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Recent Advances in Biophysical Stimulation of MSC for Bone Regeneration

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Abstract

Objectives: The purpose of this work was to review the recent advances in biophysical stimulation of MSC for bone regeneration with particular relevance in the tissue engineering field. **Methods/Statistical Analysis:** The review process had three steps: First, by the use of databases available, the principal findings published related to the different types of biophysical stimulation applied to Mesenchymal Stem cells (MSC) for bone tissue regeneration were compiled. Second, the principal characteristics such as historical relevance, conditions of operation, signaling, and principal results were obtained from each study. And third, considering the above characteristics, a description of each study was realized. **Findings**: This review highlighted the following findings: a) The capacity of MSC for differentiating to multiple lineages have attracted attention in regenerative medicine applications; b) Biophysical stimulation is an alternative in order to promote the osteodifferentiation of MSC; c) During the process of application of this type of stimulation, the generation of biochemical signals which is related to the changes in the environment of the cell (i.e., cell attachment, proliferation, and differentiation) are generated; and d) Despite a large number of studies published in this area, these do not explain clearly the mechanisms related to the generation of these signaling produced by the biophysical effects (i.e., mechanical, electrical, and electromagnetic). Furthermore, in this review, a compilation of the last five years was done, which emphasize in the aspect historical, conditions of operation, and biochemical signaling generated of each type of biophysical stimulation of MSC for osteodifferentiation. **Application/ Improvements:** Biophysical stimulation causes multiple effects on the cell environment, producing changes in its morphology, proliferation, and differentiation. The above is important in the biophysical stimulation of MSC for bone regeneration..

Keywords: Biophysical Stimulation, Bone Regeneration, Osteodifferentiation, Stem Cells, Tissue Engineering

1. Introduction

Mesenchymal Stem Cells (MSC) have attracted attention in regenerative medicine applications due to their capacity to differentiate to osteocytes, chondrocytes, adipocytes, hepatocytes, and neurocyte[1]. At present, the mechanisms used in order to direct the differentiation towards specific lineages are biochemical and biophysical cues[2,3]. Growth factors and small molecules inhibitors are used in the biochemical stimulation of MSC to the bone regeneration. Although this route is effective and easy to apply, nowadays, biophysical cues are being used to stimulate the differentiation of MSC[4]. Many studies have demonstrated that this type of stimulation causes certain effects on stem

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cell attachment, proliferation, and differentiation, but a clear conclusion about of the mechanisms of action on cells or the biochemical signal generated by these effects are not well understood[5-7]. For this reason, the changes in cell biochemistry and biology produced by biophysical effects (i.e., interactions with extracellular matrix (ECM) substrate, neighboring cells, external forces, etc.) are an important aspect to highlight. Mechanotransduction allows knowing the cell response towards these physical signals[3,8]. The application of external forces (i.e., mechanical forces) on the cell is recognized by the cellular machinery that detects biochemical signals and changes in the environment. These forces play a critical role in controlling stem cell fate and lineage determination because affecting the structure of ECM. And these effects are associated with mechanically-driven changes in adhesive cues and paracrine signals that modify the cell shape[9]. In the mechanotrasduction of bone tissue regeneration, the osteocytes play a role as sensory cells, while osteoblast and osteoclast are generated cells of the process[10]. In this review, recent findings of the different types of biophysical stimulation such as tensile strain, pressure, ultrasound, shear stress, electrical, and electromagnetic fields that promote the osteodifferentiation of MSC are described and highlighted. (See Figure 1)

2. Biophysical Stimulation Types

2.1 Tensile Strain

Applied mechanical forces have been shown to induce stem cell differentiation in a lineage-specific manner[11]. In 1976, it was demonstrated that cyclic stretching can stimulate the synthesis of extracellular matrix components. In this experiment, arterial smooth muscle cells subjected to elongation and relaxation increased their rate of collagen, hyaluronate, and chondroitin 6-sulfate synthesis[12]. In others studies, researchers found that tensile strain causes changes in the orientation and morphology of cultured cells from the anterior cruciate ligament in rabbits[13]. Later, the fundamental mechanobiology principles related to the differentiation of MSCs into bone, cartilage, or fibrous tissue were established[14] endochondral ossification, and bone remodeling. It has been shown that all these processes are influenced strongly by the local tissue mechanical loading

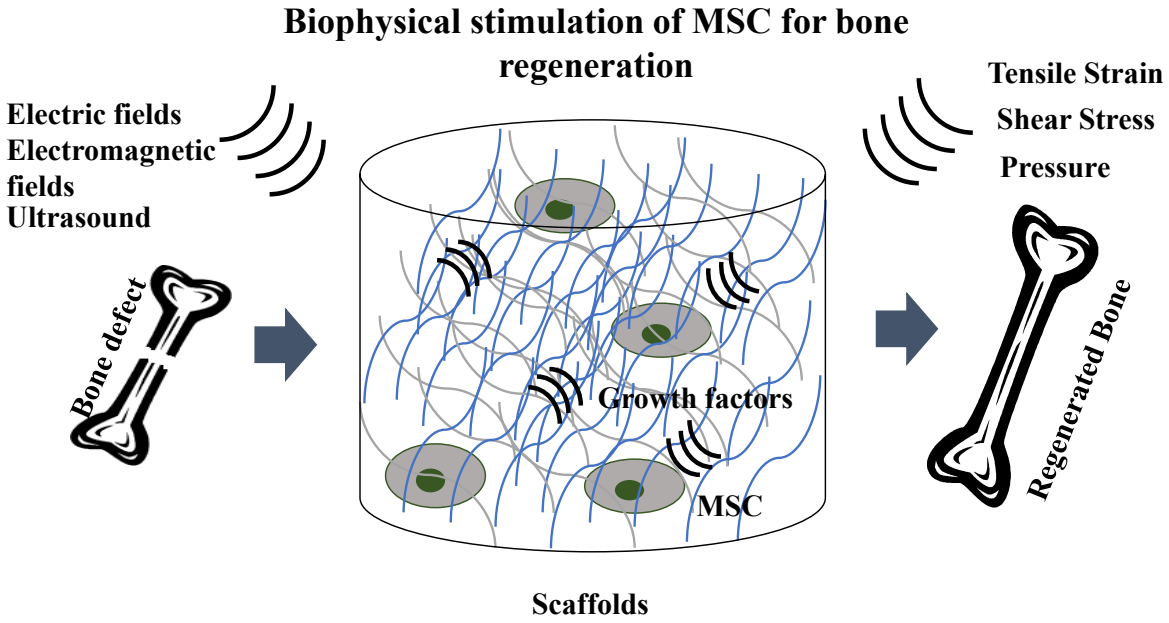

Figure 1. Biophysical stimulation of MSC for bone regeneration.

history. This article reviews some of the mechanobiologic principles that are thought to guide the differentiation of mesenchymal tissue into bone, cartilage, or fibrous tissue during the initial phase of regeneration. Cyclic motion and the associated shear stresses cause cell proliferation and the production of a large callus in the early phases of fracture healing. For intermittently imposed loading in the regenerating tissue: These principles predicted various conditions: (1) intramembranous bone formation at low stress and strain; (2) intramembranous ossification at low to moderate tensile strain and hydrostatic tensile stress; (3) chondrogenic differentiation with poor vascularity, in an osteogenic environment and exposed to an hydrostatic compressive stress; (4) fibrous tissue production at high tensile strain; and (5) fibrocartilage formation in the presence of tensile strain and hydrostatic compressive stress. Also, other researchers demonstrated that the intramembranous bone formation is activated by strain and hydrostatic pressure[15,16]. Lately, experimental studies have confirmed MSCs differentiation using mechanical strain[17-19].

Expression of the FOS family of transcription factors can be induced by mechanical loading, an effect crucial for bone remodeling and osteoblastic differentiation. Findings have demonstrated that cyclic stretch under elongation (2 to 8% elongation at 1Hz for 3 days) induce up-regulation of osteogenic transcription factors (i.e., RUNX2 and FosB) and biomarker (e.g., Type I collagen expression in MSC[20]. Similarly, human mesenchymal stem cells (hMSCs) are highly sensitive to mechanical stretching and this effect can promote the increase of the-gene expression levels of osteochondrogenic transcription factors (i.e., FOS, RUNX2, Sox9)[21]. Also, rat bone marrow mesenchymal stem cells (rMSCs) exposed to 1 Hz (2-8% elongation for 15-60 minutes) showed an increase in the proliferation and the expression of the c-Fos gene when stretching above 4%[22].

Bone morphogenetic proteins (BMPs) play an important role in the development of bone and cartilage[23]. Experimental studies have reported an increase in BMP-2 expression levels in hMSCs cultured in 3D scaffolds under uniaxial cyclic tensile strain (1 Hz and 0-12% strain) without osteogenic supplements[24]. Results showed that applied uniaxial tensile strains of 10% and 12% resulted in local strains up to 18.3% and 21.8%, respectively[25]. They also demonstrated that cyclic tensile strain can affect the expression of tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β), interleukin-6 (IL-6), and interleukin-8 (IL-8) cytokines, which are involved in bone resorption during osteogenic induction of hMSCs. It was also reported that cyclic tensile strain did not promote the expression of both IL-1β and TNF-α, but induced the expression of IL-8 that could lead to inhibition of bone resorption during osteogenesis[26]. Besides, researchers have studied the cellular mechanism of mechanotransduction in MSCs exposed to cyclic tensile mechanical strain[27]. The results of this study revealed that mechanical strain reduced the rate of MSC proliferation and the strain-induced synthesis of BMP-2 was reduced by inhibitors of the kinases, ERK, p38, and PI3 kinase. In the same direction, it was found that BMP-4 proteins are implicated in the commitment of MSCs toward adipocytes. It has reported that stretching of cells may inhibit BMP-4-induced adipogenesis[28]. In these experiments, the authors applied cyclic equibiaxial elongation (10% strain at 0.25 Hz) for 120 min/day for four days. The results demonstrated that: (1) the cell stretching suppressed BMP-4 induction of C3H10T1/2 MSC adipogenesis, (2) Both BMP-4-triggered SMAD and p38 phosphorylation were not affected by cell stretching, (3) stretching induced significant ERK1/2 phosphorylation, and (4) blocking of ERK deteriorated stretch suppression of BMP-4-induced MSC adipogenesis. The first study on the effect of cyclic tensile strain on osteodifferentiation of human adipose-derived adult stem (hASCs) was reported recently[29]. In this study, cells were subjected to 10% uniaxial cyclic tensile strain (1 Hz for 4 hours per day for up to two weeks) with cycles of 1 s tensile strain followed by a 10 s rest. The results indicated that osteodifferentiation induced by cyclic tensile strain was significantly higher

than unstrained controls. Later, another study[30] indicated that uniaxial cyclic tensile strain can promote osteogenesis in hMSC and hASCs. In this experiment, the cells were seeded in 3D type I collagen constructs and were exposed to 10% cyclic tensile strain. The results indicate that tensile strain induced expression of genes associated with migration, proliferation, musculoskeletal and cardiovascular tissue development. And, an enhanced expression of osteogenic and angiogenic factors was observed.

