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Bioreactors to Influence Stem Cell Fate: Augmentation of Mesenchymal Stem Cell Signaling Pathways via Dynamic Culture Systems

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Abstract

Background—Mesenchymal stem cells (MSCs) are a promising cell source for bone and cartilage tissue engineering as they can be easily isolated from the body and differentiated into osteoblasts and chondrocytes. A cell based tissue engineering strategy using MSCs often involves the culture of these cells on three-dimensional scaffolds; however the size of these scaffolds and the cell population they can support can be restricted in traditional static culture. Thus dynamic culture in bioreactor systems provides a promising means to culture and differentiate MSCs *in vitro*.

Scope of Review—This review seeks to characterize key MSC differentiation signaling pathways and provides evidence as to how dynamic culture is augmenting these pathways. Following an overview of dynamic culture systems, discussion will be provided on how these systems can effectively modify and maintain important culture parameters including oxygen content and shear stress. Literature is reviewed for both a highlight of key signaling pathways and evidence for regulation of these signaling pathways via dynamic culture systems.

Major Conclusions—The ability to understand how these culture systems are affecting MSC signaling pathways could lead to a shear or oxygen regime to direct stem cell differentiation. In this way the efficacy of *in vitro* culture and differentiation of MSCs on three-dimensional scaffolds could be greatly increased.

General Significance—Bioreactor systems have the ability to control many key differentiation stimuli including mechanical stress and oxygen content. The further integration of cell signaling investigations within dynamic culture systems will lead to a quicker realization of the promise of tissue engineering and regenerative medicine.

Keywords

bioreactor; mesenchymal stem cell; cell signaling; shear; oxygen tension

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1.1 Introduction

Mesenchymal stem cells (MSCs) are a multipotent stem cell population present in bone marrow as well as other tissue including adipose and can be readily differentiated *in vitro* into osteoblasts, chondrocytes, and adipocytes as well as tenocytes and myoblasts [1–3]. Therefore, these cells are a promising therapeutic cell source for regenerative medicine therapies to replace and repair these tissues. Therapies involving MSCs include direct transplantation of an MSC population, growth factor loaded scaffolds for MSC recruitment, and implantation of scaffolds containing an *in vitro* cultured MSC population [4–7]. Successful *in vitro* culture of MSCs requires an understanding of the signaling pathways that cue both the proliferation and guided differentiation of these cells. During differentiation chemical, biological, and mechanical cues induce these cells to follow a specific pathway dictating if a cell remains multipotent or differentiates into a specific cell type. These cues signal the release and uptake of cytokines, hormones, and growth factors which induce dynamic signaling pathways and mediate cell fate. Key signaling cascades for MSC differentiation include mitogen activated protein kinase (MAPK), Wnt, and SMAD. These pathways are mediated by growth factors including bone morphogenic protein 2 (BMP-2), transforming growth factor β 2 (TGF- β 2), and fibroblast growth factor (FGF) (Please see Table 1 for a complete list of abbreviations). Release of these growth factors is modulated by the environment of the cell including surrounding cell types, physical culture parameters, factors present in the media, and mechanical stimuli [8–14]. Thus the cell environment must be regulated during *in vitro* stem cell culture. Bioreactor systems represent an important tool to regulate this environment. Bioreactors provide controlled mechanical stimuli to the cell as well as regulating the cell culture medium. In that way, they provide a level of control of cell culture parameters perhaps not possible in static culture.

Bioreactors, extensively used in the culture of MSCs, include simple systems such as spinner flask and rotating wall bioreactors and more complicated systems including perfusion and dynamic loading bioreactors [15–28]. While spinner flasks and rotating wall bioreactors fail to provide full control of culture parameters, perfusion and dynamic loading systems have been demonstrated to be very effective in MSC culture. These systems have been shown to enhance both MSC chondrogenesis and osteogenesis as well as increase proliferation of these cells. By perfusing media through a porous scaffold, bioreactors can provide homogenous nutrient and oxygen concentrations to cells. As nutrient deprivation and hypoxia often occur in static culture, the ability of bioreactor systems to deliver homogenous nutrient and oxygen concentrations to cells makes these systems a key part of an *in vitro* culture strategy. This review will focus on another advantage of these systems: the potential to mediate cell signaling pathways to direct MSC proliferation and differentiation. *In vitro* these signaling pathways can be potentially triggered by environmental cues including mechanical stress and oxygen content which can be controlled using bioreactor systems. Thus this review will attempt to answer the following questions: What aspects of dynamic culture affect MSC differentiation pathways? How can bioreactors be used to augment these pathways?

