matrix-metalloproteinase-1 synthesis is not restored by reoxygenation in a three-dimensional culture of human dermal fibroblasts, J Dermatol Sci 32: 75 – 82

Kantz BS. 1991. Erythropoietin, Blood 77: 419 – 434

Katz BZ, Zamir E, Bershadsky A, Kam Z, Yamada KM, Geiger B. 2000. Physical state of the extracellular matrix regulates the structure and molecular composition of cell-matrix adhesions, Mol Bio Cell 11: 1047 – 1060

Kavalkovich KW, Boynton RE, Murphy JM, Barry F. 2002. Chondrogenic differentiation of human mesenchymal stem cells within an alginate layer culture system, In Vitro Cell Dev Biol – Anim 38: 457 – 466

Klein CE, Dressel D, Steinmayer T, Mauch C, Eckes B, Krieg T, Bankert RB, Weber L. 1991. Integrin alpha-2 beta-1is up-regulated in fibroblasts and highly aggressive melanoma-cells in 3-dimensional collagen lattices and mediates the reorganization of collagen-I fibrils, J Cell Biol 115: 1427 - 1436

Kocher AA, Schuster MD, Szabolcs MJ, Takuma S, Burkhoff D, Wang J, Homma S, Edwards NM, Itescu S, 2001. Neovascularization of ischemic myocardium by human bone-marrow-derived angioblasts prevents cardiomyocyte apoptosis, reduces remodeling and improves cardiac functions, Nat Med 7: 430 – 436

Kotch LE, Iyer NV, Laughner E, Semenza GL. 1999. Defective vascularization of HIF-1α-null embryos is not associated with VEGF deficiency but with mesenchymal cell death, Dev Biol 209: 254 – 267

Kozak KR, Abbott B, Hankison O. 1997. ARNT-Deficient mice and placental differentiation, Dev Biol 191: 297 – 305

Krishnamurthy P, Ross DD, Nakanishi T, Bailey-Dell K, Zhou S, Mercer KE, Sarkadi B, Sorrentino BP Schuetz JD. 2004. The stem cell marker Bcrp/ABCG2 enhances hypoxic cell survival through interactions with heme, J Biol Chem 279: 24218 - 24225

Kuo CK, Tuan RS. 2003. Tissue engineering with mesenchymal stem cells, IEEE Eng Biol Med Mag 22: 51 – 56

Kurz B, Domm C, Moonsoo J, Sellckau R, Schunke M. 2004. Tissue engineering of auricular cartilage under the influence of collagen I/II membranes and low oxygen tension, Tissue Eng 10: 1277 - 1296

Kuznetsov SA, Mankani MH, Gronthos S, Satomura K, Bianco P, Robey PG. 2001. Circulating skeletal stem cells, J Cell Biol 153: 1133 – 1140

Ladoux A, Frelin C. 1993. Hypoxia is a strong Inducer of Vascular Endothelial Growth Factor mRNA Expression in the Heart, Biochem Bioph Res Co 195: 1005 – 1010

Langer R, Vacanti JP. 1993. Tissue Engineering, Science 260: 920 – 925

Langer R. 1995. Biomaterials and biomedical engineering, Chem Eng Sci 50: 4109 - 4121

Lash GE, Fitzpatrick TE, Graham CH. 2001. Effect of Hypoxia on Cellular Adhesion to Vitronectin and Fibronectin, Biochem Bioph Res Co 287: 622 – 629

Lavik E, Langer R. 2004. Tissue engineering: current state and perspectives, App Microbiol Biotechnol 65: 1 – 8

Le Blanc K, Rasmusson I, Sundberg B, Götherström C, Hassan M, Uzenel M, Ringdén O. 2004. Treatment of severe acute graft-versus-host-disease with third party haploidentical mesenchymal stem cells, Lancet 363: 1439 – 1441

Lennon DP, Edmison JM, and Caplan AI. 2001. Cultivation of rat marrow-derived mesenchymal stem cells in reduced oxygen tension: effects on in vitro and in vivo osteochondrogenesis, J Cell Phys 187: 345 – 355

Lennon DP, Haynesworth SE, Bruder SP, Jaiswal N, Caplan AI. 1996. Human and animal mesenchymal progenitor cells from bone marrow: identification of serum for optimal selection and proliferation, In vitro Cell Dev Biol – Anim, 32: 602 - 611

