Journal of Bone and Mineral Metabolism (2019) 37:264–272
https://doi.org/10.1007/s00774-018-0935-y

ORIGINAL ARTICLE

MicroRNA-92a-1-5p influences osteogenic differentiation of MC3T3-E1 cells by regulating β-catenin

Zhiping Lin[1,2] · Yangyang Tang[3] · Hongchang Tan[2] · Daozhang Cai[1]

Received: 22 December 2017 / Accepted: 25 April 2018 / Published online: 17 July 2018

Abstract

Osteoblastic differentiation is a complex process that is critical for proper bone formation. An increasing number of studies have suggested that microRNAs (miRNAs) are pivotal regulators in various physiological and pathological processes, including osteogenesis. Here, we discuss the influence of miRNA-92a-1-5p on osteogenic differentiation. We found that miR-92a-1-5p was obviously downregulated during osteogenic differentiation of MC3T3-E1 cells. Gain-of-function and loss-of-function experiments revealed that miR-92a-1-5p was a negative regulator of osteogenic differentiation. Experimental validation demonstrated that β-catenin, which acts as a positive regulator of osteogenic differentiation, was negatively regulated by miR-92a1-5p. The findings of this study provide new insights into the possibility of miR-92a1-5p being a potential therapeutic target in the management of bone regeneration-related diseases.

Keywords Osteoblastic differentiation · miR-92A-1-5P · β-Catenin

Introduction

The bone is one of the organs that maintains the potential for persistent regeneration into adult life and is the only tissue that undergoes constant remodeling throughout life [1–3]. Efficacious bone differentiation can influence the management of bone and musculoskeletal-related disorders, such as fractures [4, 5]. Fracture healing is a dynamic physiological process involving a complex interplay of cells, mediators, and growth factors [6–8]. The healing process involves cellular recruitment, proliferation, and differentiation under the guidance of signaling molecules, and the deposition of extracellular matrix components, which serve as the foundation for a successful bone healing response [9]. Osteoblast differentiation and bone formation require a large number of genes to control coordinated processes, including cell proliferation, cell differentiation, bone matrix production, and mineralization. Thus, discovering novel mechanisms that are involved in the regulation of osteogenic differentiation will be meaningful for advancing our understanding of physiological and pathological osteogenesis.

MicroRNAs (miRNAs) are small noncoding single-stranded RNAs (18–25 nucleotides) that can regulate gene expression by binding to complementary sites in the 3′-untranslated regions of target messenger RNAs (mRNAs) to mediate translational inhibition or mRNA degradation [10–13]. A growing number of miRNAs have been found to be significant regulators of osteoblast differentiation and bone formation through different mechanisms [14]. For example, miR-34a can modulate the osteoblastic differentiation of human stromal stem cells [15]. miR-34c is considered important during osteogenesis partly because of its regulation of Notch signaling in bone homeostasis [16]. miR-145 inhibits osteoblast differentiation by targeting osterix and core-binding factor subunit β [17, 18]. MiR-5100 promotes osteogenic differentiation, which is modulated by binding to Tob2 [19]. Vishal et al. found that miR-590-5p can promote osteoblast differentiation by indirectly protecting and stabilizing the Runx2 protein by targeting Smad7 gene expression [20]. Several miRNAs, including miR-21, miR-23a, miR-24, miR-25, miR-100, and miR-125b, play important roles

✉ Daozhang Cai
daozhang@medmail.com.cn

1 Department of Orthopedics, The Third Affiliated Hospital of Southern Medical University, number 183, Zhong shan Road West, Guangzhou 510630, Guangdong, People's Republic of China

2 Department of Orthopedics, The Affiliated Hospital of Guangdong Medical University, Zhanjiang 524001, People's Republic of China

3 Guangdong Medical University, Zhanjiang 524023, People's Republic of China

in osteoporotic patients. Seeliger et al. [21] suggested that these miRNAs may be used as biomarkers for diagnostic purposes and may be targets for the treatment of bone loss and optimization of fracture healing in osteoporotic patients. Moreover, there may be several miRNAs that may be related to osteoblast differentiation but have not been discovered yet.

MiRNA-92a is a key miRNA that reportedly plays an important role in modulating physiological processes. For example, once stimulated with Toll-like receptor ligands, miR-92a decreases rapidly in macrophages and controls the inflammatory response by targeting the MKK4/JNK/c-Jun pathway [22]. MiR-92a controls the angiogenesis and functional recovery of ischemic tissues in mice [23]. MiR-17 is a key component of the miR-17-92 cluster that regulates the osteoblast differentiation in human periodontal ligament tissue-derived mesenchymal stem cells [24]. The miR-17-92 cluster regulates bone metabolism mainly through its function in osteoblasts. Osteoblasts derived from miR-17-92$^{+/\Delta}$ mice possessed a low proliferation rate, alkaline phosphatase (ALP) activity, and calcification [25]. However, the function of miR-17-92 during osteoblast differentiation remains unknown.

