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Stem Cell-based Tissue Engineering Approaches for Musculoskeletal Regeneration

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Abstract

The field of regenerative medicine and tissue engineering is an ever evolving field that holds promise in treating numerous musculoskeletal diseases and injuries. An important impetus in the development of the field was the discovery and implementation of stem cells. The utilization of mesenchymal stem cells, and later embryonic and induced pluripotent stem cells, opens new arenas for tissue engineering and presents the potential of developing stem cell-based therapies for disease treatment. Multipotent and pluripotent stem cells can produce various lineage tissues, and allow for derivation of a tissue that may be comprised of multiple cell types. As the field grows, the combination of biomaterial scaffolds and bioreactors provides methods to create an environment for stem cells that better represent their microenvironment for new tissue formation. As technologies for the fabrication of biomaterial scaffolds advance, the ability of scaffolds to modulate stem cell behavior advances as well. The composition of scaffolds could be of natural or synthetic materials and could be tailored to enhance cell self-renewal and/or direct cell fates. In addition to biomaterial scaffolds, studies of tissue development and cellular microenvironments have determined other factors, such as growth factors and oxygen tension, that are crucial to the regulation of stem cell activity. The overarching goal of stem cell-based tissue engineering research is to precisely control differentiation of stem cells in culture. In this article, we review current developments in tissue engineering, focusing on several stem cell sources, induction factors including growth factors, oxygen tension, biomaterials, and mechanical stimulation, and the internal and external regulatory mechanisms that govern proliferation and differentiation.

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CONFLICT OF INTEREST

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Keywords

Tissue engineering; musculoskeletal tissues; biomaterial scaffolds; stem cell regulation; oxygen; growth factors; extracellular matrix

1. INTRODUCTION

The fields of regenerative medicine and tissue engineering have grown dramatically since their early inception in the 1960s and 1970s. Early attempts simply sought to transplant somatic cells into a lesion area but typically led to little or no success. The development of biomaterial scaffolds further advanced tissue engineering by allowing for the creation of biomimetic environments that enhanced cell maintenance and differentiation [1]. While somatic cells, such as osteoblasts and chondrocytes, were among the first cell sources to be used in various tissue engineering applications, the prospects of tissue engineering were given new momentum with the addition of stem cells to the pool of cell choices. Adult tissue-derived stem cells, including mesenchymal stem cells (MSCs), became the backbone for cell therapies due to their expansion and multipotent potential [2], and demonstrated success in clinical applications. Further, stem cells may be more advantageous than somatic cells due to their tendency to favor anabolism instead of catabolism, whereas somatic cells are more so poised to maintain tissue homeostasis. Additionally, the isolation of somatic cells can induce donor site morbidity [3, 4], and somatic cells limit the potential of allogeneic cell therapy due to their immunogenicity [5]. The isolation of embryonic stem cells (ESCs) by Evans and Kaufman in 1981 [6] from mouse embryos and by Thomson from human embryos in 1998 [7] further stimulated the field by providing a cell source with seemingly infinite expansibility. Tissue engineering approaches are now conceivably able to target and derive almost any cell in the body. Stem cell-based research has exploded in recent years, attracting a great deal of scientific and public attention. An overarching goal of stem cell-based research is to understand how tissues/organs are formed and diseases develop, and in so doing, develop more effective therapies to treat diseases that are otherwise difficult to cure by current medical procedures. The isolation of ESCs is considered one of the major milestones fueling this movement, as it has provided a reliable tool to study tissue/organ formation and pathology and thus paved the way for fields like regenerative medicine and tissue engineering to emerge.

Stem cell-based tissue engineering applications aim to recapitulate key features of development or tissue repair to either promote tissue formation *ex vivo* or improve tissue regeneration *in vivo* for the replacement of malfunctioning or defective tissues/organs [8–10]. Stem cells are attractive for these applications due to their unique ability to self-renew and differentiate into multiple tissue-specific cells. In addition, stem cells secrete various kinds of trophic factors that can regulate immune response or condition the cellular microenvironment for tissue regeneration, giving them distinct advantages over terminally differentiated cells [11–14].

However, to take full advantage of the unique properties of stem cells for tissue engineering applications, it is critical to understand the underlying mechanisms controlling their self-renewal and differentiation so that researchers can precisely control cell behavior depending on the given application. As a general paradigm, tissue morphogenesis during embryonic development begins with the directed migration of progenitor cells to the site of tissue formation, followed by proliferation and condensation into a cluster of undifferentiated cells, and finally differentiation into the appropriate cell types, a process that in itself can include multiple stages. This conserved sequence of events is orchestrated by intricately timed and localized cellular interactions with soluble factors, extracellular matrix (ECM) molecules,

and other cells [15, 16]. *In vitro*, these developmental processes can be mimicked to direct the proliferation and differentiation of stem cells into the desired adult cell type. The goal of expansion culture is to obtain a large quantity of stem cells while simultaneously preventing uncontrolled or premature differentiation and the onset of senescence. Once the desired number of cells is obtained, cells are then instructed to differentiate into target tissue cells. Differentiation is complicated by heterogeneity within stem cell populations and lack of sophisticated protocols, which ultimately leads to variable efficiency and incomplete differentiation [17, 18]. Therefore, much of the current tissue engineering efforts have been focused on identifying which chemical and physical cues can efficiently drive stem cells to differentiate into target cells.

The goal of this article is to review recent research findings of the tissue engineering approaches used to induce stem cell differentiation for musculoskeletal tissue generation. We aim to provide scientists with a comprehensive, up-to-date review of how stem cells are regulated by extrinsic factors and what tissue engineering approaches, both physical and chemical, have been applied to regulate stem cell activities for tissue formation. The review begins by reviewing the properties of three promising stem cell types for musculoskeletal tissue engineering applications, ESCs, iPSCs, and MSCs, and subsequently outlines their strengths and weaknesses within this scope. We then discuss the key intrinsic and extrinsic factors controlling the behavior of stem cells throughout both phases of the tissue engineering process: expansion and differentiation culture. The discussion will specifically focus on the role of growth factors (GFs), oxygen tension, biomaterial scaffolds, and mechanical stimulation as they pertain to directing stem cell self-renewal and differentiation into musculoskeletal cell types.

2. STEM CELL TYPES, BIOLOGY, AND PROPERTIES

2.1. Embryonic Stem Cells

The discovery and isolation of human ESCs seemingly opened a new door in the research of tissue development and many different disease states. These cells were first isolated from mouse embryos at the University of Cambridge in 1981 and nearly 20 years later isolated from human embryos at the University of Wisconsin [6, 7]. These cells, isolated from preimplantation embryos, gave scientists access to some of the earliest uncommitted cells to study tissue development *in vitro*, and in so doing, allowed researchers for the first time to investigate the differentiation of hundreds of cell types, as direct study of factors directing human development is impossible.

Thomson *et al.* defined ESCs based upon their: 1) derivation from preimplantation or periimplantation embryos and 2) ability to proliferate for prolonged periods while 3) maintaining the ability to differentiate into cell types of all three germ layers. Upon isolation from the embryo and culture *in vitro*, ESCs express a subset a surface markers, including stage-specific embryonic antigen-3 (SSEA3) and SSEA4, TRA-1-60, TRA-1-81, and alkaline phosphatase (ALP). Isolated cells must also exhibit a high nucleus to cytoplasm ratio, which is further indicative of their uncommitted state [7].

The uncommitted and pluripotent state of ESCs endows them with the remarkable abilities to divide limitlessly during *in vitro* culture and differentiate into cell types from all three germ layers: ectoderm, endoderm and mesoderm. From the time of their initial isolations, ESCs have demonstrated the ability to undergo months of passage and hundreds of population doublings without evidence of cellular senescence or karyotypic abnormalities [7, 19–21]. Resistance to senescence is due, in part, to high telomerase activity, which maintains the length and integrity of telomeres as ESCs continue to divide [7, 22, 23]. The pluripotency of ESCs is regulated by transcription factors such as Oct4, Nanog, and Sox2.

The expression of these transcription factors can be upregulated through various mechanisms to maintain pluripotency, which is discussed in later sections. At the epigenetic level, these factors act by recruiting chromatin remodeling factors to target genes and promoting the expression of genes regulating self-renewal and pluripotency while suppressing genes related to differentiation [24, 25]. While the resistance of ESCs to senescence is impressive, it can be challenging to maintain them in an undifferentiated state devoid of aberrant differentiation.

The unbridled potency of ESCs is best observed when ESCs are implanted into immunodeficient mice, as they form encapsulated tumors, called teratomas, which contain cell types from all three germ layers [7]. The complication of teratoma formation requires that the differentiation of ESCs be tightly controlled if implantation is the goal. Decades of research in developmental biology have determined molecules, namely transcription factors, that are important for promoting differentiation into nearly every cell type and have since become an instrumental tool used to guide ESC differentiation. Protocols have been developed to induce ESCs to differentiate into many lineages, though most do so with low efficiency. Given the vast potential of ESCs for future clinical utility, research that is ongoing to further understand, utilize, and ensure the safety of these cells is of utmost importance.

2.2. Induced-pluripotent Stem Cells

Somatic cell nuclear transfer (SCNT) and the cloning of Dolly first demonstrated that somatic cells could be reprogrammed to an undifferentiated state [26]. The subsequent discovery and isolation of ESCs prompted researchers to question the idea of patient-specific cell therapy using SCNT. Although no human stem cell lines have been created using SCNT, the process suggested that trans-acting agents could reprogram cells and further pushed researchers to find the factors necessary to induce pluripotency in somatic cells. The first induced pluripotent stem cells (iPSCs) were generated from mouse cells using the 4 transcription factors *Oct4*, *Sox2*, *c-myc*, and *Klf4* [27]. This exact combination did not work in human cells mainly because the expression of c-Myc results in differentiation and cell death and can also transform MSCs into tumorigenic cells [28–30]. Using these successes as a guide, scientists sought to expand the procedure and develop human iPSCs.

