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Review Article

Potential mechanisms underlying the Runx2 induced osteogenesis of bone marrow mesenchymal stem cells

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Abstract: Bone marrow derived mesenchymal stem cells (BM-MSCs) belong a type of pluripotent stem cells and can be induced to differentiate into osteoblasts (OB). Runt-related transcription factor 2 (Runx2) is an osteogenesis specific transcription factor and plays an important role in osteogenesis of BM-MSCs. It can promote the expression of osteogenesis related genes, regulate cell cycle progression, improve bone microenvironment and affect functions of chondrocytes and osteoclasts, which have involvement of a large amount of signal molecules including TGF-β, BMP, Notch, Wnt, Hedgehog, FGF and microRNA. In this paper, we summarize the mechanisms underlying the Runx2 induced osteogenesis of BM-MSCs.

Keywords: Bone marrow derived mesenchymal stem cells, Runx2, osteogenic differentiation

Introduction

Marrow mesenchymal stem cells (MSCs) are a type of pluripotent stem cells with a wide range of sources. Under certain conditions, they can be induced to differentiate into other cell types including osteoblasts, cartilage cells and adipocytes. Thus, MSCs have been used as ideal seed cells in tissue engineering in recent years and widely applied in tissue engineering studies [1]. Bone marrow derived mesenchymal stem cells (BM-MSCs) are collected from bone marrow and account for no more than 0.01% of bone marrow cells. Proportion of BM-MSCs is highest at birth and thereafter reduces over age. BM-MSCs can also differentiate into osteoblasts and transform into mast cells, leading to formations of cartilage cells and osteocytes, which are crucial for maintenance of bone metabolism balance under physiological conditions and of bone repair under pathological conditions [2]. The mechanisms involved in the differentiation of BM-MSCs into osteoblasts are complex, and numerous studies have been conducted to investigate the potential mechanisms underlying this process. It has been revealed that a variety of signal molecules are involved in this process including TGF-β, BMP, Notch, Wnt, Hedgehog and FGF. Large amounts of cytokines constitute a complicated network and interact with each other to promote the osteogenic differentiation of BM-MSCs. Of these signal molecules, Runx2 plays a central role. Some investigators propose that Runx2 is the most important factor involved in the osteogenic differentiation, and to investigate the mechanisms underlying the Runx2 induced osteogenic differentiation is clinically and theoretically important.

Osteogenic activity of Runx2

Runx2/Cbfα1 is also known as polyoma virus enhancer binding protein 2/core binding factor (PEBP2) or acute myelogenous leukemia factor (AML3). Runx2/Cbfα1 belongs to the Runt domain family which includes Runx1, Runx2 and Runx3. After transcription, Runx2 is spliced into different isoforms: Cbfa1/P56 (type I), Cbfa1/P57 (type II) and Osf2/Cbfa1 (type III). These isoforms differ in the N-terminal sequence and have distinct transcriptional regulation. Runx2 is highly conservative in its structure and their homology is as high as 99% among animals. Difference in Runx2 sequence is found in only several amino acids among animals. Studies have confirmed that the transfection efficiency of murine Runx2 is comparable among human, guinea pig and mouse cells [3].

There is a nuclear matrix targeting signal (NMTS) in the C terminal of Runx2. NMTS can localize Runx2 in a specific site of the nucleus (such as osteoblast-specific cis-acting element [OSE-2]) to regulate gene expression, which controls the osteogenic differentiation of BM-MSCs. To date, OSE2 like element has been identified in some osteogenesis related genes including osteocalcin (OSC), type I collagen, bone sialoprotein (BSP) and osteopontin (OPN) [4]. In addition, the C terminal of Runx2 may also bind to other proteins (such as Smads, Yes, Groucho/TLE) to form coregulators, enhancing its regulatory effect [5].