2.1 Bioreactor Systems for MSC Culture

Many different bioreactors systems exist for the culture of mesenchymal stem cells including spinner flask [29–35], rotating wall [30, 31, 36–38], and perfusion [18, 19, 24, 26–28, 39–43] bioreactor systems (Figure 1). Recent reviews have described the role of shear stress for bone tissue engineering [22, 25] as well as detailing these systems [44–46]. All of these systems feature culture of MSCs in a three-dimensional environment.

Spinner flask culture consists of MSC containing scaffolds either suspended or free floating in a flask of culture media (Figure 1A). The media is then circulated throughout the flask using a stir bar. Rotating wall bioreactors feature scaffolds placed between two concentric cylinders in culture media (Figure 1B). While the inner cylinder remains stationary, the outer cylinder rotates moving the media in a circulatory manner. These systems have been shown to increase MSC proliferation and osteoblastic differentiation [32, 35]; however, these systems lack the ability to regulate oxygen and shear stress throughout a scaffold. This is because these systems focus primarily on media mixing while exhibiting a small amount of shear stress to the outer regions of scaffolds. Media mixing ensures a homogenous oxygen gradient in the bulk media, but non-homogenous concentrations can result throughout the scaffold. Perfusion bioreactor systems have the ability to yield a more tight control over scaffold exposure to oxygen and shear stress. The basic perfusion bioreactor design features media pumped from a media reservoir through a tubing circuit, via a pump (Figure 1C). Within the tubing circuit there is a growth chamber containing the scaffolds. In many perfusion bioreactor designs, a porous scaffold is used and is press fit into the growth chamber [15, 28, 39]. Media is then directly perfused through pores in the scaffold. An alternative type of perfusion bioreactor uses a modular design in which scaffolds are packed into a growth chamber [26, 27, 47–51]. In these designs, a collection of smaller scaffolds is cultured in a growth chamber and then can be implanted as one larger construct.

Perfusion bioreactor systems have been very effective for the culture of MSCs, being demonstrated to increase proliferation [26, 52–54], osteogenesis [15, 24, 28], and chondrogenesis [55]. These observed results are attributed to the ability of the systems to increase nutrient transport including oxygen and expose the cells to mechanical stimulus. When the effect of these two stimuli were independently evaluated, shear stress and mass transport were each shown to have an effect on human mesenchymal stem cell (hMSC) growth and osteoblastic differentiation [41]. In this study, shear and mass transport could be decoupled by changing media viscosity. In this way, it was shown that increasing shear from 0.05 to 0.15 dynes/cm² caused hMSCs to express higher levels of late osteoblastic markers osteopontin (OPN) and osteocalcin (OC) at 28 days. Increased flow rates (while keeping shear constant) also led to higher marker levels, but became inhibitory when the highest flow rate tested of 9 mL/min was reached. This demonstrates that MSCs cultured in perfusion systems respond to flow rates in two ways, through both changes in nutrient transport and shear stresses. Because of the multitude of factors influencing differentiation in bioreactor systems, the exact parameters influencing MSC differentiation may be difficult to discern. However, such studies decoupling these parameters can lead to a greater understanding of MSC culture in bioreactor systems.

3.1 Bioreactors to Mediate Shear Stress

Another powerful mechanism by which bioreactor culture can augment stem cell signaling and fate is through exposure to mechanical stresses [5]. Shear has a dramatic effect on MSC differentiation and bioreactors have the capability to regulate shear in three-dimensional constructs [22, 25, 43]. A notable demonstration of this is the ability of bioreactor systems to influence osteoblastic differentiation of MSCs without typical osteogenic induction media including the glucocorticoid steroid dexamethasone [19]. In one study, MSCs cultured in a perfusion bioreactor without osteogenic supplements exhibited elevated levels of osteogenic markers compared to static culture. Based on alkaline phosphatase (ALP) and osteopontin levels in these studies, dynamic culture can be a strong inducer of MSC osteoblastic differentiation [19]. As a possible mechanism, human MSCs exposed to 12 dynes/cm² of shear stress show an upregulation of ALP expression dependent on p38 and extracellular signal-related kinase (ERK) activation [56]. These two signaling mechanisms of the mitogen activated protein kinase (MAPK) pathway described in detail later in the review may