This process in theory would allow the development of patient specific therapy, as cells from a patient could be collected, reprogrammed, expanded, and used for specific purposes. This could potentially eliminate the need for allogenic stem cells, as an unlimited source of patient cells would be available, and would also no longer require determination of immunocompatibility in cell therapy. In 2006, independent researchers at the University of Wisconsin-Madison and Kyoto University in Japan were able to reprogram human somatic cells using *Oct4*, *Sox2*, *Nanog*, and *LIN28*. These factors were transfected into the fibroblasts and resulted in cells that fit the defining criteria for ESCs (except their derivation from embryos) [31]. In these studies, iPSCs exhibited similar morphology to ESCs and expressed the characteristic ESC cell surface markers, while the parental fibroblast line did not. Whereas the parental fibroblast lines proliferated for approximately 20 passages before undergoing senescence, iPSCs proliferated similarly to ESCs lines and had high levels of telomerase activity. Multiple cell lines have shown the ability for iPSCs to undergo extensive proliferation while maintaining their differentiation potential. Differentiation of iPSCs is seen in both embryoid bodies and through teratoma formation [31].

Despite similarities to ESCs, there remain differences between ESCs and iPSCs that have yet to be fully elucidated. The use of transgenes and viral vectors to create pluripotent cells is possibly the largest and most obvious difference between ESCs and iPSCs. The vector-

transgene method is a barrier to using iPSCs in humans as the vector and transgene are permanently integrated into the cell genome. The vectors can cause insertional mutations that may affect the function of iPSC derivatives, and the transgene may actually affect differentiation or result in tumor formation [31, 32]. To this end, researchers have developed methods to derive iPSCs without residual vectors or transgenes that possess the same properties and capabilities as ESCs [33].

Furthermore, there are other differences between ESCs and iPSCs that exist at the gene and protein levels. For example, Phanstiel *et al.* found that iPSCs displayed elevated expression of lineage specific genes, including genes for vascular and musculoskeletal system development [34]. Other studies have demonstrated that ESC lines more efficiently differentiate towards neural lineages, which may be due to post-translational modifications in proteins related to neural development [34, 35]. iPSCs may exhibit different methylation patterns around the *Oct4* promoter, and several reports have confirmed other differentially methylated gene loci compared to ESCs [36–39]. Additionally, iPSCs may maintain some of the epigenetic signatures of their parental somatic cell lines that can regulate behavior even after pluripotency is obtained. While continued passage of iPSCs can diminish these marks [40, 41], these residual epigenetic signatures in iPSCs can influence differentiation towards the tissue of origin, suggesting some constraint in reprogramming somatic cells [36–39]. Despite the ongoing research to study the differences, the current knowledge of iPSCs still places them in a position to be of great benefit to the future of developmental biology and regenerative medicine.

2.3. Adult Tissue-derived Mesenchymal Stem Cells

Adult tissue-derived stem cells are the oldest isolated stem cells to be used in research and medical therapies. Within this broad category includes hematopoietic stem cells (HSCs), tissue-specific stem cells such as cardiac, muscle, and neural stem cells, and MSCs. MSCs were first isolated from bone marrow over 50 years ago and were identified by their ability to adhere to tissue culture polystyrene and proliferate robustly, thus selecting MSCs from other cells in the bone marrow isolation [42]. The International Society for Cellular Therapy established the standard for identification of MSCs *in vitro* and determined that MSCs must adhere to plastic and express the surface antigens CD105, CD73, and CD90 while lacking expression of the hematopoietic markers CD34 and CD45, CD14 or CD11, CD79 α or CD19, and HLA class II. The surface markers allow for the simple and reproducible identification of MSCs. The third defining characteristic of MSCs is their multipotency and must at minimum differentiate into adipocytes (fat), chondrocytes (cartilage), and osteoblasts (bone) [43].

MSCs became an attractive cell source for clinical therapies for several reasons. These cells can be isolated with relative ease from multiple sources, including bone marrow and adipose, and readily expanded *in vitro*. MSCs are able to self-renew for several passages before reaching a state of senescence. They have been used to treat immune diseases due to their inherent immunomodulatory properties. Specifically, MSCs have been shown to produce anti-inflammatory cytokines and suppress the proliferation, differentiation and function of immune cells *in vitro* [44, 45]. *In vivo*, transplanted MSCs do not elicit an immune response, allowing them to be used in allogeneic stem cell therapy. MSCs were successfully used to treat graft vs. host disease and are also able to migrate to sites of inflammation and injury [46].

MSCs, like ESCs and iPSCs, proliferate and self-renew while maintaining their ability to differentiate into multiple lineages. To be defined as MSCs, cells must differentiate into the mesenchymal lineages but have shown the ability to differentiate into non-mesodermal cell types like hepatocytes and neurons [47–49]. MSCs hold a couple advantages over ESCs and

iPSCs for musculoskeletal tissue engineering applications. For one, the induction of MSC differentiation is not as complex as ESCs or iPSCs because MSCs are more restricted in their differentiation potential. Namely, they can be induced to directly differentiate into musculoskeletal cell types, whereas ESCs and iPSCs require multiple stages of differentiation. Further, they do not form teratomas when implanted *in vivo*, making them safer to use in clinical applications [50–52].

Despite the promise of MSCs, they possess some characteristics that limit their utility. MSCs exhibit significant heterogeneity between different sources and even amongst single isolations of cells. This heterogeneity can cause different behavior in regards to proliferation and differentiation [53–55]. Recently researchers have developed MSCs from ESCs (ESC-MSCs) to provide a more homogenous stem cell population [56–58]. ESC-MSCs can be derived through multiple methods and have been demonstrated to express the characteristic cell surface markers for MSCs. Further, they possess greater proliferative capacity and the ability for trilineage differentiation and potency into a wider range of cell types [44, 57, 59, 60]. ESC-MSCs were also demonstrated to have superior immunosuppressive abilities compared to MSCs [44].

The renewal of some adult tissues is undertaken by various resident stem cells, although not every tissue has this capability. ESCs and iPSCs provide the ability to develop cells and tissue of every embryonic lineage from one cell type, and similarly, MSCs can also be induced to cells and tissue of mesenchymal lineages. Utilization of stem cells in tissue engineering offers the ability to regenerate tissues, but cells require tight control to effectively differentiate into desired tissues which can be undertaken through various mechanisms covered in this review.

3. STEM CELL REGULATION DURING EXPANSION CULTURE

The tissue engineering process begins with an expansion phase, where the goal is to attain a large number of undifferentiated stem cells capable of efficiently differentiating into the desired cell type(s). Often, hundreds of millions of multipotent stem cells are necessary to grow tissues *ex vivo*. Thus, substantial cell proliferation is generally required prior to differentiation. For MSCs, extensive cell division leads to senescence, a condition that obliterates their ability to differentiate, highlighting the importance of quality, reproducible expansion conditions. For ESCs, which have the ability to proliferate indefinitely, reproducible expansion conditions are most important for preventing aberrant differentiation. Of course, to reproducibly expand stem cells, the identity and concentration of every component in the expansion medium must be well defined. Thus, the important soluble factors driving cell proliferation and maintenance of differentiation potential must be identified so that serum can be eliminated. Additionally, recent evidence has suggested that GFs can be used to purify stem cell populations and/or prime them for subsequent use in specific differentiation protocols. The following sections will highlight general mechanisms controlling stem cell self-renewal and the environmental factors that activate these mechanisms.

3.1. Proliferation and Senescence Regulation

Stem cells from various origins share similar regulatory mechanisms for self-renewal. There are many mechanisms that are involved in stem cell proliferation, and we will touch on a few in this section, including telomerase, cyclin-dependent kinase inhibitors, and the pluripotency transcription factors, Oct4 and Nanog. The self-renewal of ESCs and iPSCs is associated with telomerase activity, which prevents the shortening of telomeres and extends the life span of actively dividing cells. Telomerase is also expressed in MSCs, albeit at much lower levels than pluripotent or immortal cell lines [61]. Thus, MSCs will eventually

undergo senescence in *in vitro* culture. Nonetheless, MSCs have superior proliferation capabilities compared to most somatic cells.

The expression of Oct4 and Nanog plays a major role in the self-renewal and maintenance of potency in pluripotent and adult tissue-derived stem cells. It is well known that these factors play a critical role in maintaining ESCs and are used to induce pluripotency in other cell types. Oct4, Sox2, and Nanog promote pluripotency and inhibit differentiation of ESCs [62]. In MSCs, knocking down Oct4 or Nanog decreases the growth rate and also enhances spontaneous differentiation. Alternatively, the overexpression of *Oct4* and *Nanog* in MSCs increases proliferation of MSCs and also increases their potency. The spontaneous differentiation of MSCs was concomitantly suppressed [63].

The proliferation of somatic cells and MSCs *in vitro* is limited. MSCs begin to undergo senescence once they are taken from their quiescent state and cultured *in vitro*. ESCs and iPSCs, on the other hand, express telomerase which contributes to the phenomenal self-renewal capabilities, but the absence of telomerase does not fully explain cellular senescence in other cells [64]. Cells that lack telomerase will eventually undergo senescence as their telomeres shorten, but many other changes occur as cells are continuously passaged. After prolonged culture, MSCs will eventually stop proliferating and lose the ability to differentiate [64, 65]. The age of donors also affects MSCs in their nonproliferative state, as studies have shown that cells from older donors do not proliferate as well as those from younger donors and will more rapidly enter senescence [65, 66]. During the *in vitro* culture of MSCs, several morphological changes can be seen as the spindle shape of MSCs gives way to larger, cuboidal shaped cells with increased cell debris located in the cytoplasm [64, 66, 67]. As cell proliferation slows, deeper investigation of the MSCs reveals changes in DNA integrity that contribute to senescence. Uncorrected DNA damage and/or loss of tumor suppressor gene expression in somatic cells lead to tumor growth, while leading to senescence in MSC [64, 66]. The DNA damage may be mediated by reactive oxygen species that form in normoxic conditions, as studies have shown that culturing cells in hypoxic conditions can improve the proliferation of MSCs [68, 69].