Numerous *in vitro* and *in vivo* studies have employed Runx2 over-expression to induce the differentiation of BM-MSCs into OB and to induce the activation of some osteogenesis related genes including OSC, col-1, OPN, BSP and Osterix [6]. Further studies confirm C2C12 cells have a high Runx2 expression in early stage of osteogenic induction, but Runx2 expression reduces significantly during the differentiation into mature OB, and Runx2 is undetectable during the differentiation of OB into osteocytes [7]. Thus, Runx2 may be involved in the initiation of differentiation into OB, its expression reduces in late stage and low Runx2 expression is crucial for the maintenance of OB function. Liu et al. [8] over-expressed Runx2 in OB at late stage of differentiation, and they found it inhibited the maturation of OB and reduced bone mass, resulting in multiple fractures. Thus, we speculate that the osteogenic effect of Runx2 on BM-MSCs is time and concentration dependent.

In addition, Runx2 is also involved in the regulation of cell cycle progression. Galindo et al. [9] found Runx2 expression increased significantly during the slow cell proliferation (such as in G0 phase, during serum-free culture or during contact inhibition), but reduced markedly during the rapid cell proliferation. Runx2 deficient osteogenic progenitor cells show rapid proliferation, but cell proliferation is strictly controlled following trasnfection of Runx2. This suggests that Runx2 may serve as an inhibitor of cell proliferation. In normal OB, Runx2 expression varies in different phases of cell cycle. In G1/S transition, G2 phase and M phase, Runx2 expression reduces, but it increases after mitosis. Currently, it is speculated that Runx2 may regulate cell cycle progression via auto-formation or degradation. Runx2 may affect and cooperate with cyclins and relevant regulatory cytokines to arrest cells in G0/G1 phase, which further activates the expressions of genes related to osteogenic phenotype, inducing the osteogenic differentiation.

In addition, Runx2 may also enhance the tolerance of BM-MSCs to hypoxia. Li et al. [10] transfect adenovirus expressing Runx2 into rabbit BM-MSCs and found the osteogenic potency was increased significantly under the hypoxia condition without evident adverse effects. Runx2 is also involved in the regulation of generation of chondrocytes and osteoclasts, promotes the angiogenesis, improves the osteogenic microenvironment, and induces the osteogenesis and bone growth. The bone of Runx2$^{-/-}$ mice is composed of cartilages. The maturation of chondrocytes is significantly impaired, few blood vessels form in the bone, and osteoclasts reduce significantly in Runx2$^{-/-}$ mice [11].

Role of Runx2 in the osteogenesis related signaling pathways

The osteogenic differentiation of BM-MSCs is a complicated process. First, bone progenitor cells differentiate into preosteoblasts which then form mature osteoblasts. These osteoblasts can secret a variety of extracellular matrix (ECM) and osteoblasts are embedded in mineralized ECM and become mature osteocytes. During the osteogenic differentiation of BM-MSCs, Runx2 has been regarded as one of the most important osteogenic transcriptional factor, plays a determinant role in the early osteogenic differentiation and has become a marker of early osteogenic differentiation. In addition, some signaling pathways and cytokines are involved in the cellular differentiation, and osteogenic microenvironment (such as oxygen partial pressure, tension force and electromagnetic wave) also affects the osteogenic differentiation. Runx2 is involved in several major signaling pathways and serves as a join point among these pathways.

Runx2 and BMP axis

Bone morphogenetic protein (BMPs) is a member of transforming growth factor-β (TGF-β) superfamily. It is an extracellular factor extract-

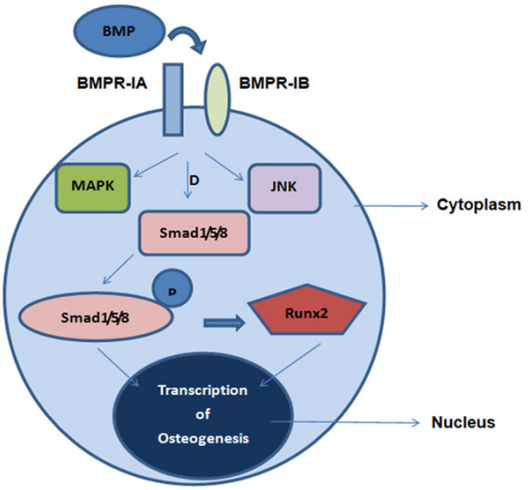

Figure 1. Signaling pathways involved in the BMP induced osteogenesis, BMPs bind to receptors on cells to activate downstream signal molecules such as Smads, MAPK and JNK. In the classic Smads signaling pathway, Smads protein is phosphorylated, leading to its activation. Activated Smads cooperate with Runx2 and then translocate into the nucleus to induce the transcriptional expression of osteogenesis related genes.