Given the roles of miRNAs in regulating osteoblast differentiation and bone formation, we hypothesized that miR-92a is involved in both osteoblast differentiation and pathogenesis. In this study, we illustrated that miR-92a-1-5p was a negative regulator of osteoblast differentiation. Furthermore, we determined that β-catenin was negatively regulated by miR-92a-1-5p, and its downregulation by miR-92a-1-5p could attenuate osteoblast differentiation.

Materials and methods

Antibodies and reagents

All substances were purchased from Gibco (Grand Island, NY, USA) unless otherwise stated. α-Minimal essential medium (α-MEM) was bought from Abcam (Cambridge, MA, USA). Licl (Wnt/β-catenin signaling pathway activator) was bought from Calbiochem (San Diego, CA, USA). Rabbit anti-RUNX2 (1:200) and β-catenin (1:200) polyclonal antibodies were acquired from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Rabbit anti-β-actin (1:1000) antibodies were bought from Sigma (St. Louis, MO, USA). HRP-conjugated goat anti-rabbit antibodies (1:5000) were acquired from Abcam (Cambridge, MA, USA). BMP-2 was purchased from R&D Systems (USA).

Cell lines and cell culture

The murine pre-osteoblast cell line MC3T3-E1 was obtained from ATCC (Manassas, USA) and cultured in accordance with the established protocols. Briefly, MC3T3-E1 cells were cultured in a proliferation medium containing α-MEM supplemented with 10% fetal bovine serum. All cells were cultured at 37 °C in an incubator (Life Technologies, Baltimore, MD, USA) containing 5% CO_2. Osteoblastic differentiation was induced by the addition of 200 ng/mL of bone morphogenetic protein 2 (BMP2) for seven or 14 days (25). After being washed with distilled water twice, the cells were photographed. MiR-92a-1-5p mimics, inhibitors, and small interfering RNAs against GSK-3β were synthesized by RiboBio (Guangzhou, China).

3-(4, 5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay analysis

MC3T3-E1 cell viability was determined by MTT assay. The cells transfected with miR-92a-1-5p mimic/inhibitor and the corresponding negative control were seeded into 96-well plates and cultured under regular conditions until they reached 80% confluence. Then, the culture medium was discarded, and fresh medium containing MTT (5 mg/mL in phosphate buffered saline (PBS), 150 μL/well, Sangon, Shanghai, China) was added. Then, the cells were incubated for another 4 h. Afterward, 150 μL of DMSO (Sigma) was added per well and shaken gently for 10 min to dissolve the formazan. Absorbance at 570 nm was determined using an ELISA reader. Cell viability assay was performed in quadruplicate and repeated thrice. Cell viability was expressed as the percentages of the value of normal cells.

Quantitative reverse transcription polymerase chain reaction (qRT-PCR)

TriPure isolation reagent was used to extract the total RNA from cell monolayers in accordance with the manufacturer's instruction. The first cDNA strands were synthesized by using an oligo(dT) primer and Revoscript™ Reverse Transcription PreMix (Intron Biotechnology, Gyeonggi-do, Korea). The expressed mRNA was analyzed using Brilliant II SYBR1 Green QPCR Master Mix (Stratagene, San Diego, CA, USA) on a LightCycler Nano Machine (Roche1). A 40-cycle thermal program was used, consisting of denaturation at 95 °C for 15 s, annealing at a specific temperature for 15 s, and extension at 72 °C for 15 s. Each experiment was performed in triplicate. The primer sequences were as follows: mmT-miR-92a-1-5p-F GGTTGGGATTTGTCG CAATGC; ALP sense primer 5′-AGCGACACGGACAAG AAGC-3′ and antisense primer 5′-GGCAAAGACCGCCAC ATC-3′; osteocalcin (OSX) sense primer 5′-GGGGAAAGG AGGCACAAA-3′ and antisense primer 5′-GAGCAAAGT CAGATGGGTAAGTAG-3′; Runt-related transcription factor 2 (RUNX2) sense primer 5′- GCACCCAGCCCATAA TAGA-3′ and antisense primer 5′-TTGGAGCAAGGAGAA CCC-3′; β-catenin sense primer 5′-ATCACTGAGCCTGCC

ATCTG-3′ and antisense primer 5′-GTTGCCACGCCTTCA TTCC-3′; β-actin sense primer 5′-GTCCCTCACCCTCCC AAAAG-3′ and antisense primer 5′-GCTGCCTCAACA CCTCAACCC-3′. The 2-ΔΔC_t method was used for data analysis normalized to β-actin. Notably, U6 was used as the endogenous control in analyzing miR-92a-1-5p expression.