Many other factors in addition to telomerase have been identified as having a role in modulating cellular senescence. Multiple regulators of the cell cycle, including cyclin-dependent kinase inhibitors 1 and 2 (p21 and p16, respectively) and the p53 tumor suppressor, have been demonstrated to be upregulated as MSCs age and approach senescence [70], and modifying the expression of these genes can repress MSC senescence. Culturing MSCs in a hypoxic environment can prolong the *in vitro* life of the cells. This occurs, in part, through repression of p16 expression [71]. Other genes associated with cellular aging were found to be downregulated as cell progress towards senescence, including genes associated with DNA metabolism, DNA repair and chromosome maintenance, which seemingly reflect the mechanisms behind reduced self-renewal [64].

Epigenetic changes are prevalent as MSCs progress toward senescence, as well. Differential methylation patterns are seen as MSCs age in several gene clusters, including those involved in embryogenesis. Furthermore, methylation patterns vary between early passages of young and old donor MSCs in genes regulating limb morphogenesis and developmental pathways, citing an inherent difference in self-renewal and differentiation potential among MSCs [72]. In MSCs, Oct4 and Nanog were found to increase the expression of a DNA methyltransferase 1 (*Dnmt1*), which is required to maintain MSC proliferation and differentiation. Inhibition of *Dnmt1* in MSCs resulted in reduced proliferation and differentiation and resulted in an increase in p16 and p21 expression, indicating progression toward senescence. Conversely, overexpression of *Dnmt1* in late passage MSCs extended the life span of cells [63]. Further, understanding the mechanisms regulating senescence,

including the genetic and epigenetic regulation, will greatly improve long-term self-renewal of MSCs to maximize their potential in tissue engineering applications.

3.2. Regulation of Stem Cell Expansion by Growth Factors

During embryonic development GFs play a critical role in orchestrating the complex sequence of events driving tissue morphogenesis *in vivo*. *In vitro*, GFs can play several roles during the expansion phase that are beneficial to the tissue engineering process, such as accelerate stem cell proliferation, maintain stemness, purify specific stem cell subpopulations, and prime cells for subsequent differentiation. This section will discuss the role of key GFs during the expansion phase of the tissue engineering process.

To date, several GFs have been identified for their ability to promote MSC growth, but of these, fibroblast growth factor-2 (FGF2) has shown the remarkable ability to serve multiple roles during the expansion phase. FGF2 has been well established as a potent mitogen for many cell types of mesodermal origin [73], and not surprisingly, it has likewise been shown to accelerate the proliferation rate of MSCs [74], thus allowing researchers to more readily obtain a sufficient number of cells for subsequent differentiation. Further, FGF2 has been shown to preserve the stemness and extend the lifespan of MSCs, allowing them to maintain their differentiation potential into later passages [75, 76]. Intriguingly, FGF2 was also found to have the ability to enhance subsequent differentiation into the chondro- and osteogenic lineages when administered during the expansion phase [77, 78] either by purifying a subpopulation of immature MSCs with inherent multipotentiality [79] or by elevating the expression of key lineage-specific transcription factors, in essence priming them for subsequent differentiation [78]. Regardless, studies on FGF2 demonstrated the multiple advantageous roles that a GF can play during the expansion phase of tissue engineering approaches.

Similarly, FGF2 has been shown to be an instrumental component of expansion media for ESCs. Undifferentiated ESCs express all four FGF2 receptors that activate a plethora of signaling pathways upon exogenous FGF2 treatment to maintain ESCs in an undifferentiated and self-renewing state [80–82]. In fact, Zoumaro-Djayoon *et al.* found that approximately 40% of the 3,261 proteins investigated showed differential phosphorylation upon FGF2 treatment using a targeted phosphoproteomics approach [82]. Among the proteins phosphorylated in response to FGF2 were core pluripotency factors, like Sox2, Oct3/4 and Nanog, and their direct targets, demonstrating the immense crosstalk required to maintain ESCs in their pluripotent state. Specifically, FGF2 has been shown to activate MEK/ERK, AKT and PI3K pathways. While it is difficult to elucidate the direct roles of each pathway, inhibition studies have revealed that PI3K and AKT more directly mediate pluripotency marker expression, proliferation and survival [80, 83], whereas MAPK signaling may only have a complimentary role in maintaining ESCs in an undifferentiated state [84]. Ding *et al.* also found that FGF2 activated members of the Src kinase family, and inhibition of Src kinase led to a loss of pluripotency marker expression, suggesting that regulation of actin cytoskeletal organization or dynamics may help maintain ESCs in an undifferentiated state [81].

Another GF family that has shown promising effects during MSC expansion is the Wnt family of secreted glycoproteins. Undifferentiated MSCs express many Wnt ligands and several of their receptors and co-receptors [85], and accordingly, many groups have demonstrated a role for Wnt signaling in controlling MSC behavior during expansion culture. For instance, Boland and colleagues found that Wnt3a suppressed osteogenesis and enhanced cell proliferation and survival of undifferentiated MSCs without compromising subsequent osteogenic differentiation upon withdrawal of the factor [86]. The group also demonstrated that inhibition of canonical Wnt signaling repressed self-renewal and

enhanced MSC osteogenesis. Together, this work demonstrated that canonical Wnt signaling maintains MSCs in an undifferentiated and proliferative state. However, other studies have presented conflicting results. For example, Jullien *et al.* found that Wnt3a induced early osteoblast differentiation in MSCs through activation of β -catenin and decreased Src signaling [87]. Similar to Boland and colleagues, however, they concomitantly showed that Wnt3a had proliferative and anti-apoptotic effects, thus raising the possibility that the observed improvement of osteogenesis was a product of increased cell density rather than a direct improvement of differentiation by Wnt3a, further supporting the role of canonical Wnt signaling in maintaining MSCs in a proliferative state. Conflicting results may also be due to crosstalk between canonical Wnt and other signaling pathways. For example, Qiu and coworkers found that Wnt3a not only activated canonical Wnt signaling but also non-canonical Wnt/JNK pathway, which typically has opposing roles to canonical Wnt signaling [88]. Thus, cooperation between both Wnt pathways may ultimately determine the differentiation fate of MSCs during expansion culture.

Conversely, canonical Wnt signaling appears to promote the differentiation rather than self-renewal of ESCs, as Wnt3a or GSK3 inhibitors have been shown to induce the differentiation of ESCs into primitive streak and definitive endoderm lineages [89, 90]. Indeed, Davidson *et al.* used a sensitive reporter to show that canonical Wnt signaling is not active during ESC self-renewal, and activation of β -catenin signaling resulted in the loss of self-renewal capacity and induction of mesodermal markers [91]. Further, they showed that Oct4 represses β -catenin signaling, and targeted knockdown of Oct4 activates β -catenin in ESCs during self-renewal. Thus, current data supports a role for canonical Wnt signaling in promoting differentiation rather than self-renewal in ESCs, demonstrating key differences in the response of distinct stem cell types to the same GF depending on their stage of development.

Other GFs have been identified as having the ability to promote MSC self-renewal [92]. For instance, Ng and colleagues identified transforming growth factor (TGF) β and platelet-derived growth factor (PDGF), in addition to FGF, signaling pathways as being important for MSC self-renewal using microarray data [93]. They found that these three pathways were both necessary and sufficient for MSC expansion; inhibition of any of the three pathways slowed MSC growth, while a combination of exogenous TGF β 1, PDGF-BB and FGF2 was sufficient to expand MSCs in serum-free medium for up to five passages, demonstrating the importance of these GFs on the survival and proliferative abilities of MSCs. Mimura *et al.* also developed a serum-free expansion medium for MSCs based on a commonly used ESC expansion formulation, and similar to Ng and colleagues, they found that TGF β 1 and FGF2 were critical in supporting MSC cell growth [94]. Conversely, PDGF-BB was not crucial for efficient expansion of MSCs in their study. In addition, researchers have identified PDGF-AB, endothelial growth factor (EGF), TGF α , hepatocyte growth factor (HGF), vascular endothelial growth factor (VEGF) and even members of the BMP family as other inducers of proliferation in MSCs [95–97]. Interestingly, Tamama and colleagues similarly showed that EGF and PDGF-BB could induce MSC proliferation [98]. However, they found EGF induced MSC proliferation without interfering with their ability to later differentiate, whereas PDGF hindered the ability of MSCs to differentiate after expansion, suggesting that while many GFs may have mitogenic activity to a given stem cell population, specific GFs are likely optimal depending on the subsequent tissue engineering application.

Similarly, administration of TGF β 1 to undifferentiated ESCs maintains them in their undifferentiated state via activation of Smad2/3 [99]. Moreover, inhibition of TGF β signaling in ESCs induces cellular differentiation [100]. Another member of the TGF β superfamily, Activin A, has also been shown to maintain ESCs in a self-renewing state

[101]. Xiao *et al.* identified Nodal/Activin signaling as important for maintaining the self-renewal capacity of ESCs using microarray data, along with the FGF, Wnt and Hedgehog pathways. They found that Activin A was both necessary and sufficient for the maintenance of ESC self-renewal and pluripotency and showed that Activin A induces the expression of *Oct4*, *Nanog*, and *Nodal*, as well as various Wnts and FGFs, further demonstrating the profound crosstalk between these signaling pathways in maintaining ESCs in a pluripotent, undifferentiated state.