provide a mechanism for shear stress regulation of MSC osteoblastic differentiation. In addition to the presence and magnitude of shear, the particular shear regime may also influence stem cell fate [57, 58]. When rat bone marrow stromal cells (BMSCs), containing a heterogeneous population of MSCs, were differentiated into immature osteoblasts and exposed to a continuous shear stress of 2.3 dynes/cm² the cells underwent a rapid phosphorylation of ERK and p38. When this shear was delivered intermittently, the phosphorylation was delayed. Synthesis of prostaglandin E₂ (PGE₂) however was increased with intermittent flow, hypothesized to be a result of signaling molecules being permitted to accumulate during breaks in the flow regime. Following just 24 hours of stimulation, cells expressed higher levels of osteoblastic differentiation markers 13 days later; however the flow regime did not affect these markers. Thus shear may have a powerful effect on MSC differentiation after just a short regime, and the nature of the shear regime could have an outcome on the differentiation pathways, especially if this regime is enacted over a long period of time. Shear stress also upregulated cyclooxygenase-2 (COX-2) expression [58]. COX-2 regulates PGE₂ production. This in turn can then regulate the BMP signaling pathway through binding to cell surface receptor prostaglandin E receptor 4 (EP4) [59]. Thus osteoblastic differentiation can be upregulated by fluid shear stress through regulation of the BMP signaling pathway.

MSC chondrogenic differentiation is also highly influenced by exposure to shear stresses [42, 60–62]. Similar to osteoblastic differentiation, chondrogenic differentiation is also regulated by MAPK activation [63, 64]. While both dynamic loading and shear upregulate chondrogenic markers, blocking ERK1/2 and p38 activation diminished the effects on most of these markers. Shear induced chondrogenic differentiation is also regulated through COX-2 to regulate PGE₂ production via c-Jun N-terminal protein kinase (JNK2) and c-jun [65]. JNK as well as ERK MAPK signaling cascades have been shown to influence Runx2 activation in chondrocytes exposed to dynamic loading [66]. Dynamic culture systems present the opportunity to leverage these effects on chondrogenic differentiation pathways to optimize production of MSC derived chondrocytes. Under fluid flow of 1 dyne/cm², chondrocyte production of type II collagen greatly increased and the tensile strength of the cartilage constructs was improved [67]. Mechanical loading of MSCs during chondrogenesis increased amount of collagen markers [68]. It is important to point out that optimal leverage of these pathways in bioreactor culture will not be a trivial matter as many different factors influence these pathways. Oxygen content in combination with shear [69], variation of shear throughout constructs, influence of supplements and growth factors in the media [68], and cell-substrate interactions [70] all will modify cell response. Thus tissue engineers must carefully consider differentiation pathways along with shear and oxygen regimes to tightly control stem cell fate. Bioreactors must then be designed to accommodate osteoinductive and osteoconductive scaffolds, to utilize growth factors and supplements, and to deliver shear and oxygen in a controlled manner for the direction of MSC differentiation.

3.2 Shear Stress Mediated Signaling

In order to fully leverage the role of shear stress in dynamic culture systems, the signaling mechanisms that depend on these stresses must be understood. While the role of mechanical forces in MSC tissue engineering and regenerative medicine has been well observed, the signaling pathways involved in converting mechanical stress to biochemical signals in dynamic cultures systems is studied far less frequently.

3.2.1 Shear Stress Mediated MAPK Signaling

A primary signaling pathway influenced by shear stress is mitogen activated protein kinases (MAPKs), which are highly involved in the differentiation of MSCs and include the ERK 1/2, JNK, and p38 pathways. The mechanisms of MAPK signaling have been extensively

reviewed elsewhere and are summarized in Figure 2 [71–77]. Briefly, the general mechanism consists of three kinase molecules that bridge the cellular signal from the cell surface deep into the cytoplasm and nucleus. As an example, signaling from the cell surface, usually in the form of growth factors including the TGF- β /BMP family, bind to a receptor tyrosine kinase (RTK). The binding causes a conformational change that allows the receptor to self-phosphorylate. Adaptor proteins bind to these phosphorylated tyrosines and in turn activate a GTPase such as Ras [78]. This GTPase phosphorylates the first member of the MAPK pathway, a MAPK Kinase Kinase (MAPKKK). This MAPKKK in turn phosphorylates a MAPKK which then phosphorylates the MAP kinase ERK 1/2, p38, or JNK. This MAPK then phosphorylates the downstream targets.