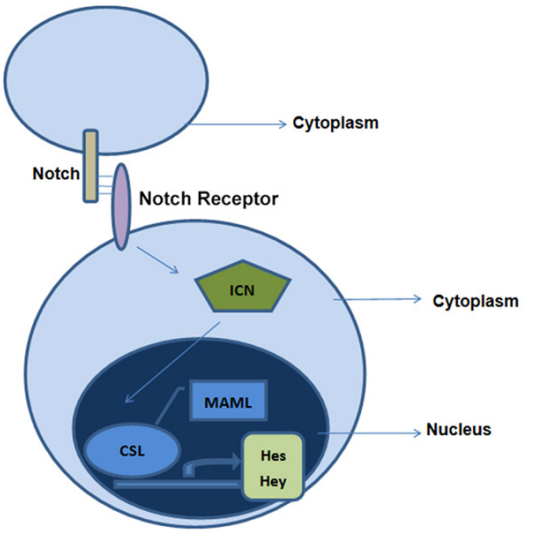

Figure 2. Notch signaling pathway [1], notch ligand on cells binds to Notch receptor on the adjacent cells, and then intracellular NICD is released, translocates into the nucleus, and binds to CSL and MAML to induce the transcriptional expression of Hes, Hey and other genes.

ed from the bone and has the activities of chondrogenesis and osteogenesis. BMP has been accepted as an osteogenic factor [12]. Numerous *in vivo* and *in vitro* studies have confirmed that BMP can enhance the osteogenic potential of BM-MSCs to induce osteogenesis. To date, more than 40 members of BMP family have been identified, and BMP-2, 4, 6, 7, 9 and 13 have been extensively investigated in available studies. BMP-2 and 7 have been approved for the clinical application due to their safety and effective osteogenic induction.

BMP may induce osteogenesis via endocrine and paracrine. Extracellular BMP may bind to and phosphorylate receptors (such as BMPR-IA and BMPR-IB) on the BM-MSCs to activate downstream cytokines including Smad1/5/8, MAPK and JNK (c-Jun N-terminal kinase), resulting in the activation of relevant signaling pathways. Of these pathways, Smad1/5/8 signaling pathway is a classic osteogenesis related pathway. Activated Smad protein may form Smads complex and then translocates into the nucleus. This complex may cooperate with Runx2 or function alone to induce the expression of osteogenesis related genes and then regulate the osteogenic differentiation of BM-MSCs at the transcriptional level [13] (**Figure 1**). Smads complex may recognize and bind to the SMID/NMTS domain in the C terminal of Runx2, which significantly increases the transcriptional specificity of Runx2. Mutation or knockout of Runx2 gene is able to block the interaction between Runx2 and Smad to inhibit the osteogenic differentiation of BM-MSCs [14].

BMPs affect the activity of Runx2. Nishimura et al. [15] found Smads complex could activate JunB (an upstream factor of Runx2) to indirectly activate Runx2 expression. BMP-2 may up-regulate Runx2 expression in a dose dependent manner, but has no influence on the promoter of Runx2. Smad6 and Smad7 may negatively regulate BMP expression to reduce Runx2 expression. In addition, Lee et al. [16] found TAK1-MKK3/6-p38 MAPK signaling pathway, a non-classic BMP signaling pathway, could promote Runx2 phosphorylation and bind to coactivator CREB (CBP) to induce the osteogenic differentiation of BM-MSCs. The Smads pathway and MAPK pathway may converge at Runx2 to affect the osteogenesis of BM-MSCs.