3.3. Regulation of Stem Cell Expansion by Oxygen Tension

The capacity of stem cells to self-renew and maintain potency depends on a complex balance of signals, and recent evidence has identified oxygen tension as a critical component influencing stem cell behavior. During embryonic development, direct measurements of tissue oxygen tensions revealed much lower oxygen levels than expected [102]. For instance, early during development prior to the establishment of a circulatory system, delivery of oxygen to developing tissues is limited by diffusion, which for oxygen is approximately 150 μm [103], thus creating a relatively oxygen poor environment. Further, oxygen measurements in adult tissues known to harbor stem cells revealed even lower oxygen tensions than those measured during embryonic development [104, 105]. In fact, oxygen concentrations between 2–9% have recently been appreciated by some researchers as physiologic normoxia, although such oxygen tensions are hypoxic compared to ambient air [106]. Taken together, it is not surprising that lower oxygen tensions greatly affect the activities of both embryonic and adult tissue-derived stem cells [107, 108], while also influencing proliferation and cell fate commitment. In this section, we will discuss the role of hypoxia in regulating stem cell activities prior to differentiation for both MSCs and ESCs.

MSCs reside in perivascular niches in virtually all tissues [109–111], but despite their close association to blood vessels, low oxygen tensions are characteristic in the various tissues where these stem cells are found. Not surprisingly, hypoxia has been found to be a key factor required to maintain MSCs in an undifferentiated state. Indeed, when cultured under hypoxic conditions *in vitro* (3–7% O_2), MSCs show an increased rate of proliferation [112] and extended lifespan [113]. In fact, Grayson and colleagues found that expansion of MSCs for seven passages resulted in approximately a 30-fold increase in population doublings over six weeks compared to MSCs expanded under normoxic conditions (20% O_2). Further, hypoxia induced an upregulation in the expression of key stemness genes, like *Oct4*, *Nanog*, *Sall4* and *Klf4* [112], suggesting that hypoxic conditions during expansion culture maintain MSCs in an undifferentiated state. Additionally, MSCs expanded under hypoxic conditions exhibited telomerase activity, maintenance of telomere length for up to 100 population doublings, normal karyotyping, and intact genetic integrity, further suggesting that hypoxia maintains MSCs in an undifferentiated state while preventing senescence [114–116]. Concurrent with the maintenance of stemness, hypoxia likewise maintains the multipotentiality of MSCs into later passages. Adesida *et al.* found that expansion of MSCs in hypoxic conditions enhanced subsequent differentiation into the chondrogenic lineage, as evidenced by increased GAG synthesis and expression of cartilage-specific gene expression [117]. More interestingly, expansion in hypoxic conditions suppressed the hypertrophic differentiation of MSCs upon differentiation in pellet culture. Similarly, Volkmer *et al.* found that expansion in hypoxic conditions enhanced subsequent osteogenesis [118]. The caveat to hypoxic conditions is that when MSCs are expanded under very low oxygen tensions (i.e. 1% O_2), they exhibit decreased proliferation and increased accumulation of cells in the G1 phase of the cell cycle [119], suggesting that proliferation and perhaps even quiescence may be regulated by gradients of oxygen tension within stem cell niches *in vivo*, with lower oxygen tensions preserving stem cells in a quiescent state and slightly higher oxygen concentrations promoting proliferation.

It is believed that hypoxic conditions maintain MSCs in an undifferentiated and senescence-free state through the activity of hypoxia-inducible factors (HIFs). HIFs belong to a family of basic Helix-Loop-Helix transcription factors that are stabilized under low oxygen tensions [120]. Upon stabilization, the subunits HIF1 α , HIF2 α , and HIF3 α individually dimerize with HIF β and translocate to the nucleus, where they regulate the expression of genes necessary to maintain oxygen homeostasis, glucose metabolism, angiogenesis, erythropoiesis, and iron metabolism [121]. In MSCs, an increase in the expression and activity of both HIF1 α and HIF2 α has been observed upon exposure to hypoxic conditions [117]. Tsai *et al.* found that an increase in HIF1 α activity led to the direct downregulation of the cell cycle inhibitor E2A-p21 in cooperation with TWIST [114]. They found that overexpression of *TWIST* could abrogate the normoxia-induced downregulation of E2A and p21, while siRNA-mediated knockdown of *TWIST* led to an upregulation of E2A and p21 expression in MSCs cultured under hypoxia. Further, overexpression of *p21* in hypoxic cells induced decreased proliferation and a loss of differentiation capacity [114], suggesting that hypoxia maintains MSCs in a proliferative and multipotential state through downregulation of p21. Similarly, Jin and colleagues found that the hypoxia-mediated prevention of senescence correlated with a downregulation of p16 and ERK1 expression [71]. Activation of HIFs has also been shown to increase the secretion of VEGF, HGF, and FGF2, which in turn may act in an autocrine or paracrine fashion to promote the self-renewal of MSCs [122].

The mammalian reproductive tract contains 1.5–5.3% O₂ [123], so stem and progenitor cells are accustomed to low oxygen tensions during embryonic development. Not surprisingly, hypoxia has been found to have a prominent role in maintaining ESCs in an undifferentiated state and enhancing the long-term self-renewal of ESCs. Several groups have found that ESC colonies contain a more homogeneous population of undifferentiated Oct4⁺ stem cells under 5% oxygen, whereas increased spontaneous differentiation is prevalent at 20% oxygen [124]. Hypoxia may induce a concomitant increase in proliferation rate, but these effects are likely only noticeable over longer culture periods [125]. Maintenance of an undifferentiated phenotype under hypoxic conditions is accompanied by increased expression of *Sox2*, *Nanog*, *Oct4*, and *SSEA4*, and reduced expression of the early differentiation marker, *SSEA1* [125, 126]. Further, embryoid body formation is enhanced under hypoxia [127], and hypoxia has been found to reduce the frequency of chromosomal abnormalities and apoptosis [123]. The antiapoptotic effect of hypoxic conditions was shown to be linked to VEGF signaling [128]. Hypoxia led to increased expression of *VEGF*, *Flk1* and *Nrp1*, and inhibition of VEGF, but not other common mitogens, increased ESC apoptosis by about 10-fold. Similarly, overexpression of *Nrp1*, a VEGF165 isoform-specific receptor, decreased apoptosis, suggesting that VEGF plays a critical role in the survival of ESCs during prolonged hypoxia through Nrp1.

Chen *et al.* found that hypoxia-mediated maintenance of the undifferentiated state was accompanied by an upregulation in genes belonging to FGF, TGF β , and Wnt signaling pathways [127]. Further, higher pSmad2/3 levels were present under hypoxic conditions, suggesting that hypoxia may be maintaining ESCs in an undifferentiated state by promoting the production of GFs, namely TGF β , known to promote stemness. Forristal *et al.* and Covello *et al.* reported that increased proliferation and expression of *Sox2*, *Nanog* and *Oct4* in response to hypoxia was regulated by activation of HIF2 α [126, 129]. Both HIF2 α and HIF3 α were upregulated by hypoxic culture and translocated to the nucleus, but only silencing of HIF2 α led to a decrease in the expression of pluripotency markers and reduced proliferation. Extending upon these findings, Das *et al.* found that, in addition to activating HIF2 α , hypoxia suppresses the activity of p53 [130]. Additionally, Prasad *et al.* found high Notch1 expression under hypoxia, and inhibition of Notch signaling completely abolished the induction of an undifferentiated phenotype by hypoxia [131]. Many of these factors seen

in ESCs also regulate cellular responses in iPSCs, and hypoxia exerts an additional effect on iPSC development. Several groups have reported that reprogramming cells under hypoxic conditions increased reprogramming efficiency [125, 132], further demonstrating the regulatory effects of hypoxia on all stem cells.

3.4. Regulation of Stem Cell Expansion by Biomaterials

Another component of *in vitro* expansion culture that can impact the ability of stem cells to maintain their undifferentiated and self-renewing state is the nature of their underlying substrate. However, unlike GFs and oxygen tension, which activate specific intra-cellular signaling programs to dictate cell behavior, biomaterials may act through a more global mechanism via control of cell morphology and actin cytoskeletal organization. As stem cells undergo senescence, they begin to exhibit actin stress fibers, which may suggest an important connection between regulation of the actin cytoskeleton and stem cell self-renewal. Indeed, biomaterial scaffolds have been shown to promote stem cell self-renewal and preserve the potency of stem cells, potentially providing state-of-the-art culture systems for the *ex vivo* expansion of stem cells. The following section will explore the regulation of stem cell self-renewal and potency in nanobiomaterials.

Three-dimensional (3D) nanofibrous scaffolds, regardless of biomaterial identity, have consistently been shown to enhance stem cell self-renewal. For instance, Nur-E-Kamal *et al.* found improved self-renewal of mouse ESCs on nanofibrous surfaces, and these observations were correlated with the activation of the small GTPase Rac and the PI3K pathway compared to two-dimensional (2D) surfaces without nanofibers [133]. Indeed, sustained activation of Rac led to cytoskeletal reorganization and increased cell proliferation [134], suggesting that Rac is an important signaling molecule in directing stem cell self-renewal in 3D nanofibrous culture. Interestingly, the 3D nanofibrillar scaffolds also enhanced the expression of *Nanog*, a transcription factor required for the maintenance of pluripotency, and inhibition of the PI3K pathway reduced the expression of *Nanog*, suggesting that nanofibers may enhance stem cell self-renewal and potency through activation of the PI3K pathway. Nanofibrous scaffolds have also been shown to upregulate the expression of *Oct3/4* and *Sox2*, two other transcription factors necessary for promoting multipotency, in MSCs [135], demonstrating the ability of nanobiomaterials to promote stem cell potency in a variety of stem cell types.

