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REVIEW

# Stimulating factors for regulation of osteogenic and chondrogenic differentiation of mesenchymal stem cells

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## Abstract

Mesenchymal stem cells (MSCs), distributed in many tissues in the human body, are multipotent cells capable of differentiating in specific directions. It is usually considered that the differentiation process of MSCs depends on specialized external stimulating factors, including cell signaling pathways, cytokines, and other physical stimuli. Recent findings have revealed other underrated roles in the differentiation process of MSCs, such as material morphology and exosomes. Although relevant achievements have substantially advanced the applicability of MSCs, some of these regulatory mechanisms still need to be better understood. Moreover, limitations such as long-term survival *in vivo* hinder the clinical application of MSCs therapy. This review article summarizes current knowledge regarding the differentiation patterns of MSCs under specific stimulating factors.

**Key Words:** Mesenchymal stem cells; Differentiation; Osteogenic; Chondrogenic; Literature review.



**Core Tip:** Mesenchymal stem cells (MSCs) are multipotent cells capable of differentiating in specific directions. The differentiation process of MSCs depends on diverse specialized external stimulating factors. The results from recent studies have revealed other underrated roles in the differentiation process of MSCs. However, several questions remain to be solved prior to stable and effective clinical treatment. Our review explores the differentiation patterns of MSCs and summarizes the relevant research according to stimulus types. Finally, future prospects are discussed with regard to their clinical applications.

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## INTRODUCTION

Mesenchymal stem cells (MSCs), which were originally identified in the bone marrow, are adult stem cells with multilineage differentiation potential. Under specific induction conditions, MSCs could differentiate into bone, adipose, muscle, neural, and endothelial tissue cells[1]. With the development of research, MSCs have been obtained from other tissues, including adipose, peripheral blood, umbilical cord blood, and periodontal membrane tissue[2-5]. Due to their multilineage differentiation potential and rich tissue sources, the application of MSCs in research on regenerative medicine is virtually limitless[6]. However, a specific number of MSCs are necessary for tissue regeneration; hence, there is a requirement for MSC amplification before therapy[7]. The question of how the differentiation of MSCs are controlled *in vitro* and *in vivo* remains unanswered, which has limited the effectiveness of MSCs in the application of regenerative medicine research. Recently, various external stimulus factors, such as biochemical stimuli, hypoxia, physical stimuli, material properties, and exosomes, have been found to have an impact on the differentiation process of MSCs (Figure 1). The purpose of this review is to discuss a variety of recent findings regarding the important external stimulus factors that influence the self-renewal and osteogenic and chondrogenic differentiation potential of MSCs.

## BIOCHEMICAL STIMULI

Growth factors, cytokines, and miRNAs are examples of biochemical stimuli that have typically been employed to control the destiny of MSCs. Growth factors and cytokines bind to the corresponding receptors and transfer signals, while miRNAs degrade mRNAs or inhibit the translation of mRNAs to regulate gene expression and thus influence the differentiation fate of MSCs. Numerous studies have examined the effects of various growth factors, cytokines, and miRNAs on the proliferation and differentiation of MSCs into other cellular phenotypes (Table 1).

### *Growth factors*

Growth factors, including fibroblast growth factor (FGF), transforming growth factor (TGF), platelet-derived growth factor, hepatocyte growth factor, granulocyte colony-stimulating factor and bone morphogenetic protein (BMP), are a class of peptides that regulate cell growth and other cell functions by binding to specific cell membrane receptors[8].

FGF-2, also known as basic bFGF, has been the subject of the majority of FGF research to date. In a concentration-dependent manner, bFGF might promote the proliferation of MSCs from several tissue sources, including bone marrow peri-adipocyte cells[9], synovial MSCs[10], adipose-derived stem cells (ADSCs)[11], umbilical cord-derived MSCs[12], and bone MSCs (BMSCs)[13,14]. Ramasamy *et al*[12] reported that cell proliferation increased accordingly with increasing bFGF concentrations in the range of 0-40 ng/mL. However, Ma *et al*[11] and Wang *et al*[14] observed that the proliferation efficiency of cells at 5 ng/mL of bFGF was higher than that at 10 ng/mL. As a result, the use of 5 ng/mL of bFGF appeared to be an appropriate choice to promote the proliferation of different MSCs. In addition to enhancing MSC proliferation, bFGF has been shown to maintain stemness, support cartilage differentiation, and influence osteogenic differentiation[9,10,13]. Intriguingly, Wang *et al*[14] reported that bFGF pretreatment inhibited osteogenic differentiation at the early stage, but promoted it in the medium phase[13]. This finding might indicate that the addition of different growth factors at different phases of osteogenesis induction could successfully promote osteogenic differentiation. Therefore, more studies are needed to clarify the mechanism of action of bFGF at different stages of osteogenic differentiation, as well as to identify the best combination of growth factors to effectively promote the osteogenic differentiation of MSCs.

Previous research has demonstrated the involvement of TGF-β in inducing chondrogenic differentiation[5]. However, while promoting cartilage differentiation, TGF-β also led to early hypertrophic maturation and the eventual formation of nonfunctional fibrocartilage[2,15]. In addition, TGF-β was also found to promote the proliferation of MSCs and their effect on osteogenic differentiation[16,17]. MSC osteogenic differentiation was influenced by TGF-β in a dose-dependent manner. According to research by Xu *et al*[17], low concentrations of TGF-β (1 ng/mL) promoted the osteogenic development of BMSCs, whereas high concentrations (10–50 ng/mL) of TGF-β inhibited osteogenic differentiation. Igarashi *et al*[18] showed that 5 ng/mL of TGF-β regulated the phenotypic differentiation of BMSCs toward osteoblasts but seemed to inhibit osteogenic differentiation at the late stage, suggesting that