It has been demonstrated that tensile strain induces differentiation in human dental pulp cells (hDPCs) and human periodontal ligament stem cells (hPDLSCs). Researchers have studied the behavior of hDPCs exposed to cyclic tensile strain (3-15% elongation) at 6 cycles/min for various periods of time. The results of this study showed that the mRNA levels for differentiation markers osteopontin (OPN), bone sialoprotein (BSP), dentin sialophosphoprotein (DSPP), and dentin matrix-protein-1 (DMP-1) were upregulated after 24 h exposure to 12% mechanical stress[31]. However, another group reported that application of extrusive forces had no influence on human pulp tissue[32]. By contrast, it was found that hPDLSCs have a sensitive response to mechanical stimulus. In this study, osteogenic transcription factors were examined under cyclic tensile strain (3,000 μstrain) with different loading durations[33]. The results showed that mRNA levels and protein expression of osteogenic transcription factors (i.e., Satb2 and RUNX2) increased significantly after 3 hours exposure to tensile strain.

Studies have indicated that physical properties of substrates where MSCs are grown could affect their differentiation. First, it was demonstrated that the application of dynamic stretch can overcome the inhibition of spreading due to the lack of matrix stiffness surrounding the cell[34]. Second, it was reported that the compressive elasticity of a 3D nanofiber matrix stimulates MSCs differentiation to vascular cells. This study was performed at a strain rate of 0.50mm m^{-1} s^{-1} up to a maximum strain of 15 % in the range 2-15 kPa and the results indicated that MSC penetrated into the graft forming a 3D matrix[35]. Third, it has used a polylactide-co-glycolide (PLGA) nanofiber-based scaffold to evaluate the synergistic effect of both chemical and mechanical stimulation on the fibroblastic differentiation of hMSCs. The results indicated that the application of both stimuli promoted ligament regeneration. However, the authors argue that a good scaffold alignment and optimized mechanical stimulation are sufficient to drive MSC differentiation, without the need for additional chemical stimuli[36,37].

Tensile strain and chemical stimulation promote osteogenic differentiation of hASCs. Researchers studied the effects of chemical and mechanical stimulation (10% cyclic tensile strain) on the response of hASC[38]. The results showed increased calcium content and upregulation of two crucial factors in bone regeneration: (1) Proinflammatory cytokine regulators IL1RN and SOCS3; (2) Angiogenic inductors FGF2, MMP2, and VEGF A. Also, other study demonstrated that both chemical and mechanical stimulation can improve osteogenic and chondrogenic differentiation in hASCs[39]. Tensile strain stimulates MSCs differentiation toward cardiac, neural and musculoskeletal tissues. It has reported the generation of tissue-engineered cardiac grafts using MSCs[40]. In this study, effects of strain intensity on cardiac-related gene expressions of rBMSCs were evaluated by cultivating them on flexible membranes subjected to 24 h of uniaxial strain (1 Hz) with different membrane elongations (i.e., 5%, 10%, 15%, and 20%). The results showed high mRNA levels of GATA-4, b-MHC, NKx2.5, and MEF2c. Afterwards, they compared the effects of cyclic strain and fluid shear stress (10 dyn/cm^2) on the cells, revealing an enhanced cardiomyogenic differentiation under cyclic strain. Similarly, others researchers analyzed the effect of mechanical forces on the development and maintenance of musculoskeletal tissues[41]. They hypothesized that mechanical loading could modulate the transcriptional behavior of MSCs, stimulate the deposition of ECM, and enhance functional properties of constructs. For this purpose, they used a nanostructured poly (e-caprolactone) scaffold and exposed it to cyclic loading at 6% strain with

Table 1. Biophysical stimulation of MSC using tensile strain

Tensile strain	Differentiation	Study Type	Comments	Ref
Cyclic stretch of 1 Hz with 2% or 8% elongation	Osteogenesis	In vitro	Cyclic stretch under elongation induced FosB expression and the up-regulation of osteoblast genes.	In[20]
Cyclic uniaxial tensile strain (3000 μstrain , 1000 cycles at 1 Hz)	Osteogenesis Chondrogenesis	In vitro	Application of mechanical strain promoted early chondrogenic and osteogenic differentiation in vitro.	In [21]
Cyclic uniaxial tensile strain (2000 μstrains at 0.5 Hz)	Osteogenesis	In vitro	Mechanical loading induced osteogenic differentiation.	In [44]
Cyclic equiaxial stretch (2–8% strain at 1 Hz)	----------	In vitro	Equiaxial stretch generated proliferation of rMSCs.	In[22]
Uniaxial cyclic tensile strain (10% or 12% s at 1 Hz)	Osteogenesis	In vitro	Cyclic tensile strain inhibited expression of both IL-1b and TNF-a, but induced the expression of IL-8 which could lead to inhibition of bone resorption during osteogenesis.	In [26]
Uniaxial cyclic stretch (0–25%, strain at range of 1–3 Hz)	Smooth muscle cells	In vitro	Cyclic stretch promoted differentiation of hMSCs to smooth muscle cells without addition of growth factors.	In [223]
Uniaxial cyclic tensile strain (10% at 1 Hz)	Osteogenesis	In vitro	Cyclic tensile strain accelerated hASC osteodifferentiation and increased calcium content.	In [29]
Cyclic tensile strain (2.5% at 0.17 Hz)	Osteogenesis	In vitro	Cyclic tensile strain modulated osteogenic differentiation of MSCs.	In [27]

Table 2 Continued

Cyclic tensile strain (3-15% elongation at 6 cycles/min)	Osteogenesis	In vitro	Cyclic tensile strain induced odontoblastic differentiation via Nrf2-regulated HO-1 expression.	In [31]
Cyclic tensile strain (6% at 1 Hz)	Fibro-chondrogenesis	In vitro	Cyclic loading stimulated the expression of matrix and matrix-associated genes.	In [41]
Equibiaxial and uniaxial cyclic tensile stretch (10% at 1Hz)	----------	In vitro	Equibiaxial stretch promoted spreading of rounded cells on soft substrates.	In [34]
Uniaxial cyclic tensile strain (10% at 1 Hz)	Osteogenesis Angiogenesis	In vitro	Cyclic tensile strain enhanced promoted angiogenesis and osteogenic differentiation of hMSC from osteoporotic donors.	In [30]
Uniaxial Cyclic tensile strain (3,000 μstrain at 0.5 Hz)	Osteogenesis	In vitro	Cyclic tensile strain stimulated osteogenic differentiation of human periodontal ligament stem cells.	In [33]
Cyclic uniaxial tensile strain (5-20%, elongation at 0.5- 2 Hz)	Chondrogenesis	In vitro	Cyclic strain is a better stimulant of rBMSC differentiation toward the cardiomyocyte lineage than shear stress.	In [40]
Uniaxial cyclic tensile strain (3% and 1 Hz)	----------	In vitro	The genetic expression of MSCs under cyclic tensile strain is different in both 2D and 3D system.	In [43]
Cyclic tensile stretch (10% strain, 0.25 Hz.	Osteogenesis	In vitro	Cyclic tensile strain suppressed adipogenic differentiation of MSCs during BMP4 treatment.	In [28]
Cyclic tensile loading (0.5, 2 and 3.5%) at (0.5, 1 and 1.5 Hz)	Neurogenesis Osteogenesis	In vitro	Cyclic tensile loading at low amplitude and frequency promoted neurogenic differentiation in MSCs.	In [42]

Table 1 Continued

Cyclic tensile stretch (0 to 4000 μstrainsat 0 and 2Hz)	Chondrogenesis	In vitro	Cyclic tensile strain induced PTHrP expression in postnatal growth plate prehypertrophic and hypertrophic chondrocytes.	In [224]
Cyclic tensile strain (1% at 1 Hz)	Fibroblast cells	In vitro	Mechanical and chemical stimulation promoted fibroblastic induction of hMSCs in nanofiber scaffold.	In [225]

a frequency of 1 Hz for 3 hours, stimulating the expression of type I collagen, Type II collagen, fibronectin, and lysyl oxidase. Also, it was demonstrated that different cyclic tensile loadings produce different microfilament rearrangement and promote neuron-like differentiation of hMSCs[42].

Experimental studies have confirmed that gene expression of MSCs under cyclic tensile strain is different in 2D and 3D culture systems[43]. In this work, gene expression of hMSCs was studied under cyclic tensile strain (3% and 1 Hz) in monolayer culture or encapsulated in a peptide hydrogel. In 2D culture, CCNL2, BAHCC1, and WDR61 were significantly downregulated. However, after 24 h strain, BAHCC1 was significantly upregulated. In contrast, in 3D culture, the BAHCC1 gene was not expressed. The authors argued that the mechanical cues affect cells differently in 3D cultures. Finally, other important advances in this field have been the study of bone regeneration during distraction osteogenesis, which involves cellular and complex molecular processes[44]. Important results have indicated that when rBMSCs were exposed to cyclic uniaxial tensile strain (0.5 Hz, 2000 μstrains) for 40 minutes, the upregulation in the expression of osteogenic markers (i.e., ALP, Cbfa1/RUNX2, and Ets-1) was generated. Table 1 shows some recent advances in biophysical stimulation of MSC generated by tensile strain.