In addition to promoting self-renewal, nanofibrous scaffolds have been shown to maintain the potency of *ex vivo*-expanded stem cells. Mauney *et al.* showed that collagen type I nanofibrillar matrices can preserve the osteogenic potential of MSCs compared to conventional 2D tissue culture plastic substrates [136]. While the mechanism underlying the retention of differentiation potential remains to be fully elucidated, they found that expansion on nanofibrillar matrices significantly reduced a prominent manifestation of cellular aging, the expression of the protective stress response protein, HSP70 [137]. Interestingly, the reduction in HSP70 expression was accompanied by an increase in proliferation and reduction in morphological changes that signify cellular senescence, such as extensive cellular spreading and formation of actin stress fibers. Given the potent ability of nanostructures to prevent stress fiber formation, it would be interesting to examine whether the enhanced proliferation and maintenance of potency in response to nanostructures is a direct effect of modulated actin cytoskeletal organization.

While nanofibrous matrices are extensively used for biomaterial scaffold research, other scaffold architectures have similarly been utilized to regulate stem cell behavior. Several scaffold designs have been investigated, including pre-made porous scaffolds, decellularized ECM, cell-secreted ECM and hydrogels. Pre-made porous scaffolds can be formed by creating pores in casted materials or by creating porous structures using technologies such as

porogen leaching, freeze-drying, laser sintering, stereolithography and 3D printing [138]. Studies using these scaffold structures have been shown to enhance stem cell expansion. For example, 3D scaffolds composed of poly(ϵ -caprolactone) (PCL), poly(lactic-co-glycolic acid) (PLGA), fibrin, and collagen were used for the *ex vivo* expansion of HSCs. During the 14 days of culture, all scaffolds, except for the PLGA, supported HSC proliferation with the fibrin scaffolds showing the most robust proliferation [139]. Similarly, these structures have been shown to support MSC proliferation, as Mygind *et al.* demonstrated with nano-sized coralline hydroxyapatite composites used to develop an engineered bone construct. These scaffolds were seeded with primary MSCs, and under dynamic culture media flow, were able to support proliferation [140]. Köllmer and colleagues also cultured MSCs in a poly(ethylene glycol diacrylate) (PEGDA) superporous hydrogel and expanded viable cells for over three weeks [141], further demonstrating the ability of 3D structures to support stem cell expansion.

4. STEM CELL REGULATION DURING DIFFERENTIATION

Once the desired number of undifferentiated stem cells is obtained, cells are then harvested for the second phase of *in vitro* culture: the differentiation phase. The goal of the differentiation phase is to produce cells and/or tissues that are phenotypically and functionally identical to those found *in vivo*. For musculoskeletal tissues, this generally means that the differentiated cell types have a similar gene expression pattern to their *in vivo* counterparts and produce an extracellular matrix of similar composition and mechanical properties as the native tissue. Indeed, a wide range of environmental factors have been shown to have a critical role in promoting differentiation into musculoskeletal lineages. Namely, biomaterial scaffolds and mechanical stimulation, through control of actin cytoskeletal organization and cell shape, and specific GFs work cooperatively to direct stem cell differentiation into musculoskeletal lineages. This section will discuss the role of GFs, oxygen tension, biomaterial scaffolds and mechanical stimulation in directing the differentiation of MSCs and ESCs into chondrocytes and osteoblasts.

4.1. Regulation of Stem Cell Differentiation by Growth Factors

A chondrocyte-like phenotype was first induced from MSCs by Johnstone and colleagues using TGF β 1 in 1998 [142]. Since then, all three TGF β isoforms, TGF β 1–3, have been identified as potent inducers of MSC chondrogenesis when administered in a 3D culture system [142]. In fact, no other single GF has been shown to consistently have the ability to initiate the chondrogenic differentiation program in MSCs, although exceptions inevitably exist [143, 144]. Upon initiation of chondrogenesis, TGF β treatment leads to phosphorylation of Smad2/3, which in turn associates with and activates Sox9. This interaction between Smad2/3 and Sox9 leads to increased association with the coactivator CBP/p300, and this complex ultimately binds to enhancer regions of cartilage-specific genes to upregulate their expression. Concomitantly, other intracellular signaling pathways are activated by TGF β treatment, including MEK/ERK, p38 and JNK, which act to further promote cell survival and cartilage-specific gene expression [145, 146].

While only TGF β isoforms are able to initiate MSC chondrogenesis, several members of the bone morphogenetic (BMP) family of proteins have been shown to have prochondrogenic effects in combination with TGF β [147]. Of these, BMP2, -4, -6, -7 and -9 have been most well studied for their ability to enhance MSC chondrogenesis. BMPs function primarily through activation of Smad1/5/8. Blocking phosphorylation of Smad1/5/8 from the onset of chondrogenesis prevents upregulation of cartilage-specific gene expression upon treatment with TGF β 1, demonstrating an important, albeit complimentary, role for BMP signaling in the initiation of chondrogenesis. Blocking Smad1/5/8 phosphorylation after the onset of chondrogenesis halts the appearance of hypertrophy markers like Col X, ALP, and matrix

mineralization, suggesting that BMP signaling acts at a later stage to promote chondrocyte hypertrophy. Interestingly, some MSC populations require stimulation with both TGF β and BMP ligands to initiate chondrogenesis. For instance, adipose-derived MSCs have consistently shown reduced chondrogenic potential in response to TGF β stimulation compared to bone marrow-derived MSCs, and Hennig and coworkers found that this may be due, at least in part, to reduced expression of TGF β R1 [148]. As a result, increased concentrations of TGF β 1 had little effect in improving cartilage-specific gene expression. However, BMP6 treatment induced TGF β R1 expression, and combined application of TGF β 1 and BMP6 restored the chondrogenic potential of adipose-derived MSCs to levels comparable to bone marrow-derived MSCs. Another report by Pan *et al.* suggests that the chondroinductive capacity of BMPs may be multifaceted [149]. BMP2 activated Sox9 transcription through histone hyperacetylation and methylation at the Sox9 promoter, thus opening the local chromatin structure. These modifications were accompanied by increased association between the transcription factor NF-Y and the histone acetyltransferase p300/CBP, and this complex showed increased recruitment to the proximal promoter of the Sox9 gene, along with PCAF and RNA Polymerase II.

Another member of the BMP family, GDF5, has likewise been investigated for its ability to improve cartilage formation from MSCs. While results have varied, several studies have reported chondroinductive properties of GDF5. In fact, Bai *et al.* found that GDF5 at a concentration of 500 ng/mL could induce the chondrogenic differentiation of bone marrow-derived MSCs even in the absence of TGF β stimulation [144]. However, their differentiation conditions included 1% FBS, which inevitably contains TGF β isoforms [150]. Similarly, Xu *et al.* observed prochondrogenic effects of GDF5 in combination with TGF β 3, although GDF5 alone had no effect on chondrogenesis under serum-free conditions [151]. The authors found that TGF β 3 treatment actually led to an upregulation of BMPRII expression, which subsequently allowed MSCs to bind GDF5.

Aside from ligands of the TGF β and BMP families, other GFs have been explored for their ability to enhance MSC chondrogenesis. The most well studied is insulin-like growth factor (IGF1), given its role as one of the primary anabolic signals acting on mature chondrocytes *in vivo*. Supporting this notion, Wang and colleagues compared the chondroinductive properties of IGF1 and TGF β 3 after three weeks of TGF β 3 treatment alone and found that IGF1 was a more potent inducer of sGAG and collagen type II production than TGF β 3 at later stages of MSC differentiation [152]. Other groups have similarly noted the prochondrogenic effects of IGF1 [153, 154]. However, it should be noted that basal chondrogenic medium contains high concentrations of insulin to promote cell survival, and given the fact that insulin and IGF1 show cross-reactivity for each other's receptors, MSCs likely do not need to be exposed to IGF1 for optimal differentiation. Another GF of great interest in the field of cartilage tissue engineering is parathyroid hormone-related protein (PTHrP), as it may be able to promote a more clinically-relevant chondrocyte phenotype. As discussed previously, MSCs have a tendency to undergo hypertrophy instead of differentiating into phenotypically stable articular chondrocytes. All the GFs discussed to this point seem to simply enhance, or accelerate, the hypertrophic differentiation process instead of shifting the ultimate chondrocytic fate of differentiating MSCs. PTHrP, on the other hand, may have the ability to promote chondrogenesis while concomitantly halting chondrocyte hypertrophy. For instance, Kim *et al.* found that PTHrP, when administered after two weeks of differentiation with TGF β 2, enhanced sGAG accumulation and upregulated the expression of *Sox9* and *Col II* while suppressing the expression of *Col I*, *Runx2* and *Col X* in both bone marrow- and adipose-derived MSCs [155]. However, few published reports exist that demonstrate such an advantageous effect of PTHrP on MSCs, with other studies finding that PTHrP represses chondrogenesis altogether [156]. Recent work by Lee and Im may resolve these discrepancies. The group found functional

differences in MSC responsiveness to PTHrP depending on the isoform of commercially available recombinant PTHrP used [157]. Of the isoforms tested, only PTHrP (1–34) enhanced chondrogenesis while concomitantly suppressing hypertrophy in MSCs.