**Table 1 Growth factors, cytokines, and their effects on the differentiation of mesenchymal stem cells**

| Factors | Amount/types | Concentration | Cell dource | Results | Ref. |
|---|---|---|---|---|---|
| FGF | FGF-2 | 10 ng/ml | BM-PACs | FGF-2 did not lead to cell differentiation into a chondrogenic lineage | Endo *et al*[9] |
| | bFGF | 5 ng/ml | SMSCs | Promoted SMSCs chondrogenic differentiation | Okamura *et al*[10] |
| | bFGF | 0-40 ng/ml | UC-MSCs | bFGF did not alter osteogenic nor adipogenic differentiation potential | Ramasamy *et al*[12] |
| | bFGF | 20 ng/ml | BMSCs | bFGF pretreatment inhibited osteogenic differentiation of BMSCs at early stage, promoted it in the medium phase, and maintained it in the later stage during osteogenic induction | Wang *et al*[13] |
| TGF-β | TGF-β3 | 10 ng/ml | SF-MSCs | Increased the expression levels of COL2A1, SOX9, ACAN, COL10A1 | Jia *et al*[15] |
| | TGF-β | 10 ng/ml | ADSCs | Promoted ADSCs chondrogenic differentiation but led to early hypertrophic maturation | Hesari *et al*[2] |
| | TGF-β1 | 1, 10, 20 or 50 ng/ml | BMSCs | Low concentration of TGF-β1 (1 ng/ml) promoted osteogenic differentiation of BMSCs while high concentration of TGF-β1 (10 to 50 ng/ml) significantly inhibited osteogenesis | Xu *et al*[17] |
| | TGF-β | 5 ng/ml | BMSCs | Promoted osteogenic differentiation of BMSCs but suppressed the maturation of ostroblastic MSC differentiation at the last stage of osteogenic process | Igarashi *et al*[18] |
| | TGF-β3 | 10 μg/L | PDLSCs | Induced chondrogenesis | Choi *et al*[5] |
| IL | IL-6 | 100 ng/mL | BMSCs | Promoted BMSCs osteogenic differentiation | Xie *et al*[21] |
| | IL-17A | 5-40 ng/ml | BMSCs | Promoted the neuronal-associated gene expression of BMSCs | Chen *et al*[24] |
| | IL-17 | 50 ng/mL | Mouse MSCs | Enhanced the osteogenic differentiation of mMSCs | Liao *et al*[22] |
| | IL-6 | 100 ng/mL | hMSCs | IL-6/soluble IL-6R promoted chondrogenic differentiation of MSCs | Kondo *et al*[20] |
| | IL-17A | 50 ng/ml | BMSCs | Inhibited osteogenic differentiation of BMSCs | Wang *et al*[23] |
| | IL-22 | 10 ng/ml | MSCs | Upregulated osteogenic and adipogenic transcription factors | El-Zayadi *et al*[25] |

FGF: Fibroblast growth factor; FGF-2/bFGF: Basic fibroblast growth factor; TGFβ: Transforming growth factor β; IL: Interleukin; BMSCs: Bone mesenchymal stem cells; BM-PACs: Bone marrow peri-adipocyte cells; ADSCs: Adipose-derived stem cells; hMSCs: Human mesenchymal stem cells; SMSCs: Synovial mesenchymal stem cells; UC-MSCs: Umbilical cord-derived mesenchymal stem cells; SF-MSCs: Synovial fluid-derived mesenchymal stem cells; PDLSCs: Periodontal ligament stem cells; COL2A1: Collagen type II alpha 1 chain; SOX9: Sex-determining region Y-box 9; ACAN: Aggrecan protein; COL10A1: collagen type X alpha 1 chain.

additional cellular signals were necessary for the osteogenic differentiation of some types of MSCs. Therefore, it is crucial to determine how to prevent hypertrophy during TGF-β promoted cartilage differentiation.

### *Cytokines*

The fate of MSCs might be influenced by many cytokines, such as interleukin (IL), tumor necrosis factor (TNF) and interferons (IFN). Studies have previously examined how various cytokines affected osteogenic differentiation. IL-10, IL-11, IL-18, and IFN-γ promoted osteogenesis, while TNF-α, TNF-β, IL-1α, IL-4, IL-7, IL-12, IL-13, IL-23, IFN-α and IFN-β inhibited osteogenesis[19]. In this article, we focus on recently discovered cytokines such as IL-6, IL-17, and IL-22 that have the potential to affect the fate of MSCs.

MSCs both produced IL-6 and reacted to it. Furthermore, the gradual reduction in IL-6 secretion by MSCs during chondrogenic differentiation suggested that IL-6 was one of the distinguishing characteristics of undifferentiated MSCs[20]. Nevertheless, the addition of exogenous IL-6 was found to be

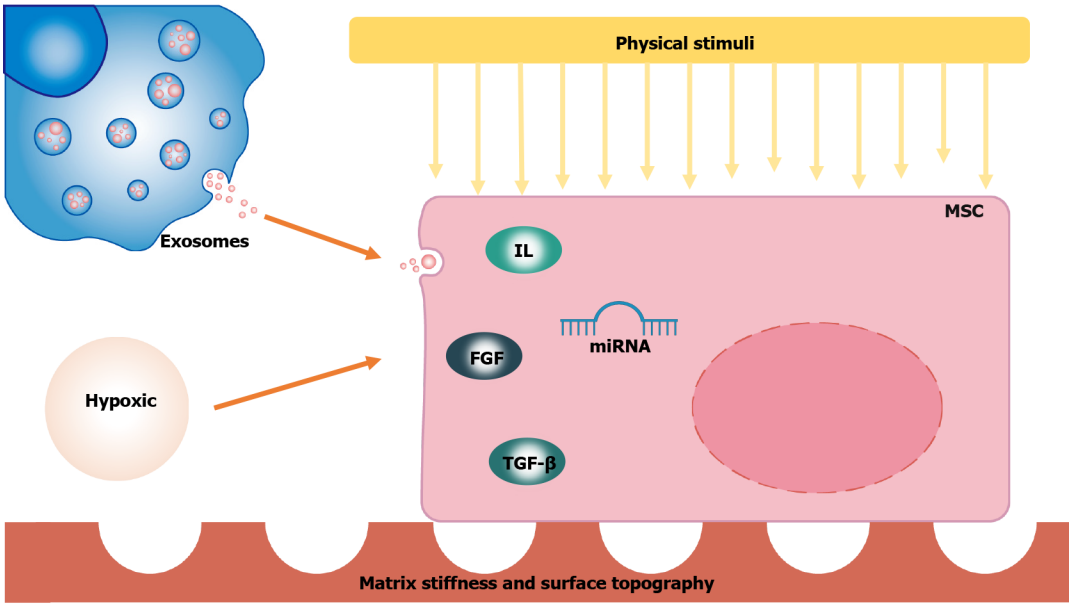


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**Figure 1 Overview of stimulating factors in differentiation of mesenchymal stem cells.** MSC: Mesenchymal stem cell; IL: Interleukin; FGF: Fibroblast growth factor; TGF-β: Transforming growth factor-β.