2.2 Hydrostatic Pressure and Compression

Mechanical stimulation is considered as the fourth strategy in bone tissue engineering along with the use of cells, scaffolds and growth factors[45]. It activates MSCs function in different manners[46] causing changes in morphology, proliferation, and differentiation[47]. In the last fifteen years, researchers have confirmed that the fluid mechanical forces activate signaling transduction in osteoblasts[48]. Researchers found a correlation between characteristic parameters of cyclic pressure and cellular functions in osteoblasts and osteoclasts[49-51]. A first study demonstrated the dependence of osteoblast proliferation on the duration of the applied cyclic pressure stimulus. They used osteoblasts, fibroblasts, and endothelial cells, which were exposed to a range of pressure of 10-40 kPa at 1 or 0.25 Hz frequency for one hour each day for five days. The results showed that osteoblast proliferation decreased at 1Hz frequency while decreasing mRNA expression of ALP after five days. Also, under this condition fibroblasts showed an increase in cell proliferation, while endothelial cells were not affected. A second study showed evidence of a correlation between mechanical loadings on osteoclast formation using the same range of pressure for 1 Hz for the same time frame to stimulate progenitor bone marrow cells. These results display a decrease of osteoclast cell formation and lower bone resorption under cyclic pres-

sure, which was supported by down-regulation of mRNA expression for IL-1-α, IL-1β, and TNF-α. In a third study, they used the same conditions of cyclic pressure for one hour daily for different time periods up to 19 days to validate the effects on certain functions of osteoblasts relevant to osteogenesis. The results showed that osteocalcin (OCN) mRNA expression did not increase while Type I collagen mRNA expression increased only when cells were exposed for 19 consecutive days. In addition, they observed that the amount of acid-soluble collagen and calcium content increased after 19 days of exposure.

Investigations have confirmed that mechanical loading plays an important role in the differentiation, maturation, and senescence of hMSCs[52]. An important finding in this field has reported the study of the gene expression patterns of stimulated cells under both dynamic tension and dynamic compression at 0.1 Hz frequency[53]. The results indicated that dynamic tension up-regulated genes associated with bone formation and inhibited chondrogenesis, while dynamic compression regulated chondrocyte proliferation and upregulated genes associated with chondrogenesis. Similarly, other studies have demonstrated that chondrogenic differentiation of hMSCs can be modulated by frequency and amplitude of dynamic compression and shear stress[54-57]. Additionally, it has shown evidence of chondrogenesis in hMSCs using fibrin scaffolds under cyclic compression[58]. Also, it has compared the effect of dynamic hydraulic compression (DHC) stimulation on hASCs and hMSCs[59]. The results of this study indicated that DHC (1 psi at 1Hz frequency) increased osteogenic gene expression in both types of cells with hMSCs being more susceptible. Besides, it has investigated the role of estrogen and its receptors in the mechanobiological effects in bone mesenchymal stem cells (BMSCs)[60]. The results of this study demonstrated that both mechanical compression and estrogens stimulated the proliferation and differentiation of BMSCs via F-actin. The application of pressure on the system caused alterations in the cytoskeleton via the orientation and alignment of fibers, forming thick fibrous structures. First, it was reported that static (23 kPa) and dynamic (10–36 kPa and at 0.25 Hz frequency) hydraulic pressure stimulated osteodifferentiation of MSCs[61,62]. In this study was found that both types of pressure promoted the expression osteogenesis-related factors of MSCs and also induced osteoclastogenesis. Second, it has investigated the effects of the low-intensity intermittent negative pressure effects on the proliferation and differentiation of hMSCs[63]. The results of this study indicated that under these conditions, proliferation was inhibited while inducing osteogenic differentiation. Researchers has demonstrated that hydrostatic pressure (HP) affects cell response during co-culture[64-67]. In a first study, it was monitored the degree of differentiation of MSCs into nucleus pulpous (NP)-like cells via mechanical stimulation. It was used a 3D co-culture system with 0.2 MPa of applied pressure with intervals of 2 min for pressurizing and 15 minutes for resting. The results showed that MSCs did not differentiate under mechanical stimulation when cultured alone, but tended to differentiate immediately when NP cells were nearby. In a second study, it was monitored the migration of MSCs with or without neighboring endothelial cell under the effects of intermittent HP (100 and 200 mm Hg, 5 minutes pressure and 10 min rest). The results displayed that HP only stimulated the migration of MSCs when endothelial cells were not nearby. In a third study, chondrogenic differentiation of MSCs in 3D co-culture under mechanical stimulation was analyzed. In this study, MSCs were co-cultured with primary chondrocytes into separate alginate beads divided by a membrane. Afterwards, they were stimulated with different conditions of intermittent hydrostatic pressure (IHP). The results indicated that the stimulation using higher magnitudes IHP promoted the proliferation and differentiation of co-cultured MSCs even without biochemical agents. Finally, in a recent study, it was found that both dynamic compression and co-culture with nucleus pulpous cells stimulated the proliferation and chondrogenic differentiation of hASCs.

Hydrostatic pressure can play a key role in regulating the chondrogenic differentiation of MSCs. It has found the following findings:

- The long-term exposure to HP stimulates the formation of cartilaginous tissue, but this effect varies depending on the donor[68].

- Chondrogenic differentiation of hASCs in collagen scaffolds under cyclic HP stimulation[69].

- Chondrogenic differentiation of hASCs using intermittent HP and biochemical stimulation[70]. The interaction of both biochemical and biophysical stimulation might regulate chondrogenesis of joint tissue-derived stem cells[71]. In this study, the cells were stimulated with different concentrations of TGF-β3 and 10 MPa of cyclic HP. The results showed that physical stimulation with low concentrations of TGF-β3 acts synergistically to increase chondrogenesis.

- A comparative study with hASCs and hMSCs to examine cell viability in 3D agarose constructs without soluble growth factors under the application of cyclic HP[72]. In this study, the cells were exposed to 7.5 MPa at a frequency of 1 Hz for up to 21 days. The results showed that at day 7 both cell types initiated chondrogenic differentiation, but at day 14 a decrease in cell metabolic activity was presented by both cells indicating that perhaps the agarose hydrogel was not an appropriate 3D structure for chondrogenic differentiation of hASCs in long-term culture.

- Dynamic HP acts to maintain a chondrogenic phenotype in cartilaginous grafts engineered[73]. In this study, it was monitored the phenotypic stability of chondrogenic differentiation of multipotent stromal cells, and infrapatellar fat pad derived multipotent stromal cells (FPSCs) seeded on agarose hydrogels subject to 10 MPa of cyclic HP (1Hz). The results displayed an increase in the accumulation of reduced sulfated glycosaminoglycan content in both cell types, an increase of the collagen content in multipotent stromal cells but not in FPSCs, a decrease in calcium deposition within multipotent stromal cells seeded constructs maintained in chondrogenic medium, and no evidence of calcium deposition on FPSCs.

- A study about of the biochemical properties and gene expression of MSCs on hybrid scaffold exposed to cyclic HP (5 MPa, 0.5 Hz)[74]. Researchers demonstrated that hydrostatic pressure increased type II collagen mRNA levels but no aggrecan and Sox9 levels. These results differed from others researchers who reported an increase in mRNA expression of aggrecan, type II collagen, and Sox9 in hBMSCs under HP. In addition to HP stimulation, also is crucial a favorable environment for the MSCs differentiation[75].

- Chondrogenic differentiation of MSCs is regulated by matrix stiffness, integrin binding and cytoskeletal organization, necessary for mechanotransduction of hydrostatic pressure[76].

- Cyclical uniaxial compressive stress affects the morphology, cytoskeleton rearrangement, and the production of proteoglycans by the expression of osteogenic markers (i.e., RUNX2 and ALP activity) via phosphorylation of myosin light chain II (MLCII)[77].

The use of three-dimensional (3D) bone constructs and hydrostatic pressure stimulation have proven to be a good option to promote osteogenic differentiation of MSCs. Researchers demonstrated that the combined effects of biochemical and biophysical stimulation encourage osteogenic differentiation of hMSCs in scaffolds[78]. In this study, hMSCs were cultured on collagen

Table 2. Biophysical stimulation of MSC using hydrostatic pressure and compression

Conditions	Differentiation	Study Type	Comments	Ref
Cyclic Pressure (10 - 40) kPa at 0.25 or 1.0 Hz.	----------	In vitro	Duration applied cyclic pressure affected osteoblast proliferation.	In[49]
Cyclic Pressure (10 - 40) kPa at 0.25 or 1.0 Hz.	----------	In vitro	Duration applied cyclic pressure stimulus affected osteoclast formation and bone resorption activity.	In[50]
Cyclic Pressure (10 - 40) kPa at 0.25 or 1.0 Hz.	----------	In vitro	Osteoblast functions related to new bone formation were promoted by cyclic pressure stimulus.	In[51]
Compression or tension under displacement controlled sinusoidal dynamic loading at 0.1 Hz	Osteogenesis Chondrogenesis	In vitro	Dynamic tension up-regulated genes associated with bone formation and inhibited chondrogenesis and dynamic compression regulated chondrocyte proliferation and upregulated genes associated with chondrogenesis.	In[53]
Intermittent Hydrostatic Pressure at 0.2 MPa	Chondrogenesis	In vitro	Hydrostatic pressure stimulated chondrogenic differentiation of MSCs co-culturing with NP cells.	In[64]
Intermittent Hydrostatic Pressure(100 and 200 mm Hg)	----------	In vitro	Hydrostatic pressure stimulated the migration of MSCs in the absence of endothelialcells neighboring.	In[65]
Static Pressure (23 kPa) or Dynamic Pressure (10–36 kPa at 0.25 Hz)	Osteogenesis	In vitro	Static and dynamic pressure promoted the expression osteogenesis-related factors of MSCs during the initial process of osteoblastic differentiation.	In[61]
Static Pressure (23 kPa) or Dynamic Pressure (10–36 kPa at 0.25 Hz)	Osteogenesis	In vitro	Static and dynamic pressure promoted osteoclastogenesis with the up-regulation of RANK/OPG ratio during the initial process of osteoblastic differentiation	In[62]
Cyclic Hydrostatic Pressure (300 – 375 kPa at 0.5 Hz).	Osteogenesis	In vitro	Biophysical and Biochemical stimulation promoted osteogenic differentiation of hMSCs.	In[78]