The ERK, JNK, and p38 pathways are all activated in osteogenic differentiation of MSCs [79, 80] and can be impacted via shear stress. Signaling via these three pathways is also activated during TGF- β induced chondrogenesis, and inhibition of any of the pathways result in partial or complete inhibition of chondrogenesis [81–83]. Another aspect crucial to augmenting MAPK signaling pathways is the specific time of activation along the MSC differentiation progression. While ERK 1/2 remains relatively active during a substantial portion of the differentiation process, the JNK and p38 pathways are more specific [84]. The JNK pathway is activated much later in MSC differentiation and is associated with matrix deposition [84]. A recent study demonstrated that the JNK pathway inhibits early and late osteogenic differentiation [85]. In osteoblasts, the BMP/TGF- β activation of the p38 and ERK 1/2 pathways occurs within the first few hours, whereas the JNK pathway was activated later [86]. The p38 pathway was shown to upregulate osteogenic markers such as ALP and Osterix (Osx) but down-regulate OC, a late osteoblastic marker, indicating it is inhibitory to late osteoblast maturation [78, 87, 88]. Future studies should focus on better understanding the different activation timings of different signal pathways involved in MSC differentiation. In this way, shear could potentially be delivered in a temporal manner to best stimulate this signaling pathway for osteoblastic differentiation.

3.2.2 Mechanotransduction in Mesenchymal Stem Cells

In vivo bone and cartilage are constantly exposed to mechanical forces and the transmission of these signals is known as mechanotransduction [89–92]. Channel proteins including calcium ion channels, G-Proteins imbedded in the cell membrane, and integrins all have a significant role in mechanotransduction [93]. Mechanistically, mechanical shear created by fluid flow has been shown to promote osteogenesis via the ERK 1/2 pathway through upregulation of Runx2. Fluid flow upregulated expression of β 1 integrins via a signaling pathway involving activating nuclear factor kappa B (NF- κ B) indicating a possible pathway for fluid-induced mechanotransduction [94]. In another study, fluid shear was shown to upregulate MAP3k8, a MAPKKK as well as growth factor interleukin 1-beta. This indicates that not only are mechanical stresses involved in pathway activation, but also in the upregulation of the proteins these pathways depend on [95]. Oscillatory fluid flow was also shown to cause an influx of calcium that corresponded to enhanced differentiation and proliferation of MSCs as well as promote osteogenic genes in osteoblasts themselves. This indicates that calcium signaling, which could be mediated by a mechanosensitive protein channel, also plays a role in MSC differentiation and function [96–98]. A role for focal adhesions in MSC differentiation has also been demonstrated. When focal adhesion kinase function was inhibited, both Runx2 and osterix function was significantly impaired and the hMSCs were prevented from undergoing osteogenesis [99]. Focal adhesions are important “signaling hubs” for mechanotransduction, and this role in osteogenesis suggests that they may serve a similar function in MSCs. As MSCs are more and more frequently cultured in dynamic culture systems the specific mechanisms of *in vitro* mechanotransduction must be understood to more effectively culture and differentiate these cells.

3.2.3 Wnt Signaling as a Regulator of Stem Cell Fate

Wnt signaling has been identified as a key signaling pathway to determine MSC differentiation or proliferation (Figure 3) [100–102]. Wnt signaling is carried out through a diverse number of pathways and molecules; however there are two general classifications that Wnt signaling is divided into. If the pathway creates its effect through the accumulation of β -Catenin in the cell, and thus the nucleus, the signaling is referred to as canonical signaling. If the Wnt signal protein uses a different pathway it is categorized as non-canonical. Wnt signaling begins with the binding of a Wnt protein to cell-surface receptors which are a part of the Frizzled (FRZ) family of proteins. The major role canonical Wnt signaling in stem cells is to maintain cell potency. Differentiation of any kind is inhibited in a rate dependent manner with increased levels of canonical Wnt signaling, which promotes cellular proliferation as well as reduces cellular apoptosis [101–103]. MSCs in the process of differentiating are more resistant to the inhibitory effects of canonical signaling because signal pathways and transcription factors related to osteo/chondrogenic differentiation downregulate and inhibit canonical signaling. Sox9 inhibits activation of B-catenin promoters and promotes the degradation of the molecule [104]. Runx2 has a similar effect [105]. JNK, p38, and ERK1/2 signaling induced by TGF- β down regulates canonical Wnt signaling as seen by an increase in B-catenin after MAPK inhibition.

Unlike canonical Wnt signaling, non-canonical Wnt signaling does not use β -catenin as its method of affecting gene transcription and has been shown to directly promote both osteogenesis and chondrogenesis [102]. Mechanistically, this difference is likely due to the differences in signal cascades used. For example, several non-canonical Wnts have been shown to activate the JNK and p38 pathways as well as activate Rho; all of which are important in TFG- β /BMP signaling. Non-canonical Wnt signaling diverges from the canonical pathway at Dishevelled where in this case it activates the JNK pathway instead of events leading to the accumulation of β -catenin [73]. Non-canonical Wnt 4a promotes osteogenesis through the activation of the p38 pathway [106]. Non-canonical Wnt signaling has been shown to be upregulated by fluid shear in osteoblasts [107]; however, little work has done to study Wnt signaling in MSCs cultured in bioreactor systems. Investigation of this key pathway could lead to another avenue to direct MSCs to proliferate or differentiate in bioreactor systems.