Runx2 and notch axis

Notch signaling pathway includes Notch ligand, receptor, CSL protein and Notch effector. Notch signaling pathway is a conservative signal conduction pathway and involved in a variety of cel-

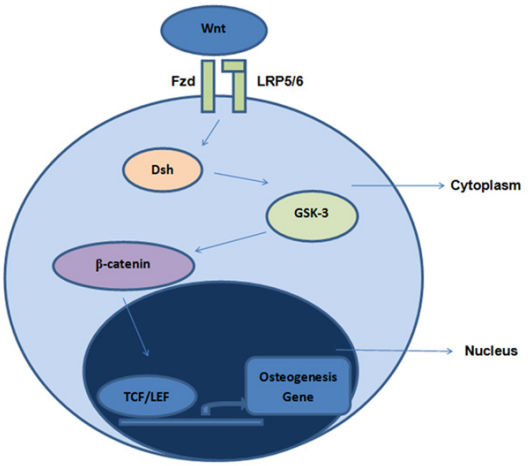

Figure 3. Wnt signaling pathway [1]. The Wnt-Fzd-LRP5/6 complex may recruit intracellular Dsh, inhibit GSK-β activity, and lead to the accumulation of β-catenin in cells, and then β-catenin translocates into the nucleus to activate the transcription of TCF/LEF.

lular processes. When Notch ligand binds to its receptor, Notch signaling pathway is activated, NICD translocates into the nucleus and binds to CSL protein to form the transcriptional activation complex which then activate HES, HEY and other genes, exerting their effects (**Figure 2**).

Available studies show Notch is able to promote or inhibit the osteogenesis. Grogan et al. [17] found the expressions of Notch-1 and Jagged-1 were detectable in the osteogenic differentiation of bMSCs, and over-expression of Notch-1 activates Notch signaling pathway to induce the osteogenesis. Hill et al. [18] found Jagged-1 knockout in mouse cranial neural crest cells caused maxillary hypoplasia, to inhibit Jagged-1 expression significantly suppressed the expression of BMPs receptor in mouse embryonic maxillary mesenchymal (MEMM) cells, and it was difficult to induce the differentiation of MEMM cells with BMPs. Some studies propose that Notch signaling pathway is able to inhibit the osteogenesis of BM-MSCs. Hilton et al. [19] found Notch knockout significantly increased the mass of trabecular bone. Several investigators propose that the osteogenic potential of Notch is time dependent: Notch might promote the osteogenic differentiation only in early stage, and cause the inhibition of osteogenic differentiation in late stage.

The Notch mediated inhibition of osteogenesis is related to the binding of effectors HEY and HES to Runx2 and the subsequent compromise of its transcriptional activity. Thus, it is feasible to inhibit Runx2 to maintain BM-MSCs in an undifferentiated status, promote their proliferation, increase the cells in the stem cell pool and elevate their osteogenic potential [20]. Hilton et al. [19] confirmed that transient transfection of NICD, HES or HEY into MSCs could reduce the transcriptional activity of Runx2. Zamurovic et al. [21] found BMP-2 could induce the HEY1 expression in preosteoblast MC3T3 cells, and the influence of HEY1 on Runx2 might negatively regulate the functions of BMP-2. In addition, Sakamoto et al. [22] found Zfp64, a coactivator of Notch, was a target gene of Runx2, and an OSE2 was identified in the promoter of zfp64 and might be involved in the Notch mediated negative regulation on Runx2.