Chondrocyte hypertrophy is perhaps the most troubling hurdle to overcome before articular cartilage tissue engineering gains clinical relevance, especially in lieu of recent evidence demonstrating that a hypertrophic cartilage template possesses all the signals necessary to recapitulate bone formation through endochondral ossification when implanted subcutaneously *in vivo* [158]. While troubling to cartilage tissue engineers, this may actually be the optimal approach for bone tissue engineers, who could exploit resident osteoprogenitors *in vivo* to instruct bone formation using an *in vitro*-developed hypertrophic cartilage template. However, the majority of efforts aimed at engineering bone tissue have focused on directly differentiating stem cells into the osteogenic lineage *in vitro*, an approach that is hampered by a lack of truly specific bone markers and production of functional tissue possessing physiological organization and vascularization. Nonetheless, much effort has been invested in differentiating MSCs into the osteogenic lineage, and not surprisingly, BMPs have been most well studied for their ability to promote osteogenesis (although the combination of β -glycerophosphate, ascorbic acid and dexamethasone is sufficient to induce osteogenesis). In one study, Kang *et al.* conducted a comprehensive analysis of 14 BMPs for their ability to induce osteogenesis from MSCs and found that BMP2, -4, -6, -7 and -9 exhibited the highest osteogenic activity *in vitro* and *in vivo* [159]. As expected, knockdown of *Runx2* expression diminished BMP-induced osteogenic differentiation, confirming the essential role of Runx2 for osteogenic differentiation. More interestingly, overexpression of *PPAR* γ 2 enhanced BMP-induced MSC osteogenesis, while knockdown of *PPAR* γ 2 reduced osteogenic differentiation, suggesting that in addition to its critical role in promoting adipo-genesis, *PPAR* γ 2 may play an important role in BMP-induced osteogenesis of MSCs. Wang *et al.* found that BMP2 induced osteogenic differentiation of MSCs through rapid and sustained activation of RhoA/ROCK activity [160]. In fact, the group found that reducing ROCK signaling, inhibiting cytoskeletal tension and restricting cell spreading using micropatterned substrates prevented Smad1 phosphorylation and translocation into the nucleus in response to BMP2, thus demonstrating a direct involvement of cell spreading and RhoA/ROCK-mediated cytoskeletal tension in the BMP-induced osteogenic differentiation of MSCs.

In another study, IGF2 was shown to potentiate BMP9-induced osteogenic differentiation [161]. IGF2 upregulated the expression of early and late bone markers and enhanced both BMP9-induced BMPR-Smad reporter activity and Smad1/5/8 nuclear translocation. Further, pharmacological inhibition of PI3K abolished the effect of IGF2 on BMP9-induced osteogenic differentiation, demonstrating a potential crosstalk between Smad1/5/8 and PI3K in promoting osteogenic differentiation in MSCs. Further crosstalk has been demonstrated between signaling components downstream of BMP9 induction and canonical Wnt signaling [162]. For example, β -catenin knockdown in MSCs has been shown to inhibit ALP activity, reduce osteocalcin reporter activity, and downregulate the expression of late osteogenic markers in response to exogenous BMP9 treatment. Furthermore, β -catenin knockdown inhibited BMP9-induced mineralization *in vitro* and ectopic bone formation *in vivo*, resulting in the formation of a chondrogenic matrix. ChIP analysis confirmed that both Runx2 and β -catenin were recruited to the osteocalcin promoter upon induction with BMP9, suggesting that interactions between β -catenin and Runx2 may be important for promoting the osteogenic differentiation of MSCs. It is unclear to what extent canonical Wnt signaling plays in promoting MSC osteogenesis, however, most reports demonstrate a proliferative and osteosuppressive role for canonical Wnt signaling. Perhaps canonical Wnt signaling serves a supportive role in promoting osteogenic differentiation when BMP signaling is active. Conversely, it is possible that Wnt signaling has differential effects on osteogenesis

depending on the stage of differentiation. For instance, Quarto and colleagues found that canonical Wnt signaling inhibited osteogenesis when added to undifferentiated MSCs, but when added to calvarial osteoblasts, high doses of Wnt3a induced bone matrix production [163]. Non-canonical Wnt signaling has a more well established role in enhancing MSC osteogenesis [164]. For instance, Wnt5a has been shown to enhance osteogenic differentiation through activation of non-canonical Wnt signaling by increasing PKC activity [165]. Similarly, Wnt4 has been shown to enhance MSC osteogenesis through activation of p38 MAPK signaling [166]. Additionally, GDF5 [167] and VEGF [168] have been shown to enhance osteogenic differentiation, as has the cytokine IL3 [169].

Of course, the task of controlling differentiation becomes much more complicated with pluripotent cell types, like ESCs and iPSCs, which are believed to be very similar to epiblast cells present during early embryonic development [170]. In such cases, tissue engineers must also elucidate the key signals that direct stages of development preceding differentiation into mature cell types, such as mesendoderm differentiation and mesoderm specification at the primitive streak and later lateral plate mesoderm formation. Thus, several stages of differentiation are likely required to attain terminally differentiated musculoskeletal cell types.

Prior to differentiation into chondrocytes and osteoblasts, ESCs must first be differentiated into mesendoderm and then mesoderm cells, a process that occurs at the primitive streak during gastrulation *in vivo* [171]. Early lineage specification into the three germ layers is ultimately driven by an intricate balance of canonical Wnt, Activin/Nodal and BMP signaling [172]. Studies have shown that Wnt3a and Activin A can induce the expression of early mesendoderm differentiation *in vitro* [173, 174]. After mesendoderm differentiation, high concentrations of Activin A promote endoderm specification, while BMP4, FGF2 and low concentrations of Activin A have been shown to efficiently induce mesoderm differentiation [174, 175]. From this point, Oldershaw and coworkers were able to generate Sox9⁺ chondrocyte-like cells in 75–97% of cells from mesoderm progenitors using BMP4 and GDF5 [174]. Additionally, the group induced a significant increase in *Sox9*, *Col II* and *ACAN* expression without *Col X*, demonstrating the successful differentiation of chondrocyte-like cells devoid of hypertrophy from pluripotent ESCs using multiple stages of differentiation under defined conditions. While the work by Oldershaw and colleagues represents the current gold standard in ESC chondrogenesis, the expression of cartilage-specific genes remained markedly lower than that observed in MSCs after chondrogenic induction, suggesting that further improvements will be necessary if these cells are to have clinical utility.

For instance, it is possible that another stage of expansion and/or differentiation is needed prior to the production of mature chondrocytes from primitive streak-derived mesodermal progenitors. Indeed, during embryonic development, mesodermal progenitors generated at the primitive streak first coalesce into a cluster known as lateral plate mesoderm before migrating to the site of limb bud formation. At the site of lateral plate mesoderm formation, there may be signals that preferentially induce a subpopulation of mesodermal progenitors to migrate from the primitive streak, in essence purifying the progenitor pool, or conversely, paracrine signals present that may prime progenitor cells to more efficiently undergo subsequent differentiation within the limb bud. Through mimicry of this stage of differentiation, researchers may be able to enrich the progenitor pool *in vitro* and improve the efficiency of differentiation. Unfortunately, there has been no research to address this possibility.

The osteogenic differentiation of ESCs has been most commonly carried out using only dexamethasone, L-ascorbic acid and β -glycerophosphate—components of basal MSC

osteogenic medium—without additional GF supplementation [176]. Similar to MSCs, these basal medium components are sufficient to induce osteogenesis after predifferentiation as embryoid bodies in the presence of retinoic acid [177, 178] or in adherent culture in the presence of serum [176] to obtain mesenchymal progenitors. Given the relative success of osteogenic differentiation without supplementation with expensive recombinant GFs, few GFs have been tested for their ability to enhance the differentiation process. However, Hu *et al.* induced ESC osteogenesis with BMP7 and found that while BMP7 had little effect alone, it elicited a synergistic effect in promoting osteogenesis when administered with dexamethasone [179]. Additionally, multiple groups have used BMP2 to enhance the osteogenic differentiation of ESCs in basal osteogenic medium [179, 180], while Kawaguchi *et al.* found improved osteogenic differentiation upon supplementation with BMP4 [177]. Interestingly, when comparing ESC osteogenesis in the presence of basal osteogenic factors or BMP2 without dexamethasone, ascorbic acid and β -glycerophosphate, the basal osteogenic factors were more potent at inducing osteogenesis than BMP2 alone [181]. Unfortunately, unlike ESC chondrogenesis, no studies have been carried out that mimic the various stages of development to direct differentiation from ESC to osteoblast. It would be interesting to see if the differentiation scheme that Oldershaw *et al.* used to direct the differentiation of ESCs to chondrocytes could be modified to derive osteoblasts instead [174].

4.2. Regulation of Stem Cell Differentiation by Oxygen Tension

Given the critical role of hypoxia in promoting the undifferentiated state of both adult and embryonic stem cells, it is not surprising that there is less evidence linking oxygen tension to differentiation. However, nearly every tissue is established in a relatively hypoxic environment compared to ambient air, and some tissues like cartilage are hypo- or even avascular in their mature form. Thus, several groups have examined the role of hypoxia in promoting stem cell differentiation. This section will discuss the role of hypoxia in promoting the chondro- and osteogenic differentiation of MSCs and ESCs.

Given that cartilage is an avascular tissue and mesenchymal cells experience hypoxia during prechondrogenic condensation of endochondral ossification, it is not surprising that hypoxic conditions have been shown to enhance MSC and ESC chondrogenesis [182, 183]. More interestingly, some reports have shown that hypoxia can improve cartilage-specific gene expression while repressing markers of hypertrophy, as demonstrated by reduced collagen type X and Alizarin Red staining and alkaline phosphatase activity [184], although these findings have not been consistently observed. As expected, the improved chondrogenic differentiation observed in MSCs has been correlated to increased HIF1 α and HIF2 α expression and activity [185, 186]. Similarly, hypoxic conditions have been shown to improve the chondrogenic differentiation of ESCs, resulting in significantly increased collagen type II and GAG production, as well as improved biomechanical properties [187]. Taken together, hypoxia seems to have an important role in improving stem cell chondrogenesis.