Table 2 Continued

Pressure of -50 kPa at frequency of 2/d,	Osteogenesis	In vitro	Low-intensity intermittent negative pressure inhibited the proliferation of cells but induced osteogenic differentiation.	In [66]
Cyclic Hydrostatic Pressure (10 MPa of at 1 Hz)	Chondrogenesis	In vitro	Application of long-term hydrostatic pressure stimulated the formation of cartilaginous tissues but the effects change depending donor.	In [68]
Cyclic Hydrostatic Pressure (10 MPa at 1Hz)	Chondrogenesis	In vitro	The application of hydrostatic pressure with low concentrations of TGF-β3 acted synergistically to increase chondrogenesis in MSCs.	In [71]

and/or chondroitin sulfate coated polycaprolactone-co-lactide substrates under cyclic HP stimulation (300 kPa and 375 kPa) at 0.5 Hz. The results showed that osteogenic differentiation of hMSCs was promoted by both chondroitin sulfate and cyclic HP. At the same time, it was dependent on stimulation time. In another study also it was demonstrated that both hydroxyapatite scaffolds and cyclic hydrostatic pressure enhance the cellular viability, stimulate osteogenic differentiation, and period of maturation, but at the same time, decrease proliferation and self-renewal of MSCs. Similarly, it has reported chondrogenic differentiation of MSCs in hydrogels (i.e., agarose or fibrin) with the presence of growth factors under HP stimulation[79]. The results of this study indicated that agarose hydrogels better-supported chondrogenesis than fibrin hydrogels, and the application of HP increased sulfated glycosaminoglycan's synthesis in fibrin hydrogels, but not in agarose hydrogels. Also, HP did not stimulate the synthesis of collagen in either fibrin or agarose predicting that both HP stimulation and scaffold material are essential factors in the cartilage regeneration and maintenance of a chondrogenic phenotype. Recent studies have demonstrated that MSC seeded on substrates based on polycaprolactone (PCL) exposed to the combination of intermittent hydrostatic pressure (270 kPa, 1Hz for 1minute daily for 21 days) and osteogenic medium of substrates of PCL nanofibers, stimulated the production of osteogenic markers such as Collagen type I, ALP activity and RUNX2[80]. Table 2 shows recent findings that highlight osteodifferentiation of MSC using hydrostatic pressure and compression.

2.3 Ultrasound

Ultrasound is an oscillating sound pressure wave that produces local changes of the medium's density and pressure, and exerts both thermal and nonthermal effects on liquids and in soft tissues[81-83]. Low-intensity ultrasound is considered a nonthermal technique that decreases tissue heating and cavitation phenomena. It also involves acoustic streaming, acoustic cavitation, and acoustic microstreaming, which increase blood flow, stimulate the cell activity, disturb the membrane permeability, and activates the second messengers's system[81,83-85]. The effects produced by the nonthermal technique are divide into two categories(put reference 86): inertial cavitational(higher acoustic pressures) and....is in the range between 6 and 8 W/m2. These biophysical effects on the cellular plasma membrane and cytoskeleton stimulate the production of

growth factors, osteogenic differentiation, and ECM production[87]. Also, ultrasound transfers mechanical energy into tissues increasing the mechanical strength of the callus formed after bone healing, nitric oxide production, activation of transcription factors (e.g., hypoxia-inducible factor-1a). These effects induce the expression of vascular endothelial grow factor (VEGF) in osteoblasts and reduce the time to bone union[88,89]. Similarly, mathematical models have predicted that the cellular response to ultrasound depends on both frequency and specific cell properties[90]. However, the mechanisms by which ultrasound can interact with cells and/or their microenvironments during fracture healing are not clear. In the early 1900s, Low Intensity Pulsed Ultrasonic (LIPUS), a longitudinal wave with regions of rarefactions and compressions began to be used to treat fractures with 1 MHz sine waves repeating at 1 kHz, with an average intensity of 30 mW/cm^2 for 200 μs giving a 20% duty cycle, which were applied for 20 minutes per day[82]. Later, a commercial LIPUS device for fracture healing and treatment of nonunion was designated by Exogen (Smith & nephew, Inc., London, UK) and was approved by the FDA in 1994. Nowadays, this technique is being used as a mechanism to promote osteogenic and chondrogenic differentiation of MSCs[91]. This technique has been used in wide range of studies including complex tibial fractures[92-94], anterior cruciate ligaments[95], osteoporotic fractures[96], and osteonecrosis of femoral heads[97], bilateral midshaft femur fractures[98], osteoradionecrosis[83], reconstruction of patella-patellar tendons[99], dental tissue repair[87], and tibial distraction osteogenesis[100]. Nonetheless, studies have demonstrated that therapeutic ultrasound has no significant effect in severe articular cartilage injuries[89].

In the last years, researchers have reported chondrogenic differentiation of mesenchymal stem cells (MSCs) by low-intensity ultrasound (LIUS) stimulation. Some important studies are described to following.

- Chondrogenic differentiation of MSCs without the presence of transforming growth factor-beta (TGF-β), a critical factor for initiation of chondrogenic differentiation[101]. In this study, the effect of LIUS (1MHZ and 200 mW/cm^2) on rMSCs in a 3D alginate culture increased the expression of chondrogenic markers (i.e., type II collagen, aggrecan, and Sox-9). Later, it was foundthat LIUS stimulation inhibited apoptosis, improved cell viability and, increased chondrogenic differentiation of MSCs[102]. In this study, hMSCs were cultured in 3D alginate scaffolds in the presence of growth factors (i.e., TGF-β1) with/without LIUS (1MHz and mW/cm^2). The results displayed that the LIUS-stimulated cells showed balanced expression of apoptosis-related genes (i.e., p53 and bax) and antiapoptotic proteins (i.e., bc1-2, and PCNA), and enhanced the expression of chondrogenic markers (i.e., Sox-9, aggrecan, and type II collagen).

- LIUS stimulation increased the collagen and glycosaminoglycan content in vivo without the presence of chondrogenic growth factors[103]. In this study, MSCs cultured in polyglycolic acid (PGA) scaffolds were implanted in the back of nude mice and stimulated with ultrasound (0.8 MHz and 200 mW/cm^2). In contrast, in another study, it was reported enhanced chondrogenic differentiation of hMSCs in pellets cultured under both TGF-β1 treatment and LIUS stimulation[104].

- LIUS stimulation (1 MHz and 100 mW/cm^2) promotes cell adhesion and improves the colony-forming capacity of MSCs during the early cell attachment stage of primary cultures[105]. In this study, the stimulated cells and the control presented the following characteristics: (1) no changes in size distribution of colonies; (2) no changes in in the overall expression patterns of cell surface antigens (i.e., CD29, CD90, and CD106, and CD45); and (3) same differentiation capacity for three different cell lineages (i.e., osteogenic, adipogenic, and chondrogenic). Also, LIUS stimulation could induce expression of cell adhesion molecules (i.e., integrin α5, integrin β1,

Table 3. Biophysical stimulation of MSC using ultrasound

Conditions	Differentiation	Study Type	Results	Ref
1MHZ and 200 mW/cm^2 (LIUS)	Chondrogenesis	In vitro	LIUS stimulated chondrogenic differentiation in MSCs cultured on alginate beads without TGF-ß treatment.	In[101]
0.8 MHz and 200 mW/cm^2 (LIUS)	Chondrogenesis	In vivo	LIUS had great potential in stimulating the chondrogenic differentiation of MSCs in vivo without using chondrogenic growth factors.	In[103]
1MHz and 200 mW/cm^2 (LIUS)	Chondrogenesis	In vitro	LIUS inhibited apoptosis of MSCs and enhanced theirs viability during chondrogenic differentiation.	In[102]
1MHz and 100 mW/cm^2 (LIUS)	Osteogenesis Adipogenesis Chondrogenesis	In vitro	LIUS activated cell adhesion and increased the colony-forming ability of MSCs during the early stage of primary culture, without affecting their phenotypes and multipotency.	In[105]
1MHz and 200 mW/cm^2 (LIUS)	Chondrogenesis	In vitro	LIUS enhanced chondrogenesis of the MSCs cultured in fibrin-Hyaluronic Acid hydrogels.	In[226]
2, 15 and 30 mW/ cm^2 (LIPUS)	Osteogenesis	In vitro	LIPUS intensities lower than those currently used clinically showed a positive effect on osteogenic differentiation of MSCs.	In [106]
1.5 MHz, 30 mW/cm^2(LIPUS)	Osteogenesis	In vivo	LIPUS induced the homing of circulating osteogenic progenitor to the fracture site for possible contribution to new bone formation.	In[107]
(1, 100, and 1000 Hz) LIPUS	Osteogenesis	In vitro	LIPUS accelerated osteogenic differentiation of hASCs based on amount of calcium accretion normalized by total DNA.	In[91]
LIPUS / microgravity	Osteogenesis	In vitro	LIPUS treated SMG cultures had higher collagen content in ECM and more matrix calcification	In [108]

Table 3 Continued

1 MHz, 200 mW/ cm^2 (LIPUS)	Osteogenesis	In vitro	The synergistic effect of LIPUS and RGD promoted proliferation and differentiation of MSCs.	In [109]
Ultrasonic Bioreactor (5.0 MHz, 2.5 Vpp)	Chondrogenesis	In vitro	Ultrasound and TGF-ß treatment promoted chondrogenesis of MSCs seeded on polymeric scaffolds that limit cell-to-cell contact.	In [110]
1 MHz, 50 mW/ cm^2, duty cycles at 20 and 50 % (LIPUS)	Osteogenesis	In vitro	LIPUSenhanced cell viability and osteogenic differentiation.	In[111]
1.5 MHz, 30, 60, and 90 mW/cm^2 (LIPUS)	Osteogenesis	In vitro	LIPUS stimulation facilitated osteogenic differentiation associated with activation of integrin β1- and upregulation of RUNX2 expression.	In [112]

paxillin, and fibronectin) and enhance focal adhesion via phosphorylation of focal adhesion kinase (FAK).