3.2.4 Downstream Targets Influenced by Dynamic Culture

While stimuli in dynamic culture affect different steps along signaling pathways, downstream targets are typically analyzed by tissue engineers as a metric to determine study outcomes. Understanding of these downstream targets and how they relate to signaling pathways is key to understanding the mechanisms influencing dynamic culture mediated signaling pathways. The activity and upregulation of several key transcription factors including Runx2 and Osx for osteoblasts and Sox9 for chondrocytes are vital to MSC commitment down a specific lineage. Runt-related transcription factor 2 (Runx2), also known as cbfa1, is the “master control” for osteogenesis as it upregulates extracellular matrix (ECM) proteins as well as other downstream transcription factors such as Osx. In the nucleus, the SMAD complex associates with Runx2 to form a new transcription complex that is essential for both BMP and TGF-B induced osteogenesis (Figure 4) [108].

The major downstream targets of another pathway mediated by mechanical forces, the JNK pathway, are AP-1 proteins, namely C-jun [71, 73]. The SMAD and JNK signaling pathways converge at AP1-binding promoter sites where C-jun and C-fos associate with SMAD proteins to mediate TGF- β and BMP induced gene transcription [78, 109]. AP-1 proteins C-fos and C-jun have also been shown to be capable of directly interacting with Runx2 to regulate gene transcription as well [110]. Downstream of Runx2 is Osx a

transcription factor that becomes active later in the osteogenic differentiation pathway [111]. It was demonstrated that inhibition of the ERK 1/2 pathway did not inhibit Runx2 expression but did inhibit Osx expression, however this could simply be that this MAPK pathway phosphorylates Runx2 promoting it and thus its downstream targets, including Osx [87]. Inhibition of the p38 MAPK pathway was shown not to inhibit mineralization but it did significantly downregulate Osx [87]. Osx was the only transcription factor related to osteogenic differentiation that was downregulated in response to p38 inhibition [112]. Osterix is a substrate of p38, and that phosphorylation of the transcription factor improves its ability to recruit co-activators [113].

In cartilage development SRY-type high mobility group box 9, or Sox9, is the major transcription factor for the chondrogenic lineage, upregulating lineage specific genes such as aggrecan and collagen II [98, 114, 115]. SMAD complexes facilitate Sox9-dependent transcription in a similar manner to Runx2, by associating with the transcription factor and a cofactor, in this case CBP/p300, in order to regulate gene expression [116]. The p38 pathway has been shown to promote Sox9-dependent transcription by facilitating the formation of the SMAD/Sox9/p300 complex via one of its downstream substrates, MSK1. P38 Kinase phosphorylates MSK1, which in turn phosphorylates the coactivator p300 which plays a role in the remodeling of chromatin [117]. This indicates that the p38 pathway facilitates complex formation by affecting the exposure of DNA binding sites in addition to direct interaction with the complex itself. While examination of these downstream targets such as Sox9, Runx2, Osx as well as markers and growth factors including BMP-2, TGF- β , ALP, OPN and OCN, are typically assayed, upstream signaling mechanisms must also be understood to determine how dynamic culture is affecting MSC differentiation.

4.1 Bioreactors to Mediate Oxygen Content

Though shear is often the focus of dynamic culture studies, oxygen is also a powerful mediator of mesenchymal stem cell fate [118–124]. In their undifferentiated state in the bone marrow, mesenchymal stem cells are maintained in an environment of approximately 7% oxygen tension [125]. Chondrocytes reside in an avascular environments with oxygen tensions from 1–3% [126]. The oxygen tension of the native environment of bone is higher at approximately 12%, but can drop significantly during fracture [127, 128]. However, mesenchymal stem cells are cultured in an *in vitro* environment of 20% oxygen tension with any lower oxygen tension typically referred to as hypoxia. When MSCs are cultured in three-dimensional scaffolds, the oxygen tension becomes much more complex. An oxygen gradient can form as cells on the outer layers of the scaffold consume oxygen, leaving cells in the inner regions of the scaffold with an anoxic or hypoxic environment. In three-dimensional culture, oxygen content can begin to significantly drop after just hundreds of microns. In scaffolds with a minimum diameter of just 5 mm, central oxygen concentrations dropped to 0% after just 5 days of culture [52]. This drop in oxygen content led to massive cell death of the pre-osteoblast cell line being cultured. Bioreactor culture was then used to mitigate these transport insufficiencies and cell viability improved. This highlights a critical need of bioreactor systems to regulate oxygen content. However, the role of oxygen is greater than a required nutrient; it can act as signaling molecule to affect stem cell fate.