Levenberg S, Huang NF, Lavik E, Rogers AB, Itskovitz-Eldor J, Langer R. 2003. Differentiation of human embryonic stem cells on three-dimensional polymer scaffolds, Proc Natl Acad Sci 100: 12741 - 12746

Li W, Tuli R, Okafor C, Derfoul A, Danielson KG, Hall DJ, Tuan RS. 2005. A three-dimensional nanofibrous scaffold for cartilage tissue engineering using human mesenchymal stem cells, Biomaterials 26: 599 - 609

Li Y, Ma T, Kniss DA, Yang ST. 2001. Modification and characterization of PET non-woven fibrous matrix as cell culture scaffold. Biomaterials 22:609-618.

Li Y, Ma T, Kniss DA, Lasky LC, Yang ST. 2001. Effects of Filtration Seeding on Cell Density, Spatial Distribution and Proliferation in Nonwoven Fibrous Matrices, Biotechnol Prog 17: 935 – 944

Li YJ, Batra NN, You L, Meier SC, Coe IA, Yellowley CE, Jacobs CR. 2004. Oscillatory fluid flow affects human marrow stromal cell proliferation and differentiation, J Orthopaed Res 22: 1283 - 1289

Lin Z, Weinberg JM, Malhorta R, Merritt SE, Holzman LB, Brosius FC III. 2000. GLUT-1 reduces hypoxia-induced apoptosis and JNK pathway activation, Am J Phys 278: E958 – E966

Loike JD, Cao L, Brett J, Ogawa S, Silverstein SC, Stern D. 1992. Hypoxia induces glucose transporter expression in endothelial cells, Am J Phys 263: C326 – C333

Luo J, Yang ST. 2004. Effects of three-dimensional culturing in a fibrous matrix on cell cycle, apoptosis, and Mab production by hybridoma cells, Biotechnol Prog 20: 306 - 315

Ma T, Li Y, Yang ST, Kniss D. 2000. Effects of trophoblast cell organization in fibrous matrix on long-term tissue development and cell cycle. Biotechnol Bioeng 70: 606-618.

Ma T, Li Y, Yang ST, Kniss D. 1999a. Tissue engineering human placenta trophoblast cells in 3-D fibrous matrix: spatial effects on cell proliferation and function, Biotechnol Prog 15: 715 – 724

Ma T, Yang ST, Kniss D. 1999b. Development of an in vitro human placenta model by the cultivation of human trophoblast in a fiber-based bioreactor system. Tissue Eng 5:91-102.

Ma T, Yang ST, Kniss DA. 2001. Oxygen tension influences proliferation and differentiation in a tissue-engineered model of placental trophoblast cells, Tissue Eng 7: 495 – 506

Mackay AM, Beck SC, Murphy JM, Barry FP, Chichester CO, Pittenger MF. 1998. Chondrogenic differentiation of cultured human mesenchymal stem cells from marrow, Tissue Eng 4: 415 - 428

Majumdar MK, Keane-Moore M, Buyaner D, Hardy WB, Moorman MA, McIntosh KR, Mosca JD. 2003. Characterization and functionality of cell surface molecules on human mesenchymal stem cells, J Biomed Sci 10: 228 –241

Majumdar M, Wang E, Morris E. 2001. BMP-2and BMP-9 promote chondrogenic differentiation of human multipotential mesenchymal cells and overcome the inhibitory effect of IL-1, J Cell Phys 189: 275 - 284

Malda J, Woodfield TBF, van der Vloodt F, Kooy FK, Martens DE, Tramper J, van Blitterswijk CA, Riesle J. 2004. The Effect of PEGT/PBT scaffold architecture on oxygen gradients in tissue engineered cartilaginous constructs, Biomaterials 25: 5773-5780

Malhorta R, Brosius FC III. 1999. Glucose uptake and glycolysis reduce hypoxia-induced apoptosis in cultured neonatal rat cardiac myocytes, J Cell Biol 274: 12567 – 12575

Malhorta R, Lin Z, Vicenz C, Brosius FC. 2001. Hypoxia induces apoptosis via two independent pathways in jurkat cells: differential regulation by glucose, Am J Phys 285: C1596 – C 1603

Marneros A, Olsen B. 2001. The role of collagen-derived proteolytic fragments in angiogenesis, Matrix Biol 20: 337 - 345