In the last years, various studies have been reported related to osteogenic and chondrogenic differentiation of MSCs using LIPUS stimulation. It has found the following studies:

- The effects on rBMSC at early, middle, and late stages of osteogenic differentiation caused by LIPUS with lower intensities used clinically (2, 15, and 30 mW/ cm^2)[106]. The results showed modulation of the ERK1/2 and p38 pathways, with the highest increase of mineralization at 2 mW/cm^2.

- New bone formation in femoral fractures in mouse using LIPUS stimulation[107]. In this study, it was reported accelerated fracture healing of transverse femoral fractures in mouse by LIPUS stimulation. Also, it was found that both local and circulating osteogenic progenitors promoted new bone formation.

- The effects produced by LIPUS stimulation on hASCs and hMSCs at different pulse repetition frequencies (PRF) (i.e. 1, 100, and 1000 Hz)[91]. The results showed osteogenic differentiation in both cell types at different PRF, obtaining the highest amount of calcium per DNA at 1 kHz.

- The effects of LIPUS stimulation on cell proliferation and osteogenic differentiation of hASCs under simulated microgravity[108]. The results showed that LIPUS stimulation increased ALP activity and the expression of osteogenic genes (i.e., ALP, OSX, RANKL, and RUNX2), and reduce the expression of OPG. It was also observed under these conditions the restoration of ALP activity, increased OSX, RUNX2, and RANKL expression,

and an increase in the production of collagen and calcium.

- The synergistic effect of LIPUS stimulation and RGD-grafted oxidized sodium alginate/N-succinyl chitosan (RGD–OSA/NSC) hydrogels, which enhanced cell proliferation and osteogenic differentiation of hMSCs[109]. In this study, it was suggested that cell differentiation resulted from an increase in cell membrane permeability, signal transduction, and improvement ofthe interaction between cytokines and RGD.

- Cell proliferation and chondrogenic differentiation of hMSCs in 3D scaffolds by ultrasonic stimulation (5MHz and 2.5 Vpp) and the presence of TGF-β3[110].

The osteogenic differentiation of dental stem cells by LIPUS stimulation has also been shown. Investigations in this field have reported that a change in duty cycle can influence migration and osteogenic differentiation of human alveolar bone-derived mesenchymal stem cells (hABMSCs)[111]. Similarly, it has demonstrated that LIPUS can promote osteogenic differentiation of hPDLCs[112]. Finally, it has reported osteodifferentiation by acoustic stimulation of MSC inabsence of fetal bovine serum[113]. Table 3 shows some important studies that demonstrate osteogenic and chondrogenic differentiation of MSC using ultrasound.

2.4 Shear Stress

Applied external forces can affect the shape and fate of stem cells[114]. In the last few years, theoretical studies have reported that fluid shear regulates MSCs differentiation by affecting the transport of bioactive factors, cell deformation and cytoskeletal strain[115]. A mathematical model based on the theory that octahedral shear strain and interstitial fluid flow was designed to estimate how mechanical stimulation affects tissue differentiation towards cartilage[116]. Data from the healing of a transverse osteotomy was found consistent and supported by this model, demonstrating that MSC differentiation is influenced by the distribution of these two components[115]. Other researchers published results showing MSC differentiation under specific tensile strains and fluid perfusion flows. They determined that low shear stress induces the production and release of a number of paracrine factors which inhibit MSCs apoptosis and contribute to quiescence[117,118].

ERK 1/2 and p38 activity are members of the mitogen-activated protein kinase (MAPK) family which serve as focal points in response to a variety of extracellular stimuli such as environmental stresses and inflammatory cytokines[119,120]. In this topic some researchers have found the following:

- hBMSCs are influenced by fluid shear stress, inducing cellular responses related to bone cell differentiation[121]. In this study, bone marrow stromal cells were exposed to a fluid shear of 12 dynes/cm^2 for 30 and 90 min, showing an important increase in ALP expression regulated by p38 activity and a decrease of type I collagen expression downregulated by ERK1/2. Nonetheless, fluid shear exposure did not affect Cbfa1/RUNX2 expression, suggesting that it could not be related to ALP activity[122].

- Furthermore, connexin 43 (CX43) expressions was confirmed indicating cell-to-cell communication in hBMSCs through gap junctions[6].

Fluid shear stress activates ERK1/2 signaling[123]. In this study, intermittent loads (mean value: 4.2 dyn/cm^2 for 1h at intervals of 0.34 dyn/cm^2 for 11 h) of fluid shear stress were applied to hBMSCs increasing the expression of osteogenic genes (i.e., RUNX2, ALP, Collagen and OCN) and ALP activity via two novels signaling pathways. In addition, other researchers have presented evidence suggesting that shear stress alone, without induction factors, can stimulate hMSCs towards the osteoblastic pheno-

type[124]. In this study hMSCs were exposed to 4, 15 and 22 dyn/cm^2 of shear stress for 24 h showing an increase in the expression of the osteogenic markers ALP, BMP-2 and Osteopontin (OPN).

MSCs gene expression can be affected by the shear stress magnitude and exposure time, with the latter being the most influential at an early stage of osteogenesis[125]. In a study, cells were exposed to 0.2 or 1 dyn/cm^2 for 30 or 60 min resulting in upregulation of RUNX2, Type I collagen, and Sox9 markers with no change in the expression of aggrecan, PPARγ, and Osterix (OSX). However, others researchers confirmed that the magnitude of shear stress is crucial for the differentiation of MSCs[126]. They found that the expression of myocardin, myosin heavy chain, and SM-22a was higher when exposed to 10 dyn/cm^2 compared with 2.5 dyn/cm^2. Also, it has indicated that the variation in shear stress levels affects gene expression on hMSCs[127]. In this study, applied shear stresses of 0.015, 0.030, 0.045 and 0.060 Pa caused upregulation of type I collagen and OPN expression. Lately, DNA microarray and quantitative real-time reverse transcription-PCR analysis showed up-regulation of MAP3K8 and interleukin-1 beta expression in MSCs exposed to different magnitudes and duration of flow-induced shear stress[128], while uniaxial tensile strain and magnetic forces did not induce any effect[129].

Fluid shear stress can regulate MSCs differentiation into cardiomyogenesis. Researchers have reported an increase in cardiomyogenic differentiation in rBMSCs exposed to laminar shear stress (i.e., 5, 10, 15 and 20 dyn/cm^2 for 24 hours)[130]. The results of this study showed various issues: (1) An increase in the expression of GATA4, b-MHC, NKx2.5 and MEF2c at < 10 dyn/cm^2 and a decrease in their expression at 15 dyn/cm^2, (2) An increase of the expression of cTnT, CX43, desmin and a-sarcomeric actinin, (3) Enhanced activity of the L-type calcium channel; and (4) an increase in the level of Atrial Natriuretic Peptide (ANP) protein. Similarly, it has been demonstrated that the combination of shear stress and compression has a higher influence on chondrogenic differentiation of MSCs than either stimulus alone[131]. For instance, a pin-on-ball bioreactor system was used on porous polyurethane scaffolds under the following conditions: (a) Compression: 1 Hz, 0.4 mm (amplitude); (b) Shears: 1 Hz, ±25% (amplitude); (c) Both compression and shear. A mechanical load was applied during 1 h per day for 5 consecutive days per week over 3 weeks. The results demonstrated that this combination causes progression of MSCs towards a chondrogenic phenotype. Also, it has demonstrated an increase in chondrogenesis of hBMSCs by exposure to cyclic axial compression and surface shear stress[132].

Fluid shear stress may induce MSC differentiation into endothelial cells. Some important findings are described to following.

- Differentiation of MSC into endothelial cells[133]. In this study, hMSCs differentiated into endothelial cells creating a capillary network in 3D culture under both, in vitro and in vivo conditions.

- Differentiation of MSCs into endothelial cells using a 3D scaffold and a pulsatile flow bioreactor[134]. In this study, the scaffold was subjected to 1 to 15 dyne/cm^2 for two days and at 15 dyne/cm^2 for two days. Under these conditions, the expression levels of VE-cadherin, PECAM-1, and CD34 were increased while smooth muscle markers were downregulated. On the other hand, some researchers have exposed hASCs to 10 dyn/cm^2 for 24, 48, and 96 h showing an increase in VEGF expression, due to an increase in nitric oxide production, with no expression of endothelial cell markers (i.e., CD31, vWF, Flk-1)[135].

Important findings have compared osteogenic differentiation of MSCs under different fluid shear conditions. Some important findings are showed to following.