Despite strong evidence that oxygen tension can regulate stem cell proliferation and differentiation, regulation of oxygen content throughout a three-dimensional scaffold is difficult. Bioreactor culture can be used to create a regulated oxygen environment for the maintenance or differentiation of mesenchymal stem cells. In a modular bioreactor system equipped with a probe to measure oxygen, a 20% oxygen level was used for the adipogenic differentiation of hMSCs, while a 5% oxygen content was used for chondrogenic differentiation [49]. These different oxygen regimes proved to increase the expression of

adipogenic and chondrogenic markers respectively. Bioreactors such as this could be used as a powerful tool to control stem cell fate. Even within bioreactor cultured constructs, oxygen content can vary throughout scaffolds, thus careful monitoring throughout the scaffold may be necessary. As an alternative to continuous oxygen monitoring, mathematical models can be developed to predict oxygen content throughout scaffolds [26, 43, 129]. Mathematical models for oxygen distribution throughout scaffolds could then be combined with oxygen monitoring to offer tight control in bioreactor systems. One bioreactor using an oxygen monitoring system has been used to culture hMSCs to produce a large construct with bone forming potential [40]. This bioreactor system only actively monitored oxygen rather than also actively controlling oxygen, but technology exists to create a bioreactor that would actively monitor and control oxygen content [40, 129–131].

4.2 Signaling Mediated by Oxygen Concentration

Examples of the influence of oxygen content on mesenchymal stem cell fate are quite prevalent. When rat MSCs were cultured in 5% oxygen compared to the standard 20% oxygen, the cells proliferated more rapidly. In addition, the 5% oxygen cultures exhibited an increase in common osteoblastic markers including alkaline phosphatase (ALP), and calcium content. When implanted *in vivo*, these cells cultured in low oxygen content led to a greater amount of *in vivo* bone formation [132]. In a study culturing human mesenchymal stem cells under 2% oxygen, proliferation was greatly increased [133]. In addition to proliferation increases, an increase of Oct-4, a stemness gene, mRNA expression was noted with hypoxia. This increase was concurrent with an increase in hypoxia inducible factor 2 α (HIF-2 α) indicating a possible mechanism by which oxygen regulates stem cell fate. Though these results were obtained in two-dimensional culture, similar results were acquired when the hMSCs were exposed to 2% oxygen in three-dimensional culture [134]. In a recent study, culture in 1% oxygen was shown to increase expression of stemness genes Oct4, Nanog, Sall4 and Klf4 of hMSCs compared to 20% [135]. Interestingly, the osteogenic potential was increased when cultured under 1% oxygen conditions in induction media, but chondrogenic and adipogenic capacity were reduced. In another study utilizing hMSCs, 3% oxygen increased the proliferative lifespan of hMSCs, but was shown to reduce their differentiation potential [136].

In addition to affecting osteoblastic differentiation, oxygen concentration mediates MSC chondrogenic differentiation. Cells expanded in 2% oxygen compared to 20% have been shown to have greater chondrogenic potential than osteoblastic [137]. Similar results are ascertained when comparing 3% oxygen tension to 20% tension cultured MSCs [138]. As a possible mechanism for increased chondrogenesis of MSCs, HIF-1 α was evaluated [139]. After rat MSCs were cultured under 2% oxygen and induced to undergo chondrogenesis, differentiation was enhanced in the 2% cultured cells. However, when HIF-1 α was knocked down using siRNA, no upregulation of chondrogenesis was observed. The shift to chondrogenesis over osteogenesis was thought to occur via the p38 mitogen activated protein kinase [140]. In bioreactor culture, mechanical forces via dynamic shear or mechanical loading also play an important role [69]. When the effect of oxygen content is compared directly to the effect of dynamic compression, oxygen content played a greater role as MSCs cultured at 5% oxygen in agarose gels showed a greater tendency toward chondrogenesis than cells cultured under dynamic compression in 20% oxygen tension [69]. These findings are important in dynamic culture where both mechanical forces and oxygen regulation could be regulated concurrently.