effective in promoting the osteogenic differentiation and chondrogenic differentiation of MSCs[20,21]. In contrast to previous studies, Xie *et al*[21] discovered that IL-6 secretion by BMSCs increased rather than decreased with osteogenic differentiation. The effect of IL-17A on the osteogenic differentiation of MSCs also seemed to be contradictory. Liao *et al*[22] reported that IL-17A inhibited the osteogenic differentiation of MSCs as well as pre-osteoblast cell lines. However, the study by Wang *et al*[23] showed the opposite. The appearance of these phenomena might be due to different microenvironments and cellular sources. Additionally, different concentrations of IL-17A have been shown to promote neuronal differentiation, with the best effect at 20 ng/mL[24]. The effect of IL-22 on the proliferation and differentiation of MSCs was first reported by scholars in 2017, which showed that IL-22 alone could upregulate the levels of osteogenic and lipogenic transcription factors but needed to be combined with IFN-γ and TNF to promote the proliferation of MSCs[25].

Cytokines must bind to specific receptors to transmit signals. The amount of the relevant receptor for cytokines appeared to be the rate-limiting element regulating the differentiation of MSCs[20]. Therefore, more studies are required to determine how cytokines affect the growth and differentiation of MSCs. Moreover, a fresh approach will be to look for factors that may raise the number of cytokine receptors on the surfaces of MSCs.

### *miRNAs*

Small non-coding RNAs (approximately 20–25 nucleotides) called miRNAs are a subclass that could bind to complementary target sites in mRNA molecules to inhibit translation or decrease mRNA stability, which controls gene expression[26]. In this case, miRNAs could regulate the expression of key genes during the differentiation of MSCs in specific lineages to influence the direction of differentiation of MSCs (Table 2).

The osteogenic differentiation of MSCs was found to be regulated by micro RNA-1286[27], micro RNA-223-3p[28], micro RNA-346-5p[29], micro RNA-21[4] and micro RNA-130a[30], whereas the chondrogenic differentiation of MSCs was found to be regulated by micro RNA-130b[31], micro RNA-218[32], micro RNA-495[33] and micro RNA-30a[34]. In addition to this, some miRNAs also exhibited roles in regulating the adipogenic differentiation[30], endothelial differentiation[26], neuronal differentiation[35], and myocardial differentiation[36,37] of MSCs.

In conclusion, investigating the impact of biochemical stimuli on the growth and differentiation of MSCs has aided our understanding of the patterns of the aberrant differentiation of MSCs in diseased situations and aided in identifying novel therapeutic targets. It appears to be a promising avenue to examine the impact of the combination of diverse biochemical stimuli on the fate of MSCs, since distinct biochemical stimuli in the microenvironment in which MSCs are positioned function in a compound manner. Additionally, since the functions of cytokines and growth factors are dependent on binding to the appropriate receptors and some studies have indicated that receptor expression might be the rate-limiting factor, it would be preferable to determine methods to boost receptor expression as opposed to raising cytokine and growth factor concentrations.

**Table 2 Micro RNA and their effects on the differentiation of mesenchymal stem cells**

| Amount/types | Expression | Cell source | Results | Ref. |
|---|---|---|---|---|
| micro-RNA-1286 | Over expression | hMSCs | ↓ Osteogenic differentiation | Zhou *et al*[27] |
| micro-RNA-223-3p | Low expression | BMSCs | ↑ Osteogenic differentiation | Long *et al*[28] |
| micro-RNA-346-5p | Over expression | BMSCs | ↓ Osteogenic differentiation | Zhang *et al*[29] |
| micro-RNA-21 | Over expression | hucMSCs | ↑ Osteogenic differentiation | Meng *et al*[4] |
| micro-RNA-130a | Over expression | BMSCs | ↑ Osteogenic differentiation ↓ adipogenic differentiation | Lin *et al*[30] |
| micro-RNA-130b | Low expression | BMSCs | ↑ Chondrogenic differentiation | Zhang *et al*[31] |
| micro-RNA-218 | Over expression | SDSCs | ↑ Chondrogenic differentiation during the eraly stage | Chen *et al*[32] |
| micro-RNA-495 | Over expression | hMSCs | ↓ Chondrogenic differentiation | Lee *et al*[33] |
| micro-RNA-30a | Over expression | BMSCs | ↑ Chondrogenic differentiation | Tian *et al*[34] |
| micro-RNA-145 | Low expression | ADSCs | ↑ Endothelial differentiation | Arderiu *et al*[26] |
| micro-RNA-124 | Over expression | ADSCs | ↑ Neuronal differentiation | Mondanizadeh *et al*[35] |
| micro-RNA-10-5p | Low expression | BMSCs | ↑ Myocardial differentiation | Li *et al*[36] |
| micro-RNA-499a-5p | Over expression | BMSCs | ↑ Cardiomyogenic differentiation | Neshati *et al*[37] |

↑: Increase; ↓: Decrease; hMSCs: Human mesenchymal stem cells; BMSCs: Bone mesenchymal stem cells; hucMSCs: Human umbilical cord mesenchymal stem cells; SDSCs: Synovium-derived mesenchymal stem cells; ADSCs: Adipose-derived stem cells.