Martin I, Obradovic B, Freed L, Vunjak-Novakovic G. 1999. Method for quantitative analysis of glycosaminoglycan distribution in cultured natural and engineered cartilage, Ann Biomed Eng 27: 656 – 662

Martin I, Shastri VP, Padera RF, Yang J, Mackay AJ, Langer R, Vunjak-Novakovic G, Freed L. 2001. Selective differentiation of mammalian bone marrow stromal cells cultured on three-dimensional polymer foams, J Biomed Mat Res 82: 229-235

Martin I, Wendt D, Heberer M. 2004. The role of bioreactors, Trends Biotechnol 22: 80 – 85

Marx J. 2004. Cell biology – how cells endure low oxygen, Science 303: 1454 - 1456

Mc Beath R, Pirone DM, Nelson CM, Bhadriraju K, Chen CS. 2004. Cell shape, cytoskeletal tension and RhoA regulate stem cell lineage commitment, Dev Cell, 6: 483 – 495

Meerovitch K, Bergeron F, Leblond L, Grouix B, Pourier C, Bubenik M, Chan L, Gourdeau H, Bowlin T, Attardo G. 2003. A novel RGD antagonist that targets both αVβ3 and α5β1 induces apoptosis of angiogenic endothelial cells on type I collagen, Vasc Pharmacol 40: 77 - 89

Meinel L, Hofmann S, Karageorgiou V, Zichner L, Langer R, Kaplan D, Vunjac-Novakovic G. 2004. Engineeering cartilage-like tissue using human mesenchymal stem cells and silk protein scaffolds, Biotechnol Bioeng 88: 379 -391

Menasché P. 2004. Cellular transplantation: hurdles remainng before widespread clinical use, Curr Opin Cardiol 19: 154 – 161

Mezey E, Key S, Vogelsang G, Szalayova I, Lange GD, Crain B. 2003. Transplanted Bone Marrow Generates New Neurons in Human Brains, Proc Natl Acad Sci 100: 1364 –1369

Minchenko A, Salceda S, Bauer T, Caro J. 1994. Hypoxia Regulatory Elements of the Human Vascular Endothelial Growth Factor Gene, Cell Mol Biol Res 40: 35 –39

Minguell JJ, Erices A, Conget P. 2001. Mesenchymal stem cells, Exp Biol Med 226: 507 - 520

Mizuno M, Fujisawa R, Kuboki Y. 2000. Type I collagen-induced osteoblastic differentiation of bone-marrow cells mediated by collagen-α2β1 integrin interaction, J Cell Phys 184: 207 - 213

Mizuno M, Shindo M, Kobayashi D, Tsuruga E, Amemiya A, Kuboki Y**.** 1997. Osteogenesis by bone marrow stromal cells maintained on type I collagen matrix gels in vivo, Bone 20: 101 – 107

Morrison SJ. 2001. Stem cell potential: can anything make anything, Curr Biol 11: R7 – R9

Mostafa SS, Miller WM, Papoutsakis ET. 2000. Oxygen tension influences the differentiation, maturation and apoptosis of human megakaryocytes, Br J Haematol 111: 879 – 889

Mueller SM, Glowacki J. 2001. Age-related decline in the osteogenic potential of human bone marrow cells cultured in three-dimensional collagen sponges, J Cell Biochem 82: 583-590

Munoz-Elias G, Woodbury B, Black IB. 2003. Marrow stromal cells, mitosis, and neuronal differentiation: stem cell and precursor functions, Stem Cells 21: 437 - 448

Muschler GF, Nitto H, Matsukura Y, Boehm C, Valdevit A, Kambic H, Davros W, Powell K, Easley K. 2003. Spine fusion using cell matrix composites enriched in bone marrow-derived cells, Clin Orthopaed 407: 102 – 118

Nakamura T, Shiojima S, Hirai Y, Iwama T, Tsuruzoe N, Hirasawa A, Katsuma S, Tsujimoto G. 2003. Temporal gene expression changes during adipogenesis in human mesenchymal stem cells, Biochem Bioph Res Co 303: 306 - 312

Orlic D, Kajstura J, Chimenti S, Jakoniuk I, Anderson SM, Li BS, Pickel J, McKay R, Nadal-Ginard B, Bodine DM, Leri A, Anversa P. 2001a. Bone marrow cells regenerate infarcted myocardium, Nature 410: 701 – 705