- Osteogenic commitment produced by cytoskeletal remodeling is correlated with vibration but not fluid shear[136]. To demonstrate this, adipose-derived hMSCs were subjected to vibration frequencies and acceleration magnitudes that

Table 4. Biophysical stimulation of MSC using shear stress

Conditions	Differentiation	Study Type	Comments	Ref
12 dyn/cm^2	Osteogenesis	In vitro	ERK1/2 and p38 signaling are both required for hBMSCs to respond shear stress and regulate osteoblastic phenotype.	In[121]
4, 15 and 22 dyn/cm^2	Osteogenesis	In vitro	Shear stress stimulated osteogenic differentiation without chemical induction.	In [124]
4.2 dyn/cm^2	Osteogenesis	In vitro	Fluid shear stress activates ERK1/2 signal and induces osteogenic differentiation of hMSCs.	In [123]
0.2 and 1 dyn/cm^2	Chondrogenesis	In vitro	Duration of exposure to mechanical stress provides a more powerful stimulus for differentiation of multipotent cells than stress magnitude.	In [125]
2.5 and 10 dyn/cm^2.	Smooth muscle cells	In vitro	High shear stress may disturb the differentiation of MSCs into Endothelial Cells in the presence of endothelial growth medium, but may promote differentiation to Smooth Muscle Cells.	In [126]
5,10,15 and 20 dyn/cm^2	Cardyomiogenesis	In vitro	Fluid shear stress induced cardyomiogenic differentiation of rBMSCs.	In [130]
10 dyn/cm^2	Endothelial cells	In vitro	Fluid shear stress did not induce the expression of endothelial cell markers in hASCs.	In [135]
2.3 dyn/cm^2 and 4.3 dyn/cm^{2}at 0.015, 0.044, and 0.074 Hz.	Osteogenesis	In vitro	Pulsatile flow enhanced osteoblastic differentiation of osteoprogenitor cells.	In [138]

Table 4 Continued

0.34 and 4.2 dyn/cm^2	Osteogenesis	In vitro	Intermittent fluid shear stress promoted enhanced osteogenic differentiation compared to continuous fluid shear stress and up-regulated the activity of ERK1/2 and FAK.	In [137]
1 and 15 dyn/cm^2	Endothelial cells	In vitro	Shear stress upregulated the expression of endothelial cell-related markers and downregulated smooth muscle-related markers in canine MSCs.	In [134]
Parallel flow (1.0x10^{-4}dyn/ cm^2) Transverse flow (5.5x10^{-3}dyn/ cm^2)	Osteogenesis	In vitro	Parallel flow allowed the effective retention of de novo ECM proteins and growth factors and promoted osteogenic differentiation of hMSCs.	In [139]
0.15, 0.30, 0.45 and 0.60 dyn/cm^2	Osteogenesis	In vitro	Shear stress associated with vascular flow may have the potential to significantly direct non-adherent stem cell expression towards osteogenic phenotypic expression.	In [127]
0.41 – 0.51 dyn/cm^2	Osteogenesis	In vitro	Osteogenic differentiation by Rocker culture method.	In [142]
0.01 - 0.0205 dyn/cm^2	Osteogenesis	In vitro	Osteogenic differentiation by Rocker culture method.	In [141]
0.231 and 1.089dyn/ cm^2	Osteogenesis	In vitro	YAP expression in MSCs and chondrocytes is regulated by fluid shear stress.	In [140]
0.5 – 3 dyn/cm^2.	Chondrogenesis	In vitro	Chondrogenic differentiation of MSCs was observed in the presence of chondrogenic supplements under both static and laminar flow cultures.	In [227]

induced fluid shear stress ranging from 0.04 Pa to 5 Pa and vibrations were applied using frequencies of both 100 and 30 Hz during 30 min/day.

- Intermittent fluid shear stress promotes enhanced osteogenic differentiation compared to continuous fluid shear stress[137]. This effect increases the expression levels of osteogenic markers (i.e., ALP, RUNX2, OCN, and Type I collagen) and promoted the activity of ERK1/2 and FAK.

- Osteoblastic gene expression when osteoprogenitor cells were cultured in a perfusion bioreactor[138]. In this study, BMSCs were exposed to steady (2.3 dyn/cm^2) and pulsatile flow (range: 2.3 - 4.3 dyn/cm^2, frequencies: 0.015 - 0.074 Hz) for 24 h and maintained in static osteogenic medium for an additional 13 days. They found a significant increase in gene expression of type 1 collagen, osteopontin (OPN), osteocalcin (OCN), and bone sialoprotein (BSP) under both conditions and for TGF-β and BMP-2, BMP-7 under pulsatile flow alone.

- Studies performed in a bioreactor with two perfusion flow conditions (a) parallel (shear stress: ~1×10^{-5} Pa) and transverse (shear stress: ~5.5×10^{-4} Pa) showed osteogenic differentiation of MSC[139]. In this experiment, MSCs were exposed to these conditions using normal growth media during the first 7 days, then to osteogenic induction media for an additional 7 days. Results revealed that cell proliferation was maintained during both the pre-induction and osteogenic induction stage under parallel flow conditions. In contrast, under transverse flow system, a similar cell proliferation rate was seen during the pre-induction stage, which was reduced after osteogenic induction due to the convective removal of proteins and growth factors.

- Studies performed in a microfluidic perfusion system allowed to study the influence of fluid shear stress in the regulation of yes-associated protein (YAP) expression in MSCs and chondrocytes[140]. The results indicated increased YAP expression, osteogenic differentiation favored over adipogenesis for MSCs, and initial dedifferentiation for chondrocytes.

- Some researchers studied the behavior of different types of cell (i.e., human alveolar bone-derived MSCs, human dermal fibroblast and embryonic stem cell-derived mesenchymal progenitor cell lines) under oscillatory fluid shear stress (Rocker culture method)[141]. The results showed an increase in ALP activity and calcium deposition when osteogenesis was induced in the system.

- Finally, the effect of osteogenic media and fluid shear stresses on human progenitor dermal fibroblasts (HDFs) and an embryonic stem cell-derived mesenchymal progenitor cell line (i.e., hES-MP) also has been studied[142]. Results of this study indicated that both biochemical and biophysical stimulation promoted osteogenic differentiation on both cell types.

Lately, the effects of fluid shear stimulation on human periodontal ligament cells[143] and human Alveolar Bone-Derived Mesenchymal Stem Cells[144] have been reported. It has demonstrated that the cells under shear stress experiment a rearrange in the orientation of the cells inducing osteogenic differentiation. Similarly, oscillatory fluid flow promotes the upregulation of osteogenic gene expression gene, production of collagen, and mineral deposition[145]. Besides, the combination of fluid shear stimulation and the addition of growth factors into the system of cell culture of MSC stimulate the osteodifferentiation of human mesenchymal progenitor cells (hMPCs). Equally, an increase in perfusion velocity applied to MSC, increase the mineralized matrix growth[146]. Some important findings of biophysical stimulation of MSC using shear stress are shown in Table 4.

2.5 Electric Fields

Electrical stimulation in bone healing has been used since the 19th century to treat tibial non-unions by the use of shock of electrical fluids[147,148] and galvanic current[149]. In 1955, some researchers reported that bone healing could be induced by electrical energy[150-153]. In 1957, Fukada and Yasuda measured the piezoelectric properties of bone and their use in fracture healing[154]. Also, Bassett and coworkers proposed that electrical potentials could influence the activity of osseous cells. They indicated that the activation of the piezoelectric properties of the collagen matrix, the electro-kinetic effects, and the polarity of applied current stimulate the formation of new bone in electronegative regions, and induce resorption in the electropositive regions[155-157]. In addition, many publications have confirmed this phenomenon[158-162]. Similarly, clinical case reports confirmed the use of electrical stimulation for bone healing[163,164]. All these studies used technologies that can be classified into three types of electrical stimulation, which have been approved by FDA for clinical use: direct current (DC), and inductive coupling (IC) such as pulsed electromagnetic fields (PEMF) and combined magnetic fields (CMF), and capacitive coupling (CC)[156].

In the last year, many researchers have reported that electrical stimulation promotes MSC differentiation. Researchers have compared the aspect such as cell adhesion and orientation in 3D scaffolds in bone marrow-derived mesenchymal stem cells (BMMSCs) and fibroblast under electrical stimuli[165]. The results of this study showed that MSCs exhibited more 3D adhesion and also a minimal alteration in cell reorientation compared to fibroblasts that presented perpendicular reorientation. Also, in this study, they incubated the cells with integrin antibodies under the same conditions and found a lack of response, which indicated that integrin-mediated mechanism is likely to regulate 3D cell morphology and orientation. Also, studies have reported the behavior of cell migration of MSCs exposed to electric fields. Other study indicated that MSCs exposed to direct currents of 10 to 600 mV/mm had strong migration towards the anode with double the speed of the control[166]. In addition, it was demonstrated that the cell migration in a physiological electric field is cell passage-dependent since migration is reduced at higher passages, and the exposition to electric fields do not affect the osteogenic potential of the cells. Another important finding was the development of a 3D tissue model of osteoblast wound healing to examine the effects of electrophysiological modulation on bone regeneration[167]. Others researchers have monitored the differentiation profile and stress response of human bone marrow-derived mesenchymal stem cells (hMSCs) exposed to electric fields[168]. In this study, cells were exposed to 20 mV/cm (60 kHz) for 40 minutes daily and the results revealed overexpression of the early bone marker (ALP), mid marker (type 1 collagen), and upregulation of heat shock proteins (hsp27, hsp70) which are stress response and cellular metabolism markers, respectively. However, the authors suggested that further studies are necessary to establish possible relationships between applied electric field, stress response markers, and osteogenic markers on osteodifferentiation.