## PHYSICAL STIMULI

In addition to the previously mentioned biochemical stimuli, physical stimuli such as electromagnetic fields (EMF), microgravity (MG), fluid shear stress (FSS), and hydrostatic pressure (HP) could also have an impact on the proliferation and differentiation of MSCs (Table 3). EMF, a non-invasive biophysical therapy, is a combination of electric and magnetic fields and has been widely used in the treatment of bone diseases[38,39]. Exposure to sinusoidal EMF (1mT,15Hz,4h/d) promoted the proliferation and osteogenic and chondrogenic differentiation of BMSCs[40]. In contrast, Wang *et al*[41] found that EMF also promoted the osteogenic differentiation of MSCs but did not inhibit their proliferation under the same parameters. With the exception of 75 Hz square EMF, Asadian *et al*[42] discovered that EMFs of various frequencies and waveforms (25, 50 Hz square, and sinusoidal waveform EMFs) enabled the suppression of BMSC growth. This might imply that MSCs from different sources had different sensitivities to EMFs. Distinct EMFs had different responses to MSCs. It is crucial to investigate the most appropriate EMF parameters for the proliferation or directed differentiation of MSCs from various sources. For instance, MSCs exposed for a brief period of time to low-amplitude and low-frequency pulsed EMF could be encouraged to differentiate into chondrogenic cells[43], while sinusoidal EMF at 1 mT, 15 Hz, 4 h/d was favorable for MSCs to differentiate into osteogenic cells[40,41], and higher-frequency EMF could also encourage MSCs to differentiate into neuronal cells[42].

Another independent factor influencing the destiny of MSCs has been identified as MG. Most of the research was thus for only conducted in a simulated MG (SMG) environment produced by a clinostat or rotating vessel, since examining the proliferation and differentiation patterns of MSCs in an actual MG environment led to some technical and budgetary challenges[44]. Quynh *et al*[45] found that SMG inhibited the proliferation of human umbilical cord MSCs by blocking the cell cycle; in contrast, a study by Nakaji-Hirabayashi *et al*[46] revealed a proliferative effect. The various SMG action times could be responsible for this circumstance. Shorter SMG treatments appeared to inhibit osteogenesis[47-49] and promote endothelial cell differentiation[48], neuronal differentiation[44,48], and adipogenic differentiation[48,49]. However, extended SMG decreased the potential for chondrogenic differentiation in MSCs[50] and encouraged their differentiation toward osteogenesis[46,48]. Different SMG action times had different effects on the cytoskeleton and could even lead to the aforementioned changes through different signal transduction pathways. In this regard, further studies are needed to determine the appropriate SMG treatment time in regulating the specific lineage differentiation of MSCs.

FSS refers to the mechanical force caused by the friction of fluid flow on the apical cell membrane. It has been demonstrated that the proliferation and differentiation of MSCs are significantly influenced by the strength, timing, and rate of FSS. Jing *et al*[51] discovered that the proliferation of BMSCs could be effectively induced by 0.06 dyn/cm$^2$ of FSS stimulation, but as the intensity of the FSS increased, cell proliferation gradually decreased or was even inhibited. Meanwhile, Zhao *et al*[52] revealed that FSS

**Table 3 Physical stimuli and their effects on the differentiation of mesenchymal stem cells**

| Physical stimuli | Parameters | Cell source | Results | Ref. |
|---|---|---|---|---|
| EMF | 1 mT, 15 Hz, 4 h/day | BMSCs | BMSCs pretreated with EMF exhibited stronger osteogenic and chondrogenic differentiation potential and weaker adipogenesis capacity | Tu *et al*[40] |
| | 25, 50, 75Hz square and sinusoidal waveform EMF | BMSCs | EMF induced BMSCs differentiation to neuron cells in all treatment groups | Asadian *et al*[42] |
| | 1 mT, 15 Hz, 4 h/day | Rabbit MSCs | EMF enhanced the osteogenic potential of MSCs | Wang *et al*[41] |
| | PEMF | MSCs | Brief exposure to low amplitude PEMFs enhanced the ability of MSCs to produce and secrete paracrine factors capable of promoting cartilage regeneration | Parate *et al*[43] |
| SMG | 30 g for 72 h or 10 days | Adult rat MSCs | A shorter period of SMG promoted MSCs to differentiate into endothelial, neuronal and adipogenic cells. In comparison, a longer period of SMG promoted MSCs to differentiate into osteoblasts | Xue *et al*[48] |
| | 10 rpm, 72 h, 0.001 G | BMSCs | Inhibited osteogenic differentiation of MSCs | Liu *et al*[47] |
| | 30 rpm clinorotation, 3 d | Adult rat MSCs | Promoted the neuronal differentiation of rat MSCs | Chen *et al*[44] |
| | 7 rpm, 21 d | hMSCs | Lowered the chondrogenic potential of hMSCs | Mayer-Wagner *et al*[50] |
| Microgravity | 0.001 G | hMSCs | microgravity-cultured hMSCs showed a better ability to differentiate into osteoblasts and adipocytes compared to cells cultured under natural gravity conditions | Nakaji-Hirabayashi *et al* [46]] |
| Spare microgravity | | hMSCs | Spare microgravity reduced the osteogenic differentiation of hMSCs and shifted the osteogenesis of hMSCs into adipogenesis, even during ostergenic induction | Zhang *et al*[49] |
| FSS | 0.375 dyn/cm$^2$, 2 h/d | BMSCs | Promoted osteogenesis-related genes and proteins in BMSCs | Jiang *et al*[54] |
| | 0.06 dyn/cm$^2$, 6 h/d | BMSCs | Proper FSS stimulation obviously enhanced BMSCs osteogenesis, while the expressions of osteogenic genes decreased with higher intensity of FSS | Jing *et al*[51] |
| | 0.5, 0.8 Pa, 3 h/d | MSCs | Promoted MSCs ostegenesis | Jiao *et al*[55] |
| | 3-7 dynes/cm$^2$ | hMSCs | Enhanced osteogenic differentiation | Zhao *et al*[52] |
| | 4.2 dynes/cm$^2$ | hMSCs | FSS could lead to the osteogenic differentiation of hMSCs | Liu *et al*[53] |
| | ΔSS from 0 dyn/cm$^2$ to 10 dyn/cm$^2$ | MSCs | Fast ΔSS (0–0') profits the chondrogenic differentiation, while Slow ΔSS (0–2') advances osteogenic differentiation | Yue *et al*[57] |
| | ΔSS from 0 dyn/cm$^2$ to 10 dyn/cm$^2$ | MSCs | Fast ΔSS (0–0') profits the chondrogenic differentiation, while Slow ΔSS (0–2') advances osteogenic differentiation | Lu *et al*[56] |
| HP | 10 MPa, 1 Hz, 4 h/d, 5 d/w, 3 w | BMSCs | HP promoted BMSCs chondrogenic differentiation | Steward *et al*[60] |
| | 0-0.5 MPa, 0.5 Hz | hMSCs | HP promoted the differentiation of the hMSCs toward osteogenesis | Huang *et al*[59] |
| | 270 kPa, 1 Hz, 1 h/d, 5 d/w, 3 w | BMSCs | HP promoted chondrogenic differentiation of BMSCs | Luo *et al*[64] |
| | 100 psi | ADSCs | HP significantly increased osteogenic differentiation of AMSCs | Ru *et al*[65] |
| | 90 kPa, 1 h | BMSCs | HP promoted chondrogenic differentiation of BMSCs | Zhao *et al*[61] |
| | 90 kPa, 1 h | BMSCs | HP promoted the expression of marker genes for early osteogenic differentiation and chondrogenic differentiation of the BMSCs | Zhao *et al*[62] |