Orlic D, Kajstura J, Chimenti S, Limana F, Jakoniuk I, Quaini F, Nadal-Ginard B, Bodine DM, Leri A, Anversa P. 2001b. Mobilized bone-marrow cells repair the infarcted heart: improving function and survival, Proc Natl Acad Sci 98: 10344 – 13349

Packer L, Fuehr K. 1977. Low Oxygen Concentrations Extends the Lifespan of Cultured Human Diploid Cells, Nature 267: 423 – 425

Pei M, Solchaga LA, Seidel J, Zeng L, Vunjak-Novakovic G, Caplan AI, Freed LE. 2002. Bioreactors mediate the effectiveness of tissue engineering scaffolds. FASEB J 16: 1691 - 1694

Peng CA, Palsson BO. 1996. Determination of specific oxygen uptake rates in human hematopoietic cultures and implications for bioreactor design. Ann Biomed Eng 24:373-381.

Peng J, Zhang L, Drysdale L, Fong GH. 2000. The transcription factor HIF-2α plays an important role in vascular remodeling, Proc Natl Acad Sci 97: 8386 –8391

Peteresen EF, Spencer RGS, McFarland EW. 2002. Microengineering neocartilage scaffolds, Biotechnol Bioeng 78: 801 - 804

Petersen BE, Boewn WC, Patrene KD, Mars WM, Sullivan AK, Murase N, Boggs SS, Greenberger JS, Goff JP. 1999. Bone marrow as a potential source of hepatic oval cells, Science 284: 1168 – 1170

Pfander D, Cramer T, Schipan E, Johnson RS. 2003. HIF-1α controls extracellular matrix synthesis by epiphyseal chondrocytes, J Cell Sci 116: 1819 – 1826

Pittenger MF, Mackay AM, Beck SC, Jaiswal RK, Douglas R, Mosca JD, Moorman MA, Simonetti DW, Craig S, Marshak DR. 1999. Multilineage potential of adult human mesenchymal stem cells, Science 284: 143 – 147

Pittenger MF, Martin BJ. 2004. Mesenchymal stem cells and their potential as cardiac therapeutics. Circ Res 95: 9 –20

Plas DR, Thompson CB. 2002. Cell Metabolism in the Regulation of Programmed Cell Death, Trends Endocrinol Metabol 13: 74 –77

Poellinger L, Johnson RS. 2004. HIF-1 and hypoxic response: the plot thickens, Curr Opin Gen Dev 14: 81 – 85

Ponticiello MS, Schinagl RM, Kadiyala S, Barry FP. 2000. Gelatin-based resorbable sponge as carrier matrix for human mesenchymal stem cells in cartilage regeneration therapy, J Biomed Mat Res 52: 246 –255

Potten CS, Loeffler M. 1990. Stem cells – attributes, cycles, spirals, pitfalls and uncertainties, lessons for and from the crypt, Development 110: 1001 –1020

Prockop DJ, Gregory CA, Spees JL. 2003. One strategy for cell and gene therapy: harnessing the power of adult stem cells to repair tissue, Proc Natl Acad Sci 100: 11917 -11923

Prockop DJ. 1997. Marrow stromal cells as stem cells for nonhematopoietic tissues, Science 276: 71 – 74

Qian L, Saltzman WM. 2002. Improving the expansion and neuronal differentiation of mesenchymal stem cells through culture surface modification, Biomaterials 25: 1331 – 1337

Quarto R, Mastrogiacomo M, Cancedda R, Kutepov SM, Mukhachev V, Lavroukov A, Kon E, Marcacci M. 2001. Repair of large bone defects with the use of autologous bone marrow stromal cells, NE J Med 344: 385 –386

Ramirez-Bergeron DL, Simon MC. 2001. Hypoxia-inducible factor and the development of stem cells of the cardiovascular system, Stem Cells 19: 279 – 286

Randers-Eichorn L, Bartlett RA, Frey DD, Rao G. 1996. Noninvasive oxygen measurements and mass transfer considerations in tissue culture flasks, Biotechnol Bioeng 51: 466 - 478

Reyes M, Verfaillie CM. 2001. Characterization of multipotent adult progenitor cells, a subpopulation of mesenchymal stem cells, Ann NY Acad Sci 938: 231 – 235