Studies examining the effects of low oxygen tension on osteogenic differentiation have shown less consistency [188]. However, the general trend has been that hypoxia reduces osteogenic differentiation [189]. Yang *et al.* found that hypoxia inhibited MSC osteogenesis by inducing TWIST, a downstream target of HIF1 α [190]. TWIST subsequently acted as a transcriptional repressor of *Runx2* expression, which in turn inhibited the expression of BMP2, suggesting that endogenously-produced GFs may be interacting with hypoxic conditions to affect differentiation. There may also be crosstalk between HIF signaling and MEK/ERK signaling, as Wang *et al.* found that reduced osteogenic differentiation coincided

with an increase in phosphorylated ERK1/2 [191]. There have been no published reports of the effect of oxygen tension on the osteogenesis of embryonic stem cells.

4.3. Regulation of Stem Cell Differentiation by Biomaterial Scaffolds

Multiple factors interact to direct the differentiation of stem cells into the various lineages, with GFs having perhaps the most prominent role. For instance, TGF β ligands are indispensable for the induction of MSC chondrogenesis. Interestingly, however, TGF β ligands can only initiate MSC chondrogenesis when cells are cultured in a 3D environment, suggesting a critical role for cell shape in promoting chondrocyte differentiation. Indeed, cell shape and actin cytoskeletal organization have been shown to be key regulators of differentiation into a variety of musculoskeletal lineages. Through creation of a 3D environment and modulation of cell shape, biomaterial scaffolds have gained a prominent role in facilitating stem cell differentiation [192–194]. Additionally, biomaterials can promote differentiation depending on the biomaterial identity and mechanical properties. This section will discuss the various roles that biomaterial scaffolds can play in promoting stem cell differentiation.

The earliest demonstration that cell shape and actin cytoskeletal organization could affect differentiation came through *in vitro* studies on chondrocytes. Chondrocytes exhibit a round morphology *in vivo*, but when cultured in 2D tissue culture polystyrene *in vitro*, they dedifferentiate into fibroblast-like cells. Concomitant with this change in cell shape is the acquisition of actin stress fibers and loss of cartilage-specific gene expression, which progressively declines with continued passage *in vitro*. To avoid this loss of cartilage-specific gene expression, researchers began to culture chondrocytes in hydrogels, which appeared to facilitate the retention of a round cell shape, further linking cell shape and differentiation state [4, 195]. Since these initial observations, researchers have gone on to show that pharmacological disruption of the actin cytoskeleton with drugs like cytochalasin D can restore the observed loss of cartilage-specific gene expression during *in vitro* culture, suggesting a direct involvement of the actin cytoskeleton in promoting cartilage-specific gene expression [196]. More recently, studies have shown that attenuating the activity of Rho GTPases, which directly regulate the actin cytoskeleton, can similarly affect MSC differentiation. For instance, McBeath *et al.* demonstrated that cell morphology regulates the commitment of MSCs to adipocytic and osteoblastic fates [197]. When MSCs were allowed to obtain a well-spread and flattened morphology, they differentiated into osteoblasts. However, when cell spreading was restricted using micropatterned substrates, MSCs remained round and differentiated into adipocytes. By controlling cell shape, McBeath *et al.* regulated the organization of the actin cytoskeleton through modulation of endogenous RhoA activity. Active RhoA, which promotes the formation of actin stress fibers, led to osteoblast differentiation, while inactive RhoA encouraged differentiation into adipocytes. Similarly, inactive RhoA encouraged differentiation into chondrocytes upon TGF β stimulation [198]. Together, it is clear that cell shape and actin cytoskeletal organization have an indispensable role in directing differentiation into musculoskeletal lineages.

Given these promising findings, researchers have exploited the role of cell shape by using fibrous biomaterial scaffolds to control actin cytoskeletal organization. The most common way to do so is by controlling the fiber diameter of biomaterial scaffolds. Hsia *et al.* compared actin cytoskeletal organization on nanofibers and microfibers and found fewer actin stress fibers in fibroblasts cultured on nanofibers [199]. Similarly, Li *et al.* observed dramatic differences in morphology between chondrocytes seeded on PLLA nanofibers and chemically identical microfibers [200]. Chondrocytes seeded on the PLLA microfibers displayed a well-spread morphology that spanned several fibers, while chondrocytes on nanofibers were found to have a more round cell shape. Expectedly, the distinct cell

morphologies observed on microfibers and nanofibers occurred concomitantly with changes in actin cytoskeletal organization. The chondrocytes seeded on microfibers displayed prominent actin stress fibers that spanned the length of the cell, while chondrocytes on nanofibers displayed a less well-developed cortical actin cytoskeletal organization, reminiscent of that found *in vivo*. These studies thus demonstrate that nanofibers promote the acquisition of a more *in vivo*-like cell morphology and cytoskeletal organization, and further suggest that a similar route could be taken to direct the chondrogenesis of relevant stem cell types.

In addition to controlling cell shape and actin cytoskeletal organization, biomaterials can also direct differentiation into specific lineages through its composition. Natural ECM components bind specific cell surface receptors, which subsequently activate specific intracellular signaling cascades depending on the identity of the ECM protein bound. Even in 2D culture, which normally is repressive for the acquisition of a chondrocyte-like phenotype, different ECM proteins can induce chondrogenesis to varying degrees. In adult articular cartilage, chondrocytes interact with a pericellular matrix composed primarily of collagen type IV and laminin [201]. *In vitro*, Lindner *et al.* induced chondrogenesis on laminin-1, laminin-5, and collagen type IV and saw that MSCs differentiated more effectively on laminin-1 and collagen type IV than they did on laminin-5, demonstrating that different ECM components possess varying degrees of bioactivity to promote differentiation. Similarly, tissue culture polystyrene coated with collagen type I, but not fibronectin or laminin, enhanced MSC osteogenesis via activation of ERK and Akt, as demonstrated by an increase in ALP activity and upregulated *Runx2* and *Osteocalcin* expression [202].

The same molecules can also be used to fabricate natural scaffolds for 3D culture to promote physiologically relevant cell morphologies while taking advantage of the inherent bioactivity of certain ECM components. A variety of biomaterials derived from natural ECM components are currently being explored as 3D scaffolds to control the differentiation of MSCs into a particular musculoskeletal lineage. Among them, collagen type I has been most widely used because it is the major protein component of bone extracellular matrix and has been approved by the FDA for clinical use. Sefcik *et al.* prepared collagen type I scaffolds by electrospinning and induced osteogenesis from adipose-derived MSCs. The expression of a variety of osteogenic markers, including *Col I*, *ALP*, *Osteopontin*, *Osteonectin*, *Osteocalcin* and *Runx2*, were all upregulated in adipose-derived MSCs cultured on nanofiber scaffolds compared to 2D collagen coatings, demonstrating that the combination of a 3D environment and bioactivity of natural ECM molecules can be a potent inducer of differentiation [203]. Collagen type I-based 3D hydrogels have also been used to induce chondrogenic differentiation of MSCs, as chondroprogenitors reside in a collagen type I-rich environment prior to initiating chondrogenesis *in vivo* [204, 205].

Synthesizing scaffolds composed of multiple ECM molecules can further enhance stem cell differentiation. Cross-linking chondroitin sulfate C to collagen type II (CSC-COL II) was shown to be effective for inducing chondrogenic differentiation from MSCs. MSCs cultured *in vitro* in CSC-COL II scaffolds demonstrated upregulated chondrogenic-specific gene expression, while osteogenic-specific gene expression was suppressed. When scaffolds were transplanted and cultivated *in vivo*, the CSC-COL II scaffold demonstrated superior ability to repair cartilage lesions than did scaffolds composed only of collagen type II. Six months after transplantation, the MSCs in the CSC-COL II group showed higher deposition of collagen type II and aggrecan and lower gene expression of *Col I* at the defect sites, indicating the production of healthy articular cartilage tissue with reduced deposition of fibrous, scar-like tissue [206, 207].

Biomaterial scaffolds can also be used to attenuate stem cell differentiation by altering key structural features or mimicking mechanical properties of the native environment. To achieve this goal, synthetic biomaterials are also often utilized in tissue engineering applications due to their ease of modification and fabrication to unique specifications. This offers a degree of control over biomaterial and scaffold properties. To modulate the mechanical strength, swelling properties, or biological function of native ECM proteins or to improve the degradation properties and long-term performance of cellular constructs, natural polymers are often combined with synthetic polymers. Of the many synthetic polymers around today, the FDA has approved PCL, PLGA, poly(L-lactide-co- ϵ -caprolactone) (PLCL), poly(glycolic acid) (PGA), poly(lactic acid) (PLA), and polyethylene glycol (PEG) for MSC-based musculoskeletal tissue engineering applications. Cartilage tissue formed by MSCs on a chitosan-modified PLCL scaffold or hyaluronic acid-PLGA scaffold produced superior cartilage constructs compared to unmodified scaffolds [208, 209]. PCL has been shown to support adipose- and bone marrow-derived MSCs, and the biological efficacy of PCL nanofibers can be improved through several mechanisms [210, 211]. Liao *et al.* fabricated biomimetic electrospun PCL scaffolds by seeding chondrocytes on the scaffolds to deposit endogenously-produced ECM molecules native to articular cartilage. The resulting ECM-coated PCL scaffolds were then decellularized and subsequently seeded with MSCs. The presence of cartilaginous ECM cooperated with TGF β treatment to enhance MSC chondrogenesis, as evidenced by increased sGAG synthesis compared to unmodified scaffolds [212].