BMSCs: Bone mesenchymal stem cells; EMF: Electromagnetic field; PEMF: Pulsed electromagnetic field; ADSCs: Adipose-derived stem cells; SMG: Simulated microgravity; hMSCs: Human mesenchymal stem cells; hucMSCs: Human umbilical cord mesenchymal stem cells; FSS: Fluid shear stress; ΔSS: Rate of fluid shear stress; HP: Hydrostatic pressure.

regulated cell proliferation in a rate- and time-dependent manner, with high FSS (9–20 dyn/cm$^2$) and the continuous effect of low FSS both inhibiting MSC proliferation, but the intermittent effect of low FSS (1–9 dyn/cm$^2$) appeared to have little or no effect. Liu *et al*[53] shown that FSS (4.2 dyn/cm$^2$) could promote the proliferation of MSCs implanted on 3D poly(lactic-co-glycolic acid) scaffolds. Although the

effects of FSS on the proliferation of MSCs were differently stated, its promotion of osteogenic differentiation[52-55] seemed to be consistent. Regarding how the rate of FSS (ΔSS) affects MSCs, it was observed that quick ΔSS (From 0 dyn/cm$^2$ in 0 min) was more beneficial for MSCs' chondrogenic development, whereas slow ΔSS (From 0 dyn/cm$^2$ in 2 mins) encouraged their osteogenic differentiation [56,57]. Clearly, more research is required to confirm the impact of FSS on MSC proliferation, as well as the appropriate stimulus parameters for osteogenic differentiation and MSC proliferation.

HP, unlike other physical stimuli, applies homogeneous tension without causing cellular deformation [58]. Physiological load (0.1-10 mPa) did not affect the proliferation of MSCs[59,60], whereas a load of 90 kPa effectively promoted the proliferation of MSCs[61,62], a possibility that resulted from the initiation of the cell cycle by HP[62]. Studies conducted in the past have indicated that HP at low loads (1–50 kPa) has an osteogenic impact on MSCs, whereas HP at physiological loads efficiently promoted chondrogenic differentiation[63]. This concept was also supported by several recent research works[60,64]. Some investigations, however, discovered a facilitative effect of physiological loading on MSCs' osteogenic differentiation[59,65], and a chondrogenic effect of low loading on MSCs[61,62]. Additionally, the study by Zhao *et al*[62] discovered that HP (70 kPa) could not only stimulate RhoA activation, which in turn promoted the expression of early osteogenic differentiation genes in BMSCs, but could also upregulate Rac1 and downregulate RhoA, which further promoted cartilage development in BMSCs. These findings suggested that further studies are needed to determine the effects of different loads of HP on the spectral differentiation of MSCs and their complex mechanisms.

Overall, physical stimuli did influence MSCs' proliferation and differentiation to varying degrees, but there is still no consensus on the parameters that are most conducive to the proliferation and specific lineages' differentiation of MSCs. Cell heterogeneity, various stemness potentials, culture conditions, and techniques that simulated physical stimulation might all be contributing factors in this issue. Therefore, more studies are needed to determine the appropriate parameters of physical stimuli that promote the differentiation of MSCs. In fact, the actual microenvironment in which cells were exposed was multifactorial. Therefore, some studies are now starting to consider the effect of compound factors [50,55,61,66] on the behavior of MSCs. Compound factors could have synergistic effects that increase the benefits for MSCs or counteract the drawbacks of a single factor. This might emerge as a new trend.

## HYPOXIA

In most studies, MSCs were cultured under atmospheric oxygen tension (20%-21% $O_2$)[67]. However, MSCs in different ecological niches encounter oxygen concentrations that are significantly lower than 20% (Table 4). For instance, the $O_2$ concentration that MSCs experienced varied from 1% to 5%[68] in adipose tissue and from 1% to 7%[69] in bone marrow. As a result, MSCs from different tissue sources were in a hypoxia microenvironment *in vivo*. Hypoxia activated various signaling pathways within a cell, which could lead to either cell death or cell adaptation[70]. Theoretically, culturing MSCs at physiological oxygen concentrations facilitated their proliferation, differentiation, and the secretion of cytokines and growth factors. Ciapetti *et al*[71] discovered that hypoxic circumstances greatly boosted BMSCs' proliferation and colony-forming capacity, as well as the expression of genes relevant to bone, such as alkaline phosphatase and osteocalcin, supporting the above idea. In contrast, in a study by Xu *et al*[72], hypoxia inhibited the osteogenic differentiation of BMSCs by activating the Notch pathway. Therefore, we focus on the effect of hypoxia on the behavior of MSCs and try to explain the contradictory findings in different studies.