Reyes M, Lund T, Lenvik T, Aguiar D, Koodie L, Verfaillie CM. 2001. Purification and ex vivo expansion of postnatal human marrow mesodermal progenitor cells, Blood 98: 2615 – 2625

Romanov YA, Svintsitskaya VA, Smirnov VN. 2003. Searching for alternative sources of postnatal human mesenchymal stem cells: candidate MSC-like cells from umbilical cord, Stem Cells 21: 105 – 110

Rosethal N, Tsao L. 2001. Helping the heart to heal with stem cells, Nat Med 7: 412 – 413

Roskelley CD, Bissell MJ. 1995. Dynamic reciprocity revisited: a continuous, bi-directional flow of information between cells and the extracellular matrix regulates mammary epithelial cell function, Biochem Cell Biol 73: 391 –397

Rosso F, Giordano A, Barbarisi M, Barbarisi A. 2004. From cell-ECM interactions to tissue engineering, J Cell Phys 199: 174 –180

Roufosse CA, Direkze NC, Otto WR, Wright NA. 2004. Circulating mesenchymal stem cells, Int J Biochem Cell Biol 26: 585 – 597

Saini S, Wick TM. 2003. Concentric cylinder bioreactor for production of tissue engineered cartilage: effect of seeding density and hydrodynamic loading on construct development. Biotechnol Prog 19:510-521.

Salceda S, Caro J. 1997. Hypoxia-inducible factor-1α (HIF-1α) protein is rapidly degraded by the ubiquitin-proteasome system under normoxic conditions, J Biol Chem 272: 22642 – 22647

Santini MT, Rainaldi G, Indovina PL. 2000. Apoptosis, cell adhesion and the extracellular matrix in the three-dimensional growth of multicellular tumor spheroids, Crit Rev Oncol Hematol 36: 75 - 87

Schieker M, Pautke C, Reitz K, Hemraj I, Neth P, Mutschlre W, Milz S. 2004. The use of four-colour immunofluorescence techniques to identify mesenchymal stem cells, J Anat 204: 133 – 139

Schiro JA, Chan BMC, Roswit WT, Kassner P, Pentland AP, Hemler ME, Eisen AZ, Kupper TS. 1991. Integrin alpha-2-beta-1 (VLA-2) mediates reorganization and contraction of collagen matrices by human cells, Cell 67: 403 - 410

Schmidt CE, Baier JM. 2000. Acellular vascular tissues: natural biomaterials for tissue repair and tissue engineering, Biomaterials 21: 2215 - 2231

Sekiya I, Larson BL, Smith JR, Pochampally R, Cui J, Prockop DJ. 2002a. Expansion of human adult stem cells from bone marrow stroma: conditions that maximize the yields of early progenitors and evaluate their quality, Stem Cells 20: 530 – 541

Sekiya I, Vuoristo JT, Larson BL, Prockop DJ. 2002b. In vitro cartilage formation by human adult stem cells from bone marrow stroma defines the sequence of cellular and molecular events during chondrogenesis, Proc Natl Acad Sci 99: 4397 - 4402

Semenza GL, Wang GL. 1992. A nuclear factor induced by hypoxia via de novo protein synthesis binds to the human erythropoietin gene enhancer at a site required for transcriptional activation, Mol Cell Biol 12: 5447 –5454

Semenza GL. 1998. Hypoxia-inducible factor 1: master regulator of O2 homeostasis, Curr Opin Gen Dev 8: 88 – 594

Semenza GL. 1999. Regulation of mammalian O2 homeostasis by hypoxia-inducible factor 1, Ann Rev Cell Dev Biol 15: 551 –578

Sharma B, Elisseeff JH. 2004. Engineering structurally organized cartilage and bone tissues, Ann Biomed Eng 32: 148 – 159

Shur I, Marom R, Lokiec F, Socher R, Benayahu D. 2002. Identification of Progenitor Cells from Human Marrow Stroma, J Cell Biochem 87: 51 – 57

Sikavitsas VL, Bancroft GN, Holtorf HL, Jansen JA, Mikos AG. 2003. Mineralized matrix deposition by marrow stromal osteoblasts in 3D perfusion culture increases with increasing shear forces, Proc Natl Acad Sci 100: 14683 – 14688