HES-1 is co-expressed with Runx2 in the osteocytes of mammalians, and the mechanisms underlying the Notch induced osteogenesis might be also related to its regulatory effect on Runx2. Laabee et al. [23] found the transcriptional activity could be regulated by TLE and HES family, and HES was able to block the inhibitory effect of TLE on Runx2 to enhance the transcriptional activity of Runx2. MAML1 is a co-activator of Notch signaling pathway and can bind to CSL-NICD to form transcription activation complex and to activate the expressions of down-stream genes. Watanabe et al. [24] found that MAML-1 could significantly elevate the transcriptional activity of Runx2 to induce the expressions of osteogenesis related genes (such as ALP), and knockout of NICD binding site at the N terminal of MAML had no influence on the MAML induced activation of Runx2. This suggests that MAML1 may increase the transcriptional activity of Runx2 which is independent of downstream genes of Notch. To date, few studies have been conducted to investigate the Notch induced osteogenic differentiation, and more studies are warranted to explore the potential mechanisms.

Runx2 and wnt axis

Wnt family is composed of at least 19 conservative secreted glycoproteins and plays important roles in the osteogenic differentiation of BM-MSCs. In the classic Wnt signaling pathway, Wnt binds to Frizzled receptor to recruit LRP5/6 and then inhibit the activity of intracellular glycogen synthase kinase-β, which leads to the accumulation of β-catenin. Then, β-catenin translocates into the nucleus and activates the

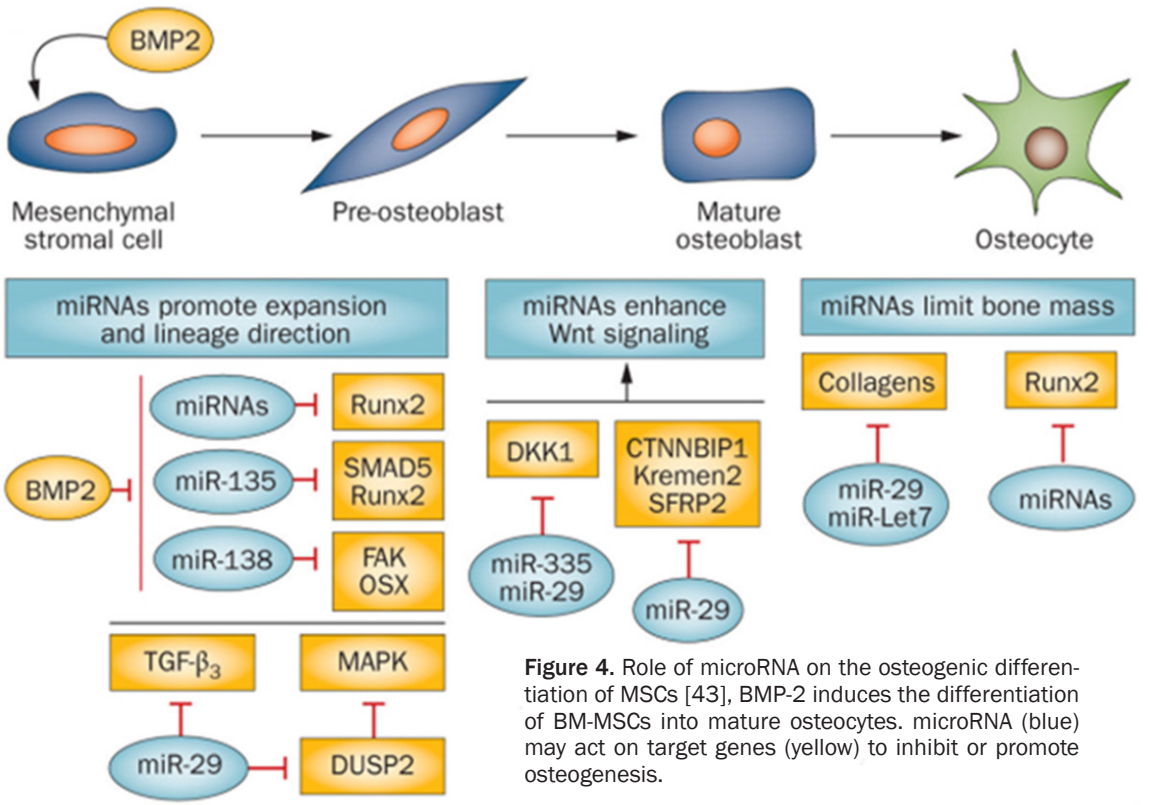

Figure 4. Role of microRNA on the osteogenic differentiation of MSCs [43], BMP-2 induces the differentiation of BM-MSCs into mature osteocytes. microRNA (blue) may act on target genes (yellow) to inhibit or promote osteogenesis.

transcription of TCF/LEF (**Figure 3**). The non-classic Wnt signaling pathway is independent of β-catenin, and PLC and PKC may release the calcium into the cytoplasm to exert effects [25].