The two primary techniques used nowadays to create *in vitro* hypoxic settings are anaerobic chambers [73] and simulation utilizing different chemicals[74]. In a study by Elabd *et al*[75], moderate hypoxia (5% $O_2$) circumstances promoted the chondrogenic and adipogenic differentiation of BMSCs but had no effect on proliferation or osteogenic differentiation. At the same oxygen concentration, Lee *et al*[76] showed that hypoxia promoted MSC proliferation and increased the chondrogenic differentiation potential. The proliferation of MSCs was also effectively promoted at a 5.5%-6.5% $O_2$ concentration simulated by 10 μM $CoCl_2$ and 4.0 mmol/L $Na_2SO_3$[74]. In contrast, Yu *et al*[77] demonstrated that a 50 M $CoCl_2$-simulated hypoxia environment appeared to prevent the growth of MSCs. Consistently, the osteogenic differentiation of MSCs was promoted in hypoxia environments simulated using different concentrations of $CoCl_2$[74,77]. Cicione *et al*[78] investigated the changes in the trilineage differentiation potential of BMSCs under severe hypoxia (1% $O_2$) and showed that the trilineage differentiation potential of BMSCs was inhibited to different degrees. Additional research demonstrated that the activation of the Notch pathway may be responsible for the suppression of the osteogenic differentiation of MSCs by severe hypoxia (1% $O_2$)[3,72]. In addition, Kim *et al*[79] found that hypoxia could inhibit the osteogenic differentiation of ADSCs by upregulating insulin-like growth factor binding-protein-3. Hypoxia has also been shown to encourage the tendon[73] and neural[80] differentiation of MSCs.

Compared to the laboratory culture environment (20%-21% $O_2$), hypoxia is more representative of the oxygen concentration in the ecological niche of MSCs. Varied oxygen concentrations had extremely different impacts on MSCs. Moderate hypoxia environment enhanced MSCs' proliferation, osteogenic differentiation, and chondrogenic differentiation. The differentiation capability of all three lineages of

**Table 4 Hypoxia and their effects on the differentiation of mesenchymal stem cells**

| Conditions | Cell source | Results | Ref. |
|---|---|---|---|
| Hypoxic culture (5%$O_2$) | BMSCs | ↑ Chondrogenic differentiation; ↑ adipogenic differentiation | Elabd *et al*[75] |
| Hypoxic culture (5.5%-6.5%$O_2$) | Balb/c mouse clonal MSCs | ↑ Osteogenic differentiation | Kim *et al*[74] |
| Hypoxic culture (50 μM $CoCl_2$ simulation) | Mice MSCs | ↑ Osteogenic differentiation | Yu *et al*[77] |
| Hypoxic culture (5%$O_2$) | ADSCs | ↑ Chondrogenic differentiation | Lee *et al*[76] |
| Hypoxic culture (1%$O_2$) | PBMSCs | ↑ Osteogenic differentiation | Yang *et al*[3] |
| Hypoxic culture (1%$O_2$) | BMSCs | ↓ Osteogenic differentiation; ↓ adipogenic differentiation; ↓ chondrogenic differentiation | Cicione *et al*[78] |
| Hypoxic culture (1%$O_2$) | BMSCs | ↑ Neuronal differentiation | Wang *et al*[80] |
| Hypoxic culture (1%$O_2$) | BMSCs | ↓ Osteogenic differentiation | Xu *et al*[72] |
| Hypoxic culture (2%$O_2$) | ADSCs | ↑ Tenocyte differentiation | Yu *et al*[73] |
| Hypoxic culture (2%$O_2$) | ADSCs | ↓ Osteogenic differentiation | Kim *et al*[79] |
| Hypoxic culture (2%$O_2$) | BMSCs | ↑ Osteogenic differentiation | Ciapetti *et al*[71] |

↑: Increase; ↓: Decrease; BMSCs: Bone mesenchymal stem cells; MSCs: Mesenchymal stem cells; ADSCs: Adipose-derived stem cells; PBMSCs: Peripheral blood mesenchymal stem cells.

MSCs was, however, somewhat hindered under severe hypoxia. The contradictory behavior in the aforementioned research might potentially be connected to the cell source of MSCs and whether they were differentiated under hypoxia conditions. In view of current studies generally focusing on hypoxia exposure either in the phase of expansion or differentiation, which have not been fully grasped, further research is necessary to comprehend the effects on MSCs specifically in these two culture forms.

## MATRIX STIFFNESS AND SURFACE TOPOGRAPHY

Two crucial material physical characteristics that have a significant impact on MSC behavior are matrix stiffness and surface topography. Matrix stiffness is a passive mechanical parameter that the cell can not directly sense. By exerting traction pressures on the cytoskeleton through focal adhesion, cells might deform the extracellular matrix (ECM), reflecting matrix stiffness[81]. Materials with ECM properties are currently being designed to simulate the actual microenvironment of cells. The ECMs of different native tissues, such as bone, cartilage, nerves, or blood vessels, are composed of micro- and nanoscale topographic patterns[82]. As a result, an increasing number of researchers have begun to look into how the substrate surface topography affects MSC behavior. Size and surface roughness are the two most fundamental parameters of surface topography[83], and the effects of these two factors, as well as substrate stiffness, on the proliferation and differentiation of MSCs are also mainly explored here.

### *Matrix stiffness*

Stiffness is one of the most common metrics in assessing a material's mechanical properties[81], and it is typically expressed in terms of Young's modulus. Matrix stiffness has been shown in many studies to affect the proliferation and differentiation of MSCs. MSCs exhibited higher proliferative behavior under a higher substrate stiffness, and Winer *et al*[84] found that MSCs inoculated in 250-Pa polyacrylamide gels that mimicked the elasticity of bone marrow and adipose tissue exhibited cell cycle arrest, but these arrested cells re-entered the cell cycle when a stiff substrate was present[84]. In comparison to lower-stiffness gels, higher-stiffness matrices could increase the number of cells by a factor of 10[85]. With fibronectin-coated polyacrylamide hydrogels, Sun *et al*[86] controlled the mechanical environment of BMSCs and discovered that BMSCs' proliferation increased with increasing stiffness. However, as opposed to firmer substrates, Lin *et al*[87] discovered that MSCs cultivated on softer substrates had greater cell proliferation rates. Gelma hydrogels with different concentrations not only had different hardness, but also showed different porosity as well. Moreover, the pore size also seemed to be one of the influencing factors for the proliferation and differentiation of MSCs. Indeed, many studies have focused on the effect of matrix stiffness on the direction of differentiation of MSCs. MSCs exhibited the upregulation of biomarkers matching tissue stiffness on polyacrylamide gels of different stiffness, such as neurogenic (0.1-1 kPa, brain), myogenic (8-17 kPa, muscle), and osteogenic (25-40 kPa, bone) markers