Even beyond the ability for oxygen content to control stem cell proliferation, oxygen content may have a role in cell organization within a construct and cell subpopulations. *In vivo* chondrocytes are organized into three zonal subpopulations the superficial, middle, and deep

[141]. Chondrocyte subpopulations in these zones have distinct phenotypes and can respond differently to substrate properties and growth factors [100, 142]. Zonal phenotype can also be retained by oxygen content [143]. Though this study was completed on adult chondrocytes, it is possible oxygen may play a role in MSC chondrogenic differentiation into zonal subpopulations and proper oxygen maintenance could yield a greater deal of phenotype control leading to a more functional cartilage construct.

It is important to point out that aside from mediating signaling mechanisms, bioreactor maintenance of oxygen in 3D scaffolds is necessary to sustain stem cell phenotype. In a recent study using human mesenchymal stem cells, exposure to oxygen tensions less than 1% for just 48 hours led to a long term downregulation of osteoblastic markers including Runx2, osteocalcin, and type 1 collagen [127]. Thus even temporary lack of oxygen can cause permanent loss of bone forming potential. If this lack of oxygen is combined with a lack of nutrients, massive cell death will occur [144]. Thus proper maintenance of oxygen concentrations is crucial to maintain stem cell viability and phenotype. Further maintenance above minimum levels may lead to an increased control of stem cell fate.

4.2.1 Oxygen Regulation of Stem Cells via HIF

Hypoxia-inducible factor (HIF) is the major transcription factor for a cellular response to hypoxia. Like other cell types, hypoxia causes the activation of HIF in MSCs [139]. HIF is made of two subunits: β and α . While HIF- β exists in stable amounts in both the nucleus and cytosol, HIF- α , which resides in the cytosol normally, is degraded under normoxia. HIF- α is allowed to stabilize in hypoxic conditions and translocates into the nucleus where it can bind to HIF- β . Once dimerized, HIF recruits coactivator proteins and facilitates the transcription of a wide variety of genes (Figure 2) [145].

A recent study has yielded a possible mechanism for the reduction in osteoblastic differentiation potential observed in cells exposed to hypoxia. When hMSCs were cultured under 1% hypoxia under osteogenic induction conditions, osteoblastic differentiation of human MSCs was downregulated compared to cells cultured at 20% oxygen [146]. Hypoxia was shown to decrease expression of Runx2, which then led to downstream reduction of osteoblastic genes including osteocalcin, osteopontin, collagen Type I alpha 1, bone sialoprotein (BSP), and alkaline phosphatase. This downregulation was shown to be mediated by HIF-1 α via TWIST, a downstream target. TWIST was discovered to bind to the Runx2 P2 promoter, suppressing transcription of Runx2. This in turn inhibits expression of Runx2 downstream targets. The same group further investigated HIF-TWIST and found that though osteoblastic differentiation was downregulated during hypoxia, MSC proliferation and phenotype maintenance were increased [147]. When cultured under 1% oxygen tension, hMSCs exhibited a more efficient expansion profile. The differentiation potential of these cells was maintained even at late passages, while cells cultured in 20% oxygen exhibited a decreased differentiation potential. In addition, hMSCs grown under hypoxia had both an increased differentiation potential and bone repair capacity when implanted into an *in vivo* mouse model. This increased potential to differentiate was shown to be regulated through p21 by transcription factor E2A. P21 activation leads to cellular senescence and was shown to be downregulated by HIF-1 α via TWIST in response to a hypoxic environment. These two studies provide evidence for a mechanism by which oxygen content regulates stem cell fate. While a low oxygen environment inhibits osteoblastic differentiation, it increases proliferation and stem cell phenotype maintenance. In addition to osteoblastic differentiation, cells under hypoxia were shown to better differentiate into adipocytes as well after culture in 5% oxygen compared to 20% oxygen [148]. This was due to a variety of genetic changes that maintained an undifferentiated state.

Though HIF can inhibit osteogenesis, it can have a stimulatory effect on chondrogenesis. Sox9 is a target of HIF regulation and chondrogenesis was shown to be significantly enhanced when MSCs were exposed to hypoxic condition [139, 149]. Hypoxia (2% oxygen) triggered the activation of the p38 pathway and inhibition of it inhibited HIF- α stabilization [139]. Activation of the JNK pathway has also been shown to be required for HIF-dependent signaling and transcription, indicating a role for MAPKs in the response of MSCs to oxygen tension [150]. When HIF- α function was silenced by siRNA, the increased upregulation of chondrogenic genes such as proteoglycan and collagen II due to hypoxia was absent [139].

HIF offers a mechanism by which oxygen can mediate stem cell fate. In bioreactors with the ability to regulate oxygen content, regimes could be developed to foster proliferation followed by osteogenic or chondrogenic differentiation. In this manner, bioreactor systems could be made even more effective for the culture of three-dimensional MSC containing constructs.