Composite scaffolds of PEG have been tested for use in cartilage regeneration, as well [213, 214]. By varying combinations of PEG, chondroitin sulfate (CS), matrix metalloproteinase-sensitive peptides (MMP-pep), and hyaluronic acid (HA), Nguyen *et al.* were able to engineer key features of the superficial, transitional, and deep zones of adult articular cartilage. For instance, a composite hydrogel of PEG:CS:MMP-pep hydrogels induced high levels of collagen type II and low levels of proteoglycan deposition, which resulted in a tissue of similar composition and compressive modulus to that of the superficial zone. PEG:CS hydrogels produced intermediate-levels of both collagen type II and proteoglycans, like the transitional zone, while PEG:HA hydrogels induced high proteoglycan and low collagen type II levels, leading to the production of a tissue with high compressive modulus, similar to the deep zone of the native tissue [215]. These results highlight the usefulness of different combinations of synthetic and natural bio-polymers to create unique niches that can stimulate a single marrow stem cell population to differentiate into chondrocytes similar to those found in the superficial, transitional, and deep zones of articular cartilage.

PLGA is one of the most popular biomaterials for cartilage regeneration. 3D scaffolds made of a PLGA-collagen hybrid have been successfully used to drive MSCs into cartilage-like tissue and generate mechanically functional cartilage grafts [216]. In one such study, a PLGA-collagen hybrid scaffold was synthesized that consisted of a central collagen sponge bound by a bi-layered PLGA mesh cup. The central collagen sponge supported cell adhesion and acquisition of round cell morphology, while the bi-layered PLGA mesh cup protected against cell leakage and provided mechanical support for the collagen sponge to maintain its shape during cell culture. The cell retention efficiency was 90.0% in the hybrid scaffolds, and MSCs readily differentiated and formed cartilage-like tissue, as evidenced by an upregulation in the expression of *Col II*, *ACAN*, and *Sox9*.

Utilizing collagen-glycosaminoglycan scaffolds as an analogue of the ECM, Murphy *et al.* determined the effect of scaffold stiffness and composition on MSC differentiation in the absence of differentiation supplements. The scaffolds with the lowest stiffness (0.5 kPa) facilitated a significant upregulation in *Sox9* expression, suggesting that MSCs are progressing toward the chondrogenic lineage in more compliant scaffolds. In contrast, the

greatest level of *Runx2* expression was found in the stiffest scaffolds (1.5 kPa), suggesting that MSCs are directed towards the osteogenic lineage in stiffer scaffolds. These results indicated that matrix stiffness has a significant influence on the fate of MSCs whereby the stiffest scaffolds directed MSCs toward the osteogenic lineage and the most compliant scaffolds directed MSCs toward the chondrogenic lineage. This also demonstrated the possibility of tailoring the intrinsic scaffold properties to control MSC differentiation into specific musculoskeletal lineages [217].

An issue that is pertinent in tissue engineering, especially in developing cartilage implants, is tissue integration with host tissue. MSC-derived cartilage constructs demonstrate poor ability to integrate with host cartilage upon implantation [218], thus limiting the healing potential. Several groups have undertaken the task of improving integration between the engineered and native tissues by generating biphasic osteochondral tissues. For example, Cheng *et al.* used an MSC-collagen microsphere-based approach to produce an osteochondral interface [206]. The osteochondral interface resembled the native tissue in terms of the presence of hypertrophic chondrocytes, calcium phosphate deposits, collagens type II and X, GAGs, and vertically-oriented collagen bundles. Similarly, Zhou *et al.* fabricated multilayer biomimetic scaffolds with an upper collagen layer and a lower collagen-hydroxyapatite (COL-HA) layer [209]. The upper collagen layer was more efficient in inducing MSC chondrogenic differentiation, while the COL-HA layer possessed superior osteogenic induction over either the collagen layer or pure HA.

4.3.1. Regulation of Stem Cell Differentiation by Mechanical Stimulation—

Given the important role that physical parameters, like matrix stiffness or dimensionality, can play in regulating stem cell differentiation and the fact that structural tissues perform their task in relatively harsh mechanical environments, it is not surprising that mechanical stimulation has been shown to be a potent inducer of stem cell differentiation [219]. Stem cells sense and respond to different mechanical stimuli through mechanotransduction, whereby activation of various cell surface mechanoreceptors and intracellular signaling proteins transfer the mechanical cues exerted on stem cells into a cascade of biochemical signals, which ultimately direct gene expression within the nucleus [220–222]. *In vitro*, bioreactors are used to provide mechanical stimulation to enhance differentiation. Bioreactors are enclosed systems capable of circulating bioactive molecules and vital nutrients in and cellular waste products out of the local microenvironment of tissue engineering products while providing compressive or tensile strains depending on the application. Indeed, these mechanical forces have been shown to enhance the differentiation of both pluripotent stem cells and MSCs [223–226]. This section will discuss the basics of mechanotransduction and how mechanical stimulation has been used to enhance stem cell differentiation into musculoskeletal lineages.

The initial step of mechanotransduction is the detection of mechanical stimuli, which primarily occurs through integrins and ion channels [227]. Integrins present on the plasma membrane physically connect the actin cytoskeleton of cells to ECM components and transmit mechanical stimulation across the plasma membrane. As the upstream molecules of mechanotransduction, integrins sense matrix stiffness and regulate the osteogenic phenotype of MSCs [228]. Upon integrin attachment to ECM at focal adhesion sites, focal adhesion kinase (FAK) is activated. FAK is a cytoplasmic tyrosine kinase and is a key integrator of intracellular mechanotransduction. It forms multi-protein complexes with intracellular signaling proteins to initiate downstream signaling events. Mechanical stimulation activates multiple signaling cascades, including MAPK, ERK1/2, JNK, and RhoA/ROCK, ultimately leading to the expression of lineage-specific genes. Some of the same pathways can be activated during mechanical stimulation for osteogenesis, tenogenesis, or other lineages, but affect different end targets depending on the presence of other differentiation inducers.

Taking MSCs as an example, RhoA/ROCK, cytoskeletal organization, and FAK compose a signaling assembly that integrate mechanical stretching and likewise drive mechanical stretch-induced tenogenic differentiation of MSCs [229]. Stretch-activated cation channels act as tensile strain sensors involved in the expression of the osteogenic transcription factor, Runx2, and biosynthesis of collagen type I and osteocalcin, during the strain-induced differentiation of MSCs [230]. Conversely, dynamic compression of MSC scaffolds for chondrogenesis has been shown to enhance MSC differentiation into chondrocyte-like cells in comparison to static culture and may better represent the *in vivo* environment seen by chondrocytes [231, 232].

Microarray analysis and pathway inhibition assays suggest that upregulation of downstream signaling targets of FAK, like JNK and ERK1/2, occurs in conjunction with elevated Wnt signaling [233]. Cyclic mechanical stretching induces the phosphorylation of FAK and upregulation of the expression and phosphorylation of Runx2, which subsequently increases ALP activity and mineralized matrix deposition [234]. Osteogenic differentiation of MSCs induced by compressive or fluid shear stress involves a dynamic cascade of responses through several signaling pathways such as MAPK, NO/cGMP/PKG and Ca²⁺ signaling pathway. As a result, the expression of osteogenic-specific genes, such as *ALP*, *Osteocalcin*, *Col I*, and *Osteopontin*, is upregulated, and the actin cytoskeleton is reorganized as MSCs differentiate into osteoblasts [235–238].

Huang *et al.* investigated the combined effects of mechanical stretching and ECM composition in driving MSCs into the osteogenic lineage [234]. To avoid the influence from osteogenic supplements, MSCs were cultured in basal medium and subjected to cyclic mechanical stretching on substrates coated with various ECM proteins, including collagen type I, vitronectin, fibronectin, and laminin. They found that all of the ECM proteins supported MSC differentiation into osteogenic phenotypes. Moreover, cyclic mechanical stretching activated the phosphorylation of FAK, upregulated the transcription and phosphorylation of Runx2, and subsequently increased ALP activity and mineralized matrix deposition. Among the ECM proteins tested, fibronectin and laminin exhibited greater effects in supporting stretch-induced osteogenic differentiation than did collagen type I and vitronectin.

5. CONCLUSION

Multiple factors regulate the self-renewal and differentiation of relevant stem cell types into musculoskeletal lineages, and elucidation of environmental cues directing appropriate cell activities has greatly advanced the field of tissue engineering. However, for *in vitro* tissue engineering products to become a clinical reality, studies investigating the combined effect of multiple environmental cues will need to be conducted. For example, studies investigating the role of GFs during a specific stage of the tissue engineering process are typically carried out under normoxic conditions, and it is entirely possible that the observed effects from these studies would not persist under hypoxic conditions, which are more physiologically relevant. More importantly, however, researchers will need to find conditions that can improve the resulting phenotype of differentiated musculoskeletal cell types. For chondrogenesis, we still need to determine how to reproducibly repress the hypertrophic and fibrocartilaginous characteristics of chondrocytes derived from MSCs, and for ESCs, we likely need to expand upon the three-step differentiation protocol from Oldershaw and colleagues to further enhance the chondrogenic differentiation program. For osteogenesis, MSCs appear quite adept at differentiating into osteoblasts *in vitro*, but the challenge will be engineering a vascularized tissue of physiologically relevant architecture. To this end, the most promising avenue may be exploiting the ability of hypertrophic chondrocytes to recapitulate endochondral ossification when implanted *in vivo*. For ESC

osteogenesis, much work is still needed directing their differentiation into the osteogenic lineage, but the early stages of differentiation will likely follow that of early attempts at chondrogenic differentiation. Nonetheless, much progress has been made in the last 5–10 years, and it is only a matter of time before *in vitro*-engineered musculoskeletal tissues become a clinical reality.

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