[88]. BMSCs could be driven to develop into an osteogenic phenotype and expressed increased quantities of bone-derived biomarkers including Runt-related transcription factor 2 (RUNX2), alkaline phosphatase (ALP), and bone-bridging proteins when grown on polyacrylamide hydrogels (62-68 kPa) [86]. Rowlands *et al*[85] found that the osteogenic differentiation of MSCs occurred mainly on polyacrylamide gels of 80 kPa stiffness and that RUNX2 was also expressed at high levels. This might be due to the fact that the 80 kPa collagen I coating could well simulate the microenvironment of the bone tissue. Without an induction medium, the stiffness of the hydrogel itself had a substantial impact in controlling MSC differentiation early on, with softer substrates encouraging the adipogenic differentiation of MSCs, while harder substrates encouraged the osteogenic differentiation of MSCs[89]. However, this effect seemed to be gradually attenuated by biochemical effects in the culture medium, implying that the effects of different factors on the differentiation behavior of MSCs might occur at different stages of differentiation. On 22 kPa gels, as opposed to softer matrices, MSCs produced larger quantities of ALP, which was consistent with the effect of matrix stiffness on osteogenic fractionation shown in the previous work[90]. Although more disagreement has emerged regarding the effect of softer matrices on the differentiation fate of MSCs, such as adipogenic differentiation[84,90-92], myogenic differentiation[85,88], neurogenic differentiation[88], and endothelial differentiation[87], there seems to be a consensus on the osteogenic role of harder matrices for MSCs. The Stiffer matrix enabled cells to produce more cytoskeletal tension and sent differentiation signals *via* transmembrane proteins such as integrins[81,85], which promoted osteogenic differentiation. Furthermore, the nuclear localization of Yes-associated protein (a key mediator of mechano-transduction) and RUNX2 could be impacted by the substrate stiffness[89,90].

### *Surface topography*

Zhao *et al*[93] produced nanotubes of various sizes and micro- and nano-hybrid topographies with ECM-like micro/nanostructures and examined their effects on the proliferation and osteogenic differentiation of MSCs. They discovered that larger-sized nanotubes hindered the early proliferation of MSCs, but the micro- and nano-morphology group had a greater cell number. Additionally, they discovered that MSC osteogenic differentiation might be induced by micro/nanotopographies, even in the absence of osteogenic inducers[93]. Similar results were obtained by Chen *et al*[94], who discovered that the micron/submicron hybrid topography of Ti surfaces promoted osteogenic and chondrogenic differentiation in the early stages of induced differentiation. By introducing nanoengineered topographic glass substrates with different surface roughness, Qian *et al*[95] investigated the impact of surface morphology on the osteogenic differentiation of MSCs. They found that surface roughness could replace the osteogenic inducer dexamethasone and worked in concert with ascorbic acid and β-glycerophosphate to jointly promote the osteogenic differentiation of MSCs[95]. In the past, it was generally agreed that surface roughness seemed to have a positive effect on osteogenic differentiation[95-97]. The osteogenic differentiation of MSCs, however, was more strongly influenced by the nanopore size than by the surface roughness, according to several recent studies[83,98]. The frequent coupling of size and surface roughness in many studies makes it difficult to state the degree of influence of each factor on the behavior of MSCs[83]. Moreover, the methods used to prepare rough surfaces in these studies differ, such as randomly rough surfaces produced by treatments such as mechanical polishing, acid etching, *etc*., where cells form focal attachments that differ from those seen on surfaces of the same roughness [98]. Therefore, more research is required to demonstrate how size and surface roughness affect MSC proliferation and differentiation, respectively. Through a variety of pathways, including the control of adhesion, cytoskeletal tension, and nuclear localization of transcription factors[95], MSCs appeared to be able to detect and respond to the surface topography, which in turn influenced their behavior such as proliferation and differentiation. At this stage, it has been reported that micro- and nano-surface topographies inhibit the proliferation of MSCs and promote osteogenic differentiation to some extent. However, there is no detailed elaboration on their respective effects on MSCs in terms of size and surface roughness.

## EXOSOMES

Various cells jointly create the microenvironment by secreting functional molecules, which leads to the sharing of stimuli between multiple cell lineages[99]. In addition to the ECM and growth factors, exosomes were considered to be an important component of the microenvironment[100]. Exosomes are small vesicles with a diameter of 30-150 nm that are released by cells through cytosolic action. The released exosomes could interact with target cells and translocated proteins, lipids, mRNAs and miRNAs to the cytoplasm of target cells[101]. Crosstalk existed between MSCs-osteoblasts and monocytes-macrophages and researchers used this to regulate bone homeostasis[99]. In vitro, BMSCs' behaviors were influenced variably by cell-conditioned media produced by variously polarized macrophages[102]. Previous studies had suggested that cytokines were the main contributors to the function exercised by macrophages. However, Song *et al*[103] found that lipopolysaccharide (LPS)-activated macrophage-derived exosomes inhibited the osteogenic differentiation of BMSCs by

mediating inflammatory stimuli. Therefore, the effect of exosomes secreted by monocytes-macrophages on the differentiation of MSCs should be considered (Table 5).