Researchers demonstrated for the first time that cultured human adipose tissue-derived stem cells (hASCs) can be modulated by DC electric fields[169]. They stimulated the cells with DC electric fields of 6 V/cm for 2-4 hours and observed: (1) Elongation and perpendicularly alignment to the applied electric field, (2) Disassembly of gap junctions, (3) Upregulation of certain genes (i.e., CX43, thrombomodulin (ThB), vascular endothelial growth factor (VEGF), and fibroblast growth factor (FGF); and (4) Lack of upregulation of osteopontin (OPN) and peroxisome proliferator-activated receptor gamma (PPARγ). Also, researchers have evaluated for first time the effects of sinusoidal AC electric fields on hASCs, and demonstrated that short-term (i.e., 1, 10, 100, or 1000 V/cm at 1 Hz for 5 min) and long-term (i.e., 1, 3, and 5 V/cm at 1 Hz for 4-h/day) electric field exposure increases intracellular calcium signaling and calcium deposition in osteogenic differentiation medium, respectively[170]. Likewise, the first

Table 5. Biophysical stimulation of MSC using electric fields

Conditions	Differentiation	Study Type	Comments	Ref
4, 7, and 10 V/cm	----------	In vitro	Electrical stimulus regulated mesenchymal stem cell adhesion and orientation in 3D collagen scaffold.	In[165]
6 V/cm	Fibroblastic and vasculogenic differentiation	In vitro	Direct current electric fields modulated morphological and phenotypic characteristics of hASCs.	In [169]
Wave electric stimulus: DC, CC, PEMF and DW.	Osteogenesis	In vitro	DW or CC electrical stimulus enhanced rate of bone healing at the fracture site compared to DC and PEMF.	In [171]
Short-term (1, 10, 100, or 1000 V/cm at 1 Hz) Long-term (1, 3, and 5 V/ cm at 1 Hz)	Osteogenesis	In vitro	Sinusoidal AC electric fields on hASCs increased intracellular calcium signaling and calcium deposition under osteogenic differentiation medium respectively.	In [170]
10 – 600 mV/mm	Osteogenesis	In vitro	Electric Fields directed migration of MSCs mainly to the anode.	In [166]
200 mV/cm at 60 kHz	Osteogenesis	In vitro	Electrical stimulation promoted osteogenic differentiation and activated osteogenic pathways.	In [168]
250 mV	Neurogenesis	In vitro	Electrical stimulation and exogenous Nurr1 gene expression together may induce nerve regeneration using stem cells.	In [172]
Rectangular pulses (7 ms, 3.6 mV/cm, 10 Hz)	Osteogenesis	In vitro	Combined treatment of biochemical and physical microenvironments increased osteogenic differentiation of MSCs.	In [179]
0.15 V/cm for at 1 Hz	Cardiomyogenesis	In vitro	Carbon nanotubes based polylactic acid scaffolds and electrical stimuli promoted the upregulation of cardiac markers.	In [173]

Table 5 Continued

500 V/m and 5 ms pulse width at 1 Hz.	Cardiomyogenesis	In vitro	Carbon nanotubes based poly-ε-caprolactone scaffolds and electrical stimuli promoted cardiomyogenic differentiation.	In [174]
Rectangular pulses (2 ms, 100 mV , 10 Hz)	Neurogenesis	In vitro/ in vivo	Electrically induced neural differentiation of mouse BMSCs contributed to the regeneration and recovery of motor function after transplantation into TBI model mice.	In [176]
Rectangular pulses(2 ms, 40 μA , 2 Hz)	Cardiogenesis	In vitro	Electrical stimulation promoted cardiogenesis in MSC and cardiac myocytes coculture monolayer.	In [175]
Alternating electric current (10 or 40 mA, 10-Hz)	Osteogenesis	in vitro	Alternating electric current promoted the differentiation of adult human MSCs toward the osteogenic pathway.	In [180]

study that compared the effects of various electric stimulation (ES) waveforms on MSCs cellular activities including cytotoxicity, proliferation, cell-kinetics, and apoptosis in vitro were reported in 2011[171]. In this study, they analyzed the effects of direct current (DC), capacitive coupling (CC), pulsed electromagnetic field (PEMF), and degenerate wave (DW) on BMMSCs differentiation. The results indicated that DW and CC conditions had a greater influence on invasion and cell proliferation compared to the other types of electric stimulation being relevant to bone regeneration.

Studies have presented evidence suggesting that MSCs exposed to electrical stimulation may differentiate into nerve, cardiac, and neuronal cells. Some important results are described below.

- Simultaneous electrical stimulation and exogenous Nurr1 gene expression may induce nerve regeneration using stem cells[172]. In this study, cells were exposed to electrical stimulation (250 mV for 1000 s) and exogenous Nurr1 gene delivery. The results indicated that cells transfected with exogenous Nurr1 genes plus electrical stimulation showed the greatest level of neurite outgrowth compared to one only stimulus.

- CNT based polylactic acid scaffolds and electrical stimuli promoted the upregulation of cardiac markers[173].

- CNT-poly (e-caprolactone) (PCL) substrates promoted the differentiation of hMSCs into cardiomyocytes[174].

- Design of an electric system to stimulate canine MSCs into cardiomyocytes[175].

- Electrical stimulation can induce differentiation of mouse BMSCs into neural cells[176]. In this study, the cells were transplanted into traumatic brain injury (TBI) model mice. The results indicated that these cells promoted neurogenesis and the recovery of motor function in this animal model.

The combination of electrical stimulation and biochemical agents induces osteogenic differentiation of MSCs[177,178]. Researchers have studied the synergistic effects of biochemical microenvironments (artificial matrix extracellular and osteogenic supplements), and physical microenvironments (electrical stimulation) with respect to osteogenic differentiation of hMSCs[179]. In this study, it was found that the cell exposed to both conditions exhibited an increase in the expression of ALP activity and osteogenic markers (i.e., RUNX2, ALP, and OPN). Also, it has reported MSCs osteodifferentiation by combining alternating electric current and growth factors (i.e., BMPs)[180]. In this study, MSCs were cultured within type I collagen hydrogels, and exposed to either 10 or 40 mA (10 Hz) for 6 h per day which promoted osteogenic differentiation evidenced by the expression of both early (RUNX2 and OSX) and late (OSP and OCN) osteogenic genes.

Of late, the substrates with conductive characteristic are being used to induce osteogenesis. It has found that the use of both electric current and conductive carbon nanotubes (CNTs) in cell-substrate enhance the osteoblastic activity of MSC[181]. Also, the use of electrically conductive scaffold that allows the ion fluxes further migration of MSC into the inner region of the scaffold and enhance the osteogenic differentiation[181,182]. Finally, an important finding highlights that the effects produced by electrical stimulation on the osteogenic differentiation of MSC in early stages are stronger[181]. Table 5 shows some recent advances in biophysical stimulation of MSC generated by electric fields.

2.6 Electromagnetic Fields

Electromagnetic fields (EMFs) play a role in the regeneration of several human tissues. In 1974, Bassett and coworkers were pioneers in the therapeutic use of extremely low frequency (ELF) pulsed electromagnetic fields (PEMFs) to accelerate the fracture repair and to treat congenital and acquired pseudarthroses and nonunions[183,184]. In 1979, PEMFs therapy was approved by the FDA[188] allowing clinical trials and production of commercial devices to promote bone fracture healing[186]. Since then, different effects of PEMF stimulation on differentiation and proliferation of some osteogenic cell lines in vitro have been published in the literature[187-190]. Researchers have indicated the forced-vibration of all the free ions on the surface of a cell's plasma membrane, changes in voltage, and conductivities are a possible mechanism of the application of electromagnetic fields to regulate cell process[191-193]. Since then, many investigations have focused on the use of this therapy to accelerate the cell proliferation and osteogenic differentiation of MSC.

In the last years, many studies have demonstrated the effectiveness of PEMFs in the regulation in osteogenesis in MSCs. It has reported the eddy currents induced by ELF-EMF exposure (60Hz, 3 mT) significantly stimulate collagen synthesis in osteoblast-like MC3T3- E1 by p38 MAPK pathways[194]. Additionally, it has indicated that both PEMFs and inductive stimulus like bone morphogenetic protein 2 (BMP-2) induce osteogenic differentiation and impact cells at specific states of commitment to an osteoblast phenotype and maturation period[195]. In this study, it was applied PEMF for 8 hours per day, which consisted of 4.5 ms bursts of 20 pulses repeating at 15 Hz with an increase in field strength from 0 to 16 gauss in 200 ms and decay back to 0 in 25 ms during each pulse. Results showed that under both conditions, ALP activity and osteocalcin expression increased and improved the effect of BMP-2 on PGE2, latent and active TGF-β1, and osteoprotegerin. Furthermore, it has studied the influence of PEMFs (300 ms quasi-rectangular pulses with a

Table 6. Biophysical stimulation of MSC using Electromagnetic Field

Conditions	Differentiation	Study Type	Comments	Ref
Helmholtz coil (Biomet, Parsippany, NJ) [Bassett, 1974]	Osteogenesis	In vitro	PEMF enhanced osteogenesis of hMSCs in the presence of an inductive stimulus like BMP-2.	In[195]
Quasi-rectangular pulses (7.5 Hz and 0.13 mT)	Osteogenesis	In vitro	PEMF stimulation may play a modulating role in hMSC osteogenesis	In [196]
ELF magnetic field (15 Hz, 1mT)	Osteogenesis	In vitro	Oligo osteogenesis microarray analysis.	In [201]
Helmholtz coil (Biomet, Parsippany, NJ) [Bassett, 1974]	Osteogenesis Adipogenesis Neurogenesis	In vitro	PEMF might change the expression of ion channel and induce membrane hyperpolarization of BMMSCs resulting in the alteration of cell cycle progression and the presence of osteoblasts at different stages of osteogenesis.	In [202]
Helmholtz coil (Biomet, Parsippany, NJ) [Bassett, 1974]	Osteogenesis	In vitro	PEMF increased cell proliferation in human BMMSCs during osteogenesis in the presence of osteogenic medium.	In [203]
ELF magnetic field (50 Hz , 0-20 mT)	Osteogenesis	In vitro	Extremely Low Frequency (ELF) magnetic fieldsinhibited the growth and metabolism of hMSC, but not affected osteogenic differentiation in hMSCs.	In [204]
ELF magnetic field (15 Hz, 1mT)	Osteogenesis Adipogenesis	In vitro	Extremely Low Frequency (ELF) magnetic fieldspromoted osteoblastic differentiation instead of adipogenesis in rMSCs.	In [197]
15 Hz, 1 Gauss with 5-millisecond bursts with 5-microsecond pulses.(Orthopulse® II, IMD)	Osteogenesis	In vitro	PEMF stimulated osteogenesis in BMSCs.	In [208]