Sikavitsas VL, Bancroft GN, Lemoine JJ, Liebschner MAK, Dauner M, Mikos AG. 2005. Flow perfusion enhances the calcified matrix deposition of marrow stromal cells in biodegradable nonwoven fiber mesh scaffolds, Ann Biomed Eng 35: 63 – 70

Simonsen JL, Rosada C, Serakinci N, Justesen J, Stenderup K, Rattan SIS, Jensen TG, Kassem M. 2002. Telemorase expression extends the proliferative life-span and maintains the osteogenic potential of human bone marrow stromal cells, Nat Biotechnol 20: 592 – 596

Singhvi R, Kumar A, Lopez GP, Stephanopoulos GN, Wang D, I, Whitesides GM, Ingber DE. 1994. Engineering cell shape and function, Science 264: 696 - 698

Sivitz Wl, Lund DD, Yorek B, Grover-McKay M, Schmid PG. 1992. Pretranslational regulation of two cardiac glucose transporters in rats exposed to hypobaric hypoxia, Am J Phys 263: E562 – E 569

Solchaga LA, Gao J, Dennis JE, Awadallah A, Lundberg M, Caplan AI, Goldberg VM. 2002. Treatment of Osteochondral Defects with Autologous Bone Marrow in a Hyaluronan-Based Delivery Vehicle, Tissue Eng 8: 333 – 347

Spees JL, Olson SD, Ylostalo J, Lynch P, Smith J, Perry A, Peister A, Wang M, Prockop DJ. 2003. Differentiation, cell fusion, and nuclear fusion during ex vivo repair of epithelium by human adult stem cells from bone marrow stroma, Proc Natl Acad Sci 100: 2397 - 2402

Stamm C, Kleine HD, Westphal B, Petzsch M, Kittner C, Nienaber CA, Freund M, Steinhoff G. 2004. CABG and bone marrow stem-cell transplantation after myocardial infarction, Thor Cardiovasc Surg 52: 152 – 158

Stamm C, Westphal B, Kleine HD, Petzsch M, Kittner C, Klinge H, Schumichen C, Nienaber CA, Freund M, Steinhoff G. 2003. Autologous bone-marrow stem-cell transplantation for myocardial regeneration, Lancet 361: 45 – 46

Steinbrech DS, Longaker MT, Mehrar BJ, Saadeh PB, Chin GS, Gerrets RP, Chau DC, Rowe NM, Gittes GK. 1999. Fibroblast response to hypoxia: the relationship between angiogenesis and matrix regulation, J Surg Res 84: 127 – 133

Steinman RA. 2002. Cell cycle regulators and hematopoiesis, Oncogene 21: 3403 - 3413

Streuli C. 1999. Extracellular matrix remodeling and cellular differentiation, Curr Opin Cell Biol 11: 634 – 640

Stute N, Holtz K, Bubenheim M, Lange C, Blake F, Zander AR. 2004. Autologous serum for isolation and expansion of human mesenchymal stem cells for clinical use, Exp Hematol 32: 1212 - 1225

Sutherland RM, Sordat B, Bamat J, Gabbert H, Bourrat B, Muellerklieser W. 1986. Oxygenation and differentiation in multicellular spheroids of human colon carcinoma, Cancer Res 416: 5320 - 5329

Terada N, Hamazaki T, Oka M, Hoki M, Mastalerz DM, Nakano Y, Meyer EM, Morel L, Petersen BE, Scott EW. 2002. Bone marrow cells adopt the phenotype of other cells by spontaneous fusion, Nature 416: 542 – 545

Thomas CH, Collier JH, Sfeir CS, Healy KE. 2002. Engineering gene expression and protein synthesis by modulation of nuclear shape. Proc Natl Acad Sci 99:1972-1977.