Western blots

Western blots were performed as described. Briefly, the protein concentration was measured by using the BCA Protein Assay Kit (Thermo). The samples were combined with 2× SDS loading buffer, boiled for 10 min, and loaded onto 10 or 4–20% gradient polyacrylamide-SDS gel. The proteins were then transferred to a PVDF membrane (Millipore, Billerica, MA, USA) for 2 h at 200 mA, and the membranes were incubated in Odyssey Blocking Buffer (LiCor) for 2 h at room temperature. After incubation with primary antibodies overnight at 4 °C, the blots were washed thrice in TBS containing 0.1% Tween-20 for 15 min and then incubated with peroxidase- or IRDye-conjugated secondary antibody for 1 h in TBS and 0.1% Tween-20 at room temperature. Immunoreactivity was detected by chemiluminescence with an ECL reagent or LiCor imaging system.

Oligonucleotide and siRNA transfection

MiR-92a-1-5p mimics, inhibitors, and small interfering RNAs against GSK-3β were synthesized by RiboBio (Guangzhou, China). These reagents were transfected into MC3T3-E1 at final concentrations of 50 nM for miRNA and 25 nM for siRNA in a six-well plate. All transfections were performed using Lipofectamine 2000 Transfection Reagent (Invitrogen) in accordance with the manufacturer's instructions. Sequence: 5′-CCACTCAAGAACTGTCAAGTA-3′ against GSK-3β or a scrambled siRNA (5′-UUCUCCGAA CGUGUCACGUTT-3′ was synthesized by RiboBio (Guangzhou, China).

Alizarin red staining (ARS)

Alizarin red staining was performed for 14 days to detect the osteoblast calcification after BMP2 treatment. The cells were seeded in 24-well plates, washed with PBS, fixed in 95% ethanol for 10 min, washed with distilled water, and stained with alizarin red solution (1 g Tris and 0.1 g alizarin red (Bio Basic Inc., Canada) in 100 mL ultrapure water; pH was adjusted to 8.3 by using HCl for 30 min at 37 °C). After being washed with distilled water twice, the cells were photographed.

ALP activity

Alkaline phosphatase activity was analyzed using an ALP activity colorimetric assay kit (BioVision, Milpitas, CA, USA). The cultured cells were rinsed three times with PBS and 1% Triton X100 and then scraped into distilled water. This step was followed by three cycles of freezing and thawing. ALP activity was determined at 405 nm using nitrophenyl phosphate as substrate. The total protein content of the same sample was determined through the BCA method using the Pierce protein assay kit (Thermo Fisher Scientific, Rockford, IL, USA). ALP activity relative to the control treatment was calculated after normalization to the total protein content.

Statistical analysis

The quantitative data were expressed as mean ± SD. Statistical analysis was carried out using a one-way analysis of variance followed by a Bonferroni test for multiple groups or Student's *t* test between two groups (SPSS 13.0 software; SPSS Inc., Chicago, USA). Differences with a *p* value less than 0.05 were considered statistically significant.

Results

MiR-92a-1-5p was downregulated in osteogenic differentiation

To analyze whether miR-92a-1-5p was associated with osteogenic differentiation, we introduced a well-accepted model for investigating the osteogenic differentiation. After being cultured in BMP2 for 14 days, the MC3T3-E1 cells displayed a lower proliferative capacity than those cultured in OG medium (Fig. 1a, b). Then, the expression levels of osteogenic-related genes, including OSX, ALP, and RUNX2, were significantly upregulated under differentiation-inducing conditions for seven and 14 days, as confirmed by qRT-PCR (Fig. 1c). The upregulation of RUNX2 was further confirmed at the translational level by western blot (Fig. 1d). Additionally, the activity of ALP was promoted after BMP2 treatment (Fig. 1e). Furthermore, we found that miR-92a expression was markedly decreased in induced MC3T3-E1 cells during osteogenic differentiation (Fig. 1f).

MiR-92a-1-5p inhibited the BMP2-induced osteogenic differentiation of MC3T3-E1 cells

To clarify the biological roles of miR-92a-1-5p in osteogenic differentiation, we performed gain-of-function and loss-of-function studies on MC3T3-E1 cells. We stably transfected MC3T3-E1 cells with miRNA mimics or inhibitors and