Wnt signaling pathway has a potent ability to induce the osteogenesis. Wnt3a and 10a may activate classic Wnt signaling pathway, and Wnt4a and 5a are able to activate non-classic Wnt signaling pathway. Both pathways may promote the differentiation of OB and the bone formation, and to inhibit the Wnt signaling pathway is found to suppress the bone formation. However, a few studies reveal that Wnt signaling pathway is able to inhibit the osteogenesis. Boland et al. [26] investigated the role of Wnt3a in human MSCs and found Wnt3a was able to inhibit the ALP expression and reduce matrix mineralization to inhibit osteogenesis, and Wnt3a also inhibited the expressions of osteogenesis related markers in differentiated OB. Liu et al. [27] also confirmed that the activation of Wnt signaling pathway could induce the osteogenesis of MSCs in normal medium but inhibited the osteogenesis of MSCs in osteogenic medium.

A TCF regulatory element has been identified in the promoter of Runx2 and there is an activation site for Runx2 promoter in the TCF. Both β-catenin and TCF can bind to this activation site to exert their effects. Kook et al. [28] investigated the role of classic Wnt signaling pathway in the osteogenesis of human periodontal ligament cells, and they found Wnt could promote Runx2 expression, Wnt inhibition was able to suppress Runx2 expression, but Runx2 failed to affect the phosphorylation of β-catenin and GSK-3β, two downstream molecules of Wnt signaling pathway. Thus, Runx2 might be a unidirectional regulator of downstream pathway. Gaur et al. [29] found Runx2 was still normally expressed in β-catenin deficient mouse MSCs, but β-catenin/TCF1 was likely to promote Runx2 expression.

In a study of Nemoto et al. [30], classic Wnt signaling pathway was independently activated with endogenous and exogenous stimuli in dental bone cells, which reduced Runx2 expression and inhibited the osteogenic differentiation. Some investigators propose that Wnt/β-catenin signaling pathway and LEF1 complex are responsible for the direct inhibition of

Runx2 transcription. The different effects of Wnt signaling pathway on Runx2 might be related to the different cell types and different stages of differentiation. In addition, Daniel et al. [31] found Runx2 was able to cooperate with Cbfβ to activate the expression of Sclerostin, an inhibitor of Wnt signaling pathway, in MDA-MB-231 cells to inhibit the osteogenic differentiation of these cells. This Runx2 mediated osteogenic inhibition has been found to be closely related to the pathogenesis of bone tumors.

Runx2 and microRNA

microRNA is a group of evolutionarily conserved non-encoding RNAs and can bind to the 3' untranslated region (UTR) of mRNA to inhibit the mRNA activity, which exerts post-transcriptionally inhibitory effects. In recent years, increasing microRNAs are found to be involved in the osteogenic differentiation of BM-MSCs, and some microRNAs plays important roles in the osteogenic and adipogenic differentiation of BM-MSCs. microRNA may act on pro-osteogenic factors to inhibit the osteogenesis or on anti-osteogenic factors to promote the osteogenesis [32] (**Figure 4**).