Tian H, Hammer RE, Matsumoto AM, Russell DW, McKnight SL. 1998. The hypoxia responsive transcription factor EPAS1 is essential for catecholamine homeostasis and protection against heart failure during embryonic development, Genes Dev 12: 3320 – 3324

Toma C, Pittenger MF, Cahill KS, Kessler PD. 2002. Human mesenchymal stem cell differentiate to a cardiomyocyte phenotype in the adult murine heart, Circulation 105: 93 - 98

Tremain N, Korkko J, Ibberson D, Kopen GC, DiGirolamo C, Phinney DG. 2001. MicroSAGE analysis of 2,353 expressed genes in a single cell-derived colony of undifferentiated human mesenchymal stem cells reveals mRNAs of multiple cell lineages, Stem Cells 19: 408 – 418

Tsuchiya K, Chen G, Uhsida T, Matsuno T, Tateishi T. 2001. Effect of cell adhesion molecules on adhesion of chondrocytes, ligament cells and mesenchymal stem cell, Mat Sci Eng 17: 79 – 82

Tuan RS, Boland G, Tuli R. 2003. Adult mesenchymal stem cells and cell-based tissue engineering, Arthritis Res Ther 5: 32 – 45

Turner CE. 1998. Molecules in focus paxillin, Int J Biochem Cell Biol 30: 955 - 959

Van der Bos C, Silverstetter S, Murphy M, Connolly T. 1998. $p21^{cip1}$ rescues human mesenchymal stem cells from apoptosis induced by low-density culture, Cell Tissue Res 293: 463 – 470

Vergani L, Grattarola M, Nicolini C. 2004. Modifications of chromatin structure and gene expression following induced alterations of cell shape, Int J Biochem Cell Bio. 36: 1447 - 1461

Vunjak-Novakovic G, Obradovic B, Martin I, Bursac PM, Langer R, Freed LE. 1998. Dynamic Cell Seeding of Polymer Scaffolds for Cartilage Tissue Engineering. Biotechnol Prog 14:193-202

Wagers AJ, Weissman IL. 2004. Plasticity of adult stem cells, Cell 116: 639 –648

Wang GL, Semenza GL. 1993. Desferrioxamine induces erythropoietin gene expression and hypoxia inducible factor 1 DNA binding activity: implications for models of hypoxia signal transduction, Blood 82: 3610 – 3615

Wang GL, Jiang B, Rue EA, Semenza GL. 1995a. Hypoxia-inducible factor-1 is a basic-helix-loop-helix-PAS heterodimer regulated by cellular O2 Tension, Proc Natl Acad Sci 92: 5510 – 5514

Wang GL, Jiang B, Semenza GL. 1995b. Effects of Protein Kinase and Phosphatase Inhibitors on Expression of Hypoxia-Inducible Factor-1, Biochem Bioph Res Co 216: 669 - 675

Wang JHC, Jia F, Gilbert TW, Woo SLY. 2003. Cell orientation determines the alignment of cell-produced collagenous matrix, J Biomech 36: 97 -102

Watt FM, Hogan BLM. 2000. Out of Eden: Stem cells and their niches, Science 287:1427 – 1430

Watt FM. 2001. Stem cell fate and patterning in the mammalian epidermis, Curr Opin Gen Dev 11: 410 – 417

Weaver VM, Petersen OW, Wang F, Larabell CA, Briand P, Damsky C, Bissell MJ. 1997. Reversion of the malignant phenotype of human breast cells in three-dimensional culture and in vivo by integrin blocking antibodies, J Cell Bio 137: 231 – 245

Weiner CM, Booth G, Semenza GL. 1996. In vivo expression of mRNAs encoding hypoxia-inducible factor 1, Biochem Bioph Res Co 225: 485 – 488

Wendt D, Marsano A, Jakob M, Heberer M, Martin I. 2003. Oscillating perfusion of cell suspensions through three-dimensinal scaffolds enhances cell seeding efficiency and uniformity, Biotechnol Prog 84: 205 – 214

Wenger RH. 2000. Mammalian Oxygen Sensing, Signalling and Gene Regulation, J Exp Biol 203: 1253 – 1263

Wittelsberger SC, Kleene K, Penman S. 1981. Progressive loss of shape-responsive metabolic controls in cells with increasingly transformed phenotype, Cell 24: 859 – 866

Wolf K, Mazo I, Leung H, Engelke K, von Adrian UH, Deryugina EI., Strongin AY, Bröcker EB, Friedl P. 2003. Compensation mechanism in tumor cell migration: mesenchymal amoeboid transition after blocking of pericellular proteolysis, J Cell Biol 160: 267 – 277

Woodbury D, Schwarz EJ, Prockop DJ, Black IB. Adult rat and human bone marrow stromal cells differentiate into neurons, J. Neurosci Res 61: 364 – 370