According to Liu *et al*[104], miR-21a-5p found in M1 macrophage-derived exosomes directed BMSCs toward an osteoblastic fate during the early stages of osteogenesis[104]. In their investigation of the effects of MO, M1, and M2 macrophage-derived exosomes on BMSCs, Xia *et al*[105] discovered that M1 macrophage-derived exosomes efficiently enhanced the proliferation, osteogenic differentiation, and adipogenic differentiation of BMSCs, but M2 macrophage-derived exosomes were harmful to the proliferation of BMSCs and, curiously, all three hindered the chondrogenic differentiation of BMSCs. Xiong *et al*[106] noticed that miRNA-5106, enriched in M2 macrophage-derived exosomes, promoted the osteogenic differentiation of BMSCs by suppressing the expression of salt-inducible kinase 2 (SIK2) and SIK3, which was consistent with the role of M2 macrophage-derived exosomes in promoting osteogenesis in a study by Li *et al*[107]. Kang *et al*[108] demonstrated that M0 and M2 macrophage-derived exosomes were positive for BMSC osteogenesis while M1 macrophage-derived exosomes lowered BMP expression and inhibited the osteogenic differentiation of BMSCs[108]. Despite being enriched in distinct miRNAs, primary extraction M2 macrophages[109] and RAW264.7 mouse monocyte-macrophage leukemia cell[107] derived exosomes both showed osteogenesis-promoting and lipogenic differentiation-inhibiting effects. Current research has indicated the impact of exosomes produced from monocytes[110], osteoclasts[111], and osteoblasts[112] on BMSCs, in addition to exosomes released by macrophages. Ekström *et al*[110] found that exosomes released from LPS-stimulated monocytes could be ingested by MSCs and encouraged the osteogenic differentiation of MSCs. Liang *et al*[111] showed that osteoclast-released exosomes promoted osteogenic differentiation and facilitated osteogenic mineralization by inhibiting Rho GTPase activating protein 1. This might imply that active osteoclasts release large amounts of extracellular vesicles during the resorption phase, promoting the osteogenesis of MSCs for better stabilization and bridging the transition between bone resorption and formation. The addition of osteoblast exosomes could further enhance the expression of RUNX2 and osterix, thereby promoting osteogenic differentiation, and, in addition, osteoblast exosomes could even alter adipocyte ECM-mediated lineage differentiation[112].

Exosomes, one of the recently identified microenvironment components, have unique benefits, such as a nano size, non-toxicity, low immunogenicity, biocompatibility, and versatility of use, drawing widespread attention[113]. The current work appeared to demonstrate the beneficial influence of M2 macrophage-derived exosomes on the osteogenic differentiation of MSCs. As for MO and M1 macrophage-derived exosomes, further research is required to understand their impacts on MSC differentiation and the processes at play. At the same time, research has been conducted progressively on the influence of exosomes released by cells in the same microenvironment as BMSCs on the differentiation of BMSCs, which might represent a new avenue.

## CONCLUSION

MSCs play important roles in pathological and physiological processes because of their self-renewal, migration, and pluripotency. Especially due to their multi-differentiation potential, MSCs have been considered as a new therapeutic agent in regenerative medicine. Since the detailed mechanisms involved in these regulation processes has not been fully revealed, research on intrinsic and extrinsic factors regulating MSCs' differentiation may provide new methods in manipulating the cell fate of MSCs. Here, we discussed multiple chemical and mechanical factors affecting the osteogenic and chondrogenic differentiation of MSCs, including typical differentiation promoting patterns, cell environmental factors, and other interesting research areas, such as material morphology and exosomes. After sensing these differentiation-stimulating factors, MSCs from various sources are able to differentiate into specific cell lineages. With the rising demand for MSCs in clinical treatment, noble strategies have been developed that aim at inducing the stable and directional differentiation of stem cells, and further providing efficient methods of MSC regulation in basic research and clinical application.

Meanwhile, there is much more to discover in stem cell research. Due to some limitations of MSCs, such as homing efficiency and long-term survival *in vivo*, most of the research has achieved its results at the cellular level in vitro. Moreover, discrepancies remain between single-factor experiments and synergistic effects by multiple factors. At present, extensive research on factors stimulating MSCs' differentiation has promoted our understanding of cell functional alterations. However, mechanisms involved in manipulating MSCs' cell fate have so far been incomplete. With the deepening of stem cell research alongside technology improvements, the synergistic effect of multiple factors inducing MSC differentiation is increasingly likely to be clarified, as well as providing new patterns in clinical stem cell therapy.

**Table 5 Exosomes of different cell sources and their effects on the differentiation of mesenchymal stem cells**

| Source and kind | Specific cargo | Target | Results | Ref. |
|---|---|---|---|---|
| M1 macrophages-EVs | miRNA-21a-5p | BMSCs | ↑ Osteogenic differentiation | Liu *et al*[104] |
| M0 macrophages-EVs | | BMSCs | ↓ Chondrogenic differentiation | Xia *et al*[105] |
| M1 macrophages-EVs | | BMSCs | ↑ Osteogenic differentiation; ↑ adipogenic differentiation; ↓ chondrogenic differentiation | |
| M2 macrophages-EVs | | BMSCs | ↓ Chondrogenic differentiation | |
| M2 macrophages-EVs | miRNA-5106 | BMSCs;SIK2 and SIK3 | ↑ Osteogenic differentiation | Xiong *et al*[106] |
| M2 macrophages-EVs | miRNA-690 | BMSCs | ↑ Osteogenic differentiation; ↓ adipogenic differentiation | Li *et al*[107] |
| M0 macrophages-EVs | | MSCs | ↑ Osteogenic differentiation | Kang *et al*[108] |
| M1 macrophages-EVs | miRNA-155 | MSCs | ↓ Osteogenic differentiation | |
| M2 macrophages-EVs | miRNA-378a | MSCs | ↑ Osteogenic differentiation | |
| M2 macrophages-EVs | miRNA-26a-5p | BMSCs | ↑ Osteogenic differentiation; ↓ adipogenic differentiation | Bin-bin *et al*[109] |
| Macrophages-EVs | | BMSCs | ↓ Osteogenic differentiation | Song *et al*[103] |
| Monocytes-EVs | | MSCs | ↑ Osteogenic differentiation | Ekström *et al*[110] |
| Osteoclasts-EVs | miRNA-324 | BMSCs | ↑ Osteogenic differentiation | Liang *et al*[111] |

↑: Increase; ↓: Decrease; EVs: Extracellular vesicles; MSCs: Mesenchymal stem cells; BMSCs: Bone mesenchymal stem cells; SIK2/SIK3: Salt-inducible kinase 2/3.

## FOOTNOTES

**Author contributions:** Zhou JQ and Wan HY contributed equally to this study; Jiang N conceived and designed the study; Zhou JQ searched the literature; Zhou JQ and Wan HY drew the figure; Zhou JQ and Wang ZX drafted the article; Wan HY and Jiang N made critical revisions; all the authors approved the final version of the submitted article.

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