Huang et al. [33] found both endogenous miR-204 and miR-211 were able to inhibit the transcriptional activity of Runx2 to inhibit the osteogenic differentiation of MSCs, and interfering of miR-204 expression increased the osteogenic differentiation of MSCs but compromised their adipogenic differentiation. In addition, they found the Runx2 inhibition remained unchanged in the presence of adipogenic induction after interfering of miR-204 expression. These suggest that Runx2 is not exclusively regulated by microRNA. Hassan et al. [34] found miR-23a was able to inhibit Runx2 and cooperate with the transcription of SATB2, a transcription factor, but inhibited the final maturation of OB. Hu et al. [35] found miR-205 could inhibit the transcriptional activities of Runx2 and STAB2. However, in *in vitro* experiment, Park et al. [36] over-expressed miR-23a in mice, and found there were no obvious changes in the bone as compared o wide type mice, suggesting that miR-23a has limited effect on the bone formation and the maintenance of bone balance. The discrepancy in findings of *in vitro* and *in vivo* experiments might be related to the difference in the experimental environment. Van et al. [37] found, in normal OB, p53, a tumor suppressor gene, was an upstream regulator of Runx2 and could activate miR-34c to inhibit the transcriptional activity of Runx2. Under pathological conditions, p53 is inactivated, miR-34c expression is also inhibited, and Runx2 expression is dysregulated, which might be closely related to the occurrence of osteosarcoma. In addition, some microRNA may act on Runx2. Zhang et al. [38] reported that miR-23a, miR-30c, miR-34c, miR-133a, miR-135a, miR-137, miR-204, miR-205, miR-217 and miR-338 were able to inhibit the transcriptional activity of Runx2 to inhibit the osteogenic differentiation of MC3T3 cells.

Some microRNAs act to promote the osteogenic differentiation of MSCs. Huang et al. [39] found miR-22 acted on the histone deacetylase 6 (HDAC6). HDAC6 is a co-suppressor of Runx2. miR-22 may abolish the inhibitory effect of HDAC6 on Runx2 to promote the osteogenic differentiation. Heparin-binding EGF-like growth factor (HB-EGF) is an inhibitor of osteogenic differentiation and is able to inhibit the osteogenic differentiation of MC3T3-E1 cells and human BM-MSCs cells. Yu et al. [40] found HB-EGF expression reduced in Runx2 induced osteogenic differentiation of C2C12 cells, Runx2 could specifically bind to the promoter of HB-EGF to inhibit its transcription and induce miR-1192 expression. miR1192 is able to bind to HB-EGF to inhibit its activity. Thus, Runx2 regulates HB-EGF at transcriptional and post-transcriptional levels.

Runx2 and other signaling pathways

FGF-2 is an important factor regulating the osteogenesis. FGF-2 may activate ERK signaling pathway and PKC signaling pathway to activate Runx2 and induce its expression. In addition, FGF may act on the PST region in the C terminal of Runx2 to promote the phosphorylation of Runx2 and increase its transcriptional activity. Naganawa et al. [41] found Runx2 mRNA expression reduced in mouse cells with FGF-2 knockout, but it returned to normal level after addition of FGF-2. The pro-osteogenic activities of FGF and BMPs may coordinate via Runx2.

TNF-α may counteract the BMP induced osteogenesis. Different from classic BMPs pathway, TNF-α/IL-1β may bind to and activate p38, ERK1/2 and JNK1/2 in the MAPK signaling

pathway, exerting effects contrary to those of BMPs. Huang et al. [42] confirmed that TNF-α/IL-1β activated MAPK pathway to reduce the BMP-2 induced Runx2 expression, leading to the inhibition of osteogenic differentiation.

Conclusion

Runx2 is a specific transcription factor crucial for the differentiation and maturation of osteoblasts and plays important roles in the bone formation and growth. Mutation and deficiency of Runx2 gene may significantly cause the abnormal bone development and growth. Runx2 silencing or over-expression may also affect the osteogenesis. The temporal Runx2 expression and its extent are strictly controlled. In addition, Runx2 may also be modified at post-translational level such as phosphorylation, acetylation, deacetylation and ubiquitination, which affects the structure stability and transcriptional activity of Runx2. To investigate the patterns at which Runx2 regulate other molecules and the ways in which Runx2 act on different pathways and cytokines will be helpful for the understanding of mechanisms underlying the osteogenic differentiation of BM-MSCs and for the therapy of bone related diseases.

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Disclosure of conflict of interest

None.

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