Worster AA, Brower-Toland BD, Fortier LA, Bent SJ, Williams J, Nixon AJ. 2001. Chondrocyte differentiation of mesenchymal stem cells sequentially exposed to transforming growth factor-beta 1 and insulin-like growth factor-1 in a three dimensional matrix, J Orthopaed Res 19: 738-749

Yamada KM, Geiger B. 1997. Molecular interaction in cell adhesion complexes, Curr Opin Cell Biol 9: 76 –85

Yamada KM, Clark K. 2002. Survival in three-dimensions, Nature 419, 2002 790 - 791

Yamada Y, Boo JS, Ozawa R, Nagsaka T, Okazaki Y, Hata K, Ueda M. 2003. Bone regeneration following injection of mesenchymal stem cells and fibrin glue with a biodegradable scaffold, J Cranio-Maxillofacial Surg 31: 27-33

Yen BL, Huang HI, Chien CC, Jui HY, Ko BS, Yao M, Shun CT, Yen ML, Lee MC, Chen YC. 2005. Isolation of multipotent cells from human term placenta, Stem Cells 23: 3 – 9

Ying Q, Nichols J, Evans EP, Smith AG. 2002. Changing potency by spontaneous fusion, Nature 416: 545 – 547

Young G, Butler DL, Weber W, Caplan AI, Gordon SL, Fink DJ. 1998. Use of mesenchymal stem cells in a collagenous matrix for Achilles tendon repair, J Orthopaed Res 16: 406 – 413

Yu AY, Frid MG, Shimoda LA, Weiner CM, Stenmark K, Semenza GL. 1998. Temporal, Spatial, and Oxygen-regulated expression of hypoxia-inducible factor-1 in the lung, Am J Phys 275: L818 – L826

Yu F, White SB, Zhao Q, Lec FS. 2001. HIF-1α Binding to VHL is Regulated by Stimulus Sensitive Proline Hydroxylation**,** Proc Natl Acad Sci 98: 9630 - 9635

Zahir N, Weaver VM. 2004. Death in the third dimension: apoptosis regulation and tissue-architecture, Curr Opin Gen Dev 14: 71 - 80

Zamir E, Geiger B. 2001a. Components of cell-matrix adhesions, J Cell Sci 114: 3577 – 3579

Zamir E, Geiger B. 2001b. Molecular complexity and dynamics of cell-matrix adhesions, J Cell Sci 114: 3583 - 3590

Zamir E, Katz B, Aota S, Yamada KM, Geiger B, Kam Z. 1999. Molecular diversity of cell-matrix adhesions, J Cell Sci 112: 1655 – 1669

Zandstra PW, Eaves CJ, Piret JM. 1994. Expansion of hematopoietic progenitor cell populations un stirred suspension bioreactors of normal human bone marrow cells, Biotechnol Bioeng 12: 909 - 914

Zhang S. 2004. Beyond the Petri dish, Nat Biotechnol 22: 151 - 152

Zhang S. 2003. Fabrication of novel biomaterials through molecular self-assembly, Nature Biotechnol 21: 1171 - 1178

Zhao F, Ma T. 2005. Perfusion bioreactor system for human mesenchymal stem cell tissue engineering: dynamic cell seeding and construct development, Biotechnol Bioeng *In Press*

Zhao F, Pathi P, Grayson WL, Xi Q, Locke BL, Ma T. 2005. Effect of nutrient transport on human mesenchymal stem cell tissue development in perfusion and static cultures, Biotechnol Prog *Submitted*

Zheng MH, Chen J, Kirilak Y, Willers C, Xu J, Wood D. 2005. Porcine small intestine submucosa (SIS) is not an acellular collagenous matrix and contains porcine DNA: possible implications in human implantation, J Biomed Mat Res B 73B: 61 – 67

Zhou S, Schuetz JD, Bunting KD, Colapiettro AM, Sampath J, Morris JJ, Lagutina I, Grosveld GC, Osawa M, Nakauchi H, Sorrentino BP. 2001. The ABC transporter Bcrp1/ABCG2 is expressed in a wide variety of stem cells and is a molecular determinant of the side-population phenotype Nat Med 7: 1028 - 1034

Zipori D. 2004. Mesenchymal stem cells; harnessing cell plasticity to tissue and organ repair, Blood Cells, Mol Dis 33: 211 – 215

BIOGRAPHICAL SKETCH

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