Table 6 Continued

ELF magnetic field (15 Hz, 5 mT)	Chondrogenesis	In vitro	Extremely low frequency (ELF) magnetic fields stimulated chondrogenic differentiation of hMSCs.	In [209]
ELF magnetic field (50 Hz, 0.5 mT)	Osteogenesis	In vitro/ In vivo	Extremely low frequency (ELF) magnetic fieldspromoted the proliferation, osteogenic differentiation in vitro (Bone Marrow Stromal cells) and in vivo (mice femur) experiments.	In [198]
PEMF (2 mT, 75 Hz and pulse of 1.3 msec)	Osteogenesis	In vitro	PEMF enhanced the commitment of BM-MSCs to osteoblasts more efficiently in comparison with ASCs	In [199]
ELF- PEMF (6 gauss at 10, 30, and 100 Hz)	Osteogenesis	In vitro	ELF- PEMF increased cell proliferation and osteogenic response on hAMSCs.	In [200]
ELF-Magnetic fields (50 Hz, 1 mT)	Neurogenesis	In vitro	PEMF induced neural differentiation in BMMSCs without any chemicals or differentiation factors.	In [211]
ELF-Magnetic fields (50 Hz or 100 Hz, 1 mT)	Neurogenesis	In vitro	ELF-magnetic fields accelerated neural differentiation of BMMSCs via ROS-induced EGFR activation and, subsequently, Akt and CREB phosphorylation.	In [212]
PEMF (1 Gauss at 15 Hz, 5 ms bursts with a pulse of 1 ms)	Osteogenesis	In vitro	PEMF and DHEA (prohormone) promoted the viability, proliferation, and osteogenic differentiation of MSCs.	In [213]
PEMF (1.5 mT at 75 Hz with a pulse of 1.3 ms)	Chondrogenesis	In vitro	PEMF might inhibit the catabolic activity of IL-1b during cartilage-regenerating surgical interventions	In [210]

Table 6 Continued

PEMF frequencies (1.1 mT at 5, 25, 50, 75, 100, and 150 Hz)	Osteogenesis	In vitro	Different pulsed electromagnetic field frequencies had different effects on induction of bone formation and an optimal frequency for osteogenic differentiation of hMSCs was 50 Hz.	In [219]
High PEMF(50 – 100 μV/cm at 27.1 MHz, pulsed frequency of 1000Hz, and pulse lasting 100 ms)	Osteogenesis	In vitro	PEMF stimulation without the use of chemical increased the expression of osteogenic markers in osteoprogenitor cells.	In [214]
ELF-Magnetic fields(1 mT at 30/45 Hz, and 1 mT at 7.5 Hz,)	Osteogenesis	In vitro	The effects of the electromagnetic fields on osteogenic differentiation differed depending on the electromagnetic field conditions.	In [215]
ELF-Magnetic fields(1 mT at 50 Hz)	Neurogenesis	In vitro	ELF- magnetic fields and magnetic nanoparticles promoted neural differentiation of MSCs.	In [228]

repetition rate of 7.5 Hz and 0.13 mT) on the proliferation and osteogenic differentiation of hMSCs in vitro[196]. The results of this study demonstrated a high proliferation rate, and osteogenic differentiation with the time that was supported by the gene expression of RUNX2 at early and mid-stages of culture and calcium accumulation at the highest levels of the culture period. Similarly, it has demonstrated that EMFs (15 Hz, 1mT) play a vital role in balancing the osteoblastic and adipogenic differentiation of MSCs, inhibiting adipogenesis, and stimulating osteoblastic differentiation[197]. Also, the effects of low-intensity EMFs (50 Hz, 0.5 mT) on cell proliferation, differentiation, and cycle in mouse bone marrow stromal cells (BMSCs) *in vitro* and *in vivo*has been investigated[198]. Their results showed that EMFs induce ALP secretion, and not only increase collagen I gene expression but also DNA synthesis and replication. Others researchers have reported that PEMFs (2 ± 0.2 mT, 75 ±2 Hz, and pulses of 1.3 msec) enhanced the commitment of BM-MSCs to osteoblasts more efficiently in comparison with ASCs[199]. And, cell proliferation and osteogenic response of human alveolar bone-derived mesenchymal stem cells (hABM-SCs) exposed to EMFs[200].

Mounting evidence suggests that the application of PEMFs affectthe cell proliferation and osteogenic differentiation, through modulation of growth factors, intracellular signaling molecules and, pro-or post-differentiation genes[201]. Researchers used an oligo-osteogenesis microarray to detect the effect of PEMF (15 Hz, 1mT, 8 hr/day for 2 days) on gene expression during the process of MSC cell differentiation. Their results showed that the mRNA levels of BMP1, BMP7 were significantly

higher than EGF and EGFR. Studies have demonstrated that PEMFs induce membrane hyperpolarization in MSCs resulting in the alteration of cell cycle progression[202] and the presence of osteoblasts at different stages of osteogenesis[203]. In the first study, the results indicated that the alteration of cell cycle progression promoted cell proliferation during the exponential growth phase and multi-lineage differentiation potential of bone marrow mesenchymal stem cells (BMMSCs). In the second study, the results suggested that PEMF altered early osteogenesis-related gene expression, up-regulated the expression of cbfa1/Runx2 at early stages of the culture process, and increased mineralization at early and middle stages of BMMSC osteogenic differentiation. Finally, it has demonstrated that EMFs could inhibit the growth and metabolism of hMSCs, but have no significant effect on their differentiation[204]. These results showed that the effect of EMFs on hMSCs resulted in high proliferative activity, no changes in the morphology, cell viability, higher extracellular Na^+ ions concentration, higher osmolality, and calcium deposition.

It has been demonstrated that the activation of ERK1/2, via phosphorylation regulates differentiation of MSCs towards the osteoblast lineage[205], which might be activated by mechanical stimuli (i.e., fluid flow or strain)[206]. Similarly, researchers have provided evidence of the activation of MAPK and ERK in HL-60 human leukemia cells, MCF-7 human breast cancer cells, and rat fibroblast cells exposed to a 60 Hz, 1 G EMF[207]. However, another study reported that EMF stimulated osteogenic differentiation without activating ERK phosphorylation, significantly increasing the ALP activity or the matrix mineralization timing[208]. Therefore, EMF may induce differentiation at the expense of proliferation. EMF has also been able to stimulate MSCs toward a chondrogenic and neural phenotype. Important findings have demonstrated that ELF-EMF (15 Hz, 5 mT) can stimulate chondrogenic differentiation of hMSCs in vitro and affect them at higher passages more distinctively[209]. Similarly, it has reported that PEMFs may inhibit the catabolic activity of IL-1b, during cartilage-regenerating surgical interventions[210]. In addition, it has found that ELF-EMF (50 Hz, 1 mT) can induce neural differentiation in BM-MSCs without any chemicals or differentiation factors, and accelerate neural differentiation of BM-MSCs *via* ROS-induced EGFR activation[211, 212].

Researchers demonstrated that the application both PEMF and DHEA (prohormone) promotes the viability, proliferation, and osteogenic differentiation of MSCs[213]. They suggested a therapy based PEMF early during fracture healing followed by administration of DHEA with an osteogenic differentiating effect. Moreover, it was demonstrated an increase in the osteogenic response of osteoprogenitor cells (C3H10T1/2) to high-frequency PEMF stimulation without the use of osteogenic media[214]. The results display an improvement in ALP activity and matrix mineralization, cellular proliferation, stimulation of the late stage of osteogenic differentiation, moderate expression of p38α mRNA, and an increase of mRNA expression of numerous BMPs. The effects produced by EMFs on osteogenic differentiation in MSCs can vary depending on their frequency, waveforms, and intensity. Researchers reported the effects of positive (30/45 Hz, 1 mT) and negative (7.5 Hz, 1-2 mT) EMFs on osteogenic differentiation of hASCs[215]. The results showed a higher expression level of osteogenic markers at positive EMFs and lower at negative EMFs, while both still supporting osteogenic differentiation. The authors argue that this behavior might be related to motion and higher efflux of ions (Ca^+) through the membrane allowing osteoblastic function and viability at specific frequencies[216-218]. Similarly, it has reported, that different PEMF frequencies produce distinct effects on hMSCs differentiation[219]. In this study were used different PEMF frequencies (5, 25, 50, 75, 100, and 150 Hz) each with a field intensity of 1.1 mT, for 30 minutes per day for 21 days. The results indicated that at 50-Hz PEMFs the levels of ALP and Osteocalcin are increased. Also, it was demonstrated that in a range from 5 to 50 Hz, as the frequency increased the inductive effect on bone differentiation also increased.

However, the inductive effect decreased with the increase of the frequency from 50 to 150 Hz. It was also demonstrated that waveforms of EMF are crucial parameters to induce the response of osteoblasts[220] and different electric field intensities could regulate the formation of osteoclast-like cells[221]. Lately, an important study in this field has highlighted that PEMFs promote osteodifferentiation of MSC only when they are precommitment[222]. Table 6 shows recent findings that highlight osteogenic differentiation of MSC using electromagnetic fields.

3. Conclusion

After the use of cell culture, scaffolds, and growth factors, biophysical stimulation has been used as a tool in bone regeneration. This type of stimulation causes effects on the cell morphology, proliferation, and differentiation. The different types of biophysical stimulation cause multiple effects on the cell environment and some of these effects are as follows:

- Expression of transcription factors take place during osteogenic differentiation i.e., FOS family and Bone Morphogenetic Proteins (BMPs).
- Osteodifferentiation in different types of MSC.
- Cardiac, neural, chondrogenic, and musculoskeletal differentiation of MSC.
- Difference in genetic expression of MSC with the culture system (2D and 3D).
- The combination of biophysical stimulation, the use of scaffolds, and the addition of growth factors into the system of cell culture of MSC, has resulted in an excellent option to stimulate the osteodifferentiation of MSC.
- Better conditions to promote the osteodifferentiation are reached when the stimulation is applied in early stages.
- Nowadays, the mechanisms that explain the signaling generated by biophysical stimulation (i.e., mechanical, electrical and electromagnetic) are not entirely clear. Therefore, it is necessary to conduct more research on the effects produced by this type of modulation, which will serve in the application of clinical the rapies.

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