5.1 Conclusion and Future Directions

Dynamic culture of MSCs has expanded greatly in the last ten years and bioreactor culture is now widely used. In addition, bioreactor design has advanced considerably including bioreactors to measure oxygen content [40], culture anatomically shaped grafts [28], and develop prevascular networks [49, 151]. Bioreactor culture creates a more efficient means to culture MSCs and provides for the differentiation to chondrocytes and osteoblasts in an environment more similar to native tissue. The signaling cascades covered in this review provide a mechanism for this differentiation. However, despite the volume of work on stem cell signaling mechanisms, there still is research to be done on how these pathways are affected in dynamic culture systems. This knowledge could be improved by a greater emphasis on experiments designed to characterize these pathways in clinically relevant situations. Much of the work regarding signaling pathways has been completed with cell lines and two-dimensional culture, while emphasis in the tissue engineering community is placed on primary cells grown in clinically relevant situations including bioreactors and biocompatible three-dimensional scaffolds. However, many tissue engineering experiments are designed to be outcome oriented and do not evaluate mechanisms and pathways in great detail. Since cell-substrate interactions and dynamic culture can have a profound effect on signaling mechanisms, evaluating these mechanisms in situations relevant to tissue engineering is paramount to understanding ways to leverage these mechanisms to create *in vitro* engineered tissue. Thus greater communication and collaboration between labs with expertise evaluating MSC signaling and those developing new bioreactor culture systems should be undertaken to design the proper experiments to evaluate how bioreactor culture is augmenting these signaling pathways. In this manner, a relationship between oxygen content, shear, and stem cell differentiation could be developed. Following this development, advanced bioreactor systems with the ability to temporally regulate oxygen content and mechanical stimuli could be used to augment these signaling pathways and optimize creation of *in vitro* engineered tissue.

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Highlights

- Perfusion bioreactors can regulate MSC exposure to shear stress and oxygen tension
- Shear stress and oxygen tension can enhance MSC differentiation pathways
- Bioreactors can be used to regulate oxygen and shear to direct MSC differentiation

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Fig. 1a

Fig. 1b

Fig. 1c

Figure 1. Schematic of three commonly used tissue engineering bioreactor systems. The spinner flask (a) and the rotating wall bioreactor (b) focus on mixing media around scaffolds while provided some mechanical stimulation. The perfusion bioreactor (c) provides more direct stimulation to cells by perfusing media directly through a cell containing scaffold.

Figure 2.

Demonstration of signaling influenced by culture conditions in a perfusion system. Shear stress and controlled oxygen tension provide stimulus to cells growing on three-dimensional scaffolds. This in turn influences HIF (left) and MAPK (right) signaling pathways.

Figure 3.

Overview of canonical Wnt signaling pathway. (1) Under normal conditions, β -Catenin is phosphorylated by a protein complex consisting of GSK3 β and Axin among other proteins. This phosphorylation triggers the degradation of the β -Catenin molecules. (2) Wnt proteins bind to a receptor of the frizzled family which in turn causes the recruitment of Dishevelled and other proteins such as Axin forming a receptor complex. LRP is a protein hypothesized to stabilize the complex. This recruitment causes the disassembly and inactivation of the degrading protein complex which in turn allows the buildup of β -catenin and accumulation in the nucleus where the molecule impacts gene transcription.

Figure 4.

A simplified diagram of signal pathway convergence at downstream nuclear targets. Targets including Runx2, Osx, and Sox9 are vital to MSC differentiation and are modulated by the SMAD pathway as well as upstream MAPKs including JNK, ERK1/2 and P38.

Table 1**Abbreviations**

ALP	alkaline phosphatase
BMP	bone morphogenic protein
BMSC	bone marrow stromal cell
BSP	Bone Sialoprotein
COX-2	Cyclooxygenase-2
ECM	Extracellular Matrix
ERK	extracellular signal-regulated kinase
EP4	Prostaglandin E receptor 4
FGF	Fibroblast Growth Factor
FRZ	Frizzled
HIF	Hypoxia Inducible Factor
hMSC	Human Mesenchymal Stem Cells
JNK	c-Jun N-terminal kinases
MAPK	mitogen activated protein kinase
MSC	mesenchymal stem cell
NF-KB	Nuclear factor kappa B
OC	Osteocalcin
OPN	Osteopontin
Osx	Osterix
PGE ₂	Prostaglandin E ₂
RTK	Receptor Tyrosine Kinase
Runx2	runt-related transcription factor-2
siRNA	Small interfering Ribonucleic Acid
TGF-β	Transforming Growth Factor Beta