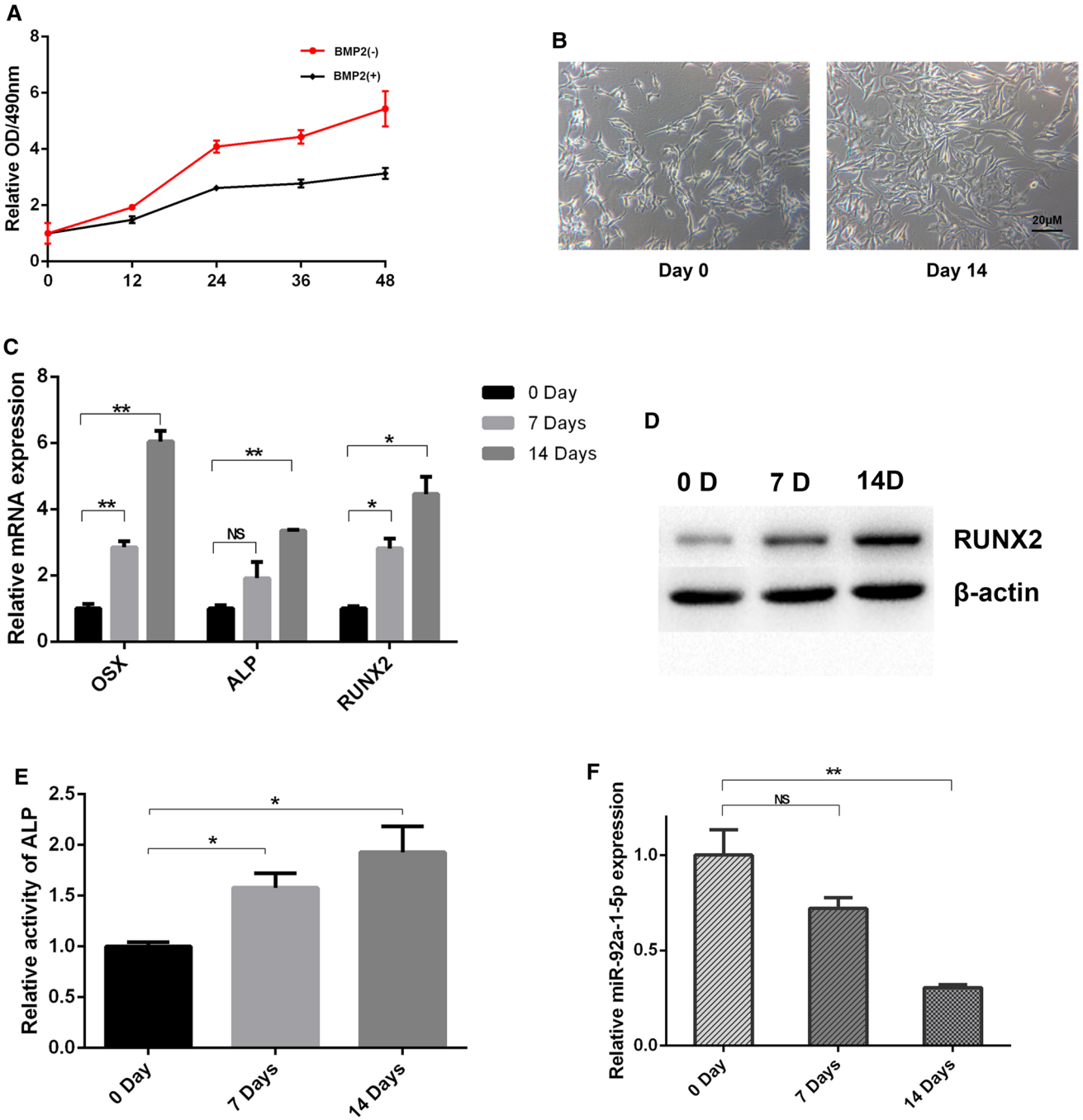


Fig. 1 MiR-92a-1-5p is downregulated in osteogenic differentiation. **a** Cell viability analysis of MC3T3-E1 cells treated with or without BMP2 after 3 weeks. **b** The morphology of MC3T3-E1 cells induced by BMP2 after 3 weeks. **c** RT-PCR analysis of osteogenic-related genes, OSX, ALP, and RUNX2, in induced MC3T3-E1 cells. **d** Expression of RUNX2 protein by western blot in MC3T3-E1 cells. **e** Activity of ALP at 0, 7, and 14 days after BMP2 treatment. **f** Expression of miR-92a-1-5p was assayed by qRT-PCR at 0, 7, 14 days after BMP2 treatment. Results are derived from at least three independent experiments. $^{*}p < 0.05$, $^{**}p < 0.01$

their negative controls (Fig. 2a). The results of qRT-PCR assay revealed that transfection with miR-92a-1-5p mimics markedly reduced the expression of ossification-related genes compared with those transfected with the negative control of the miRNA mimics. By contrast, silencing the miR-92a-1-5p expression by using miRNA inhibitors dramatically increased the levels of ossification-related genes in MC3T3-E1 cells (Fig. 2b). The results further revealed that the ALP activity in the differentiated MC3T3-E1 cells was inhibited by transfection with miR-92a-1-5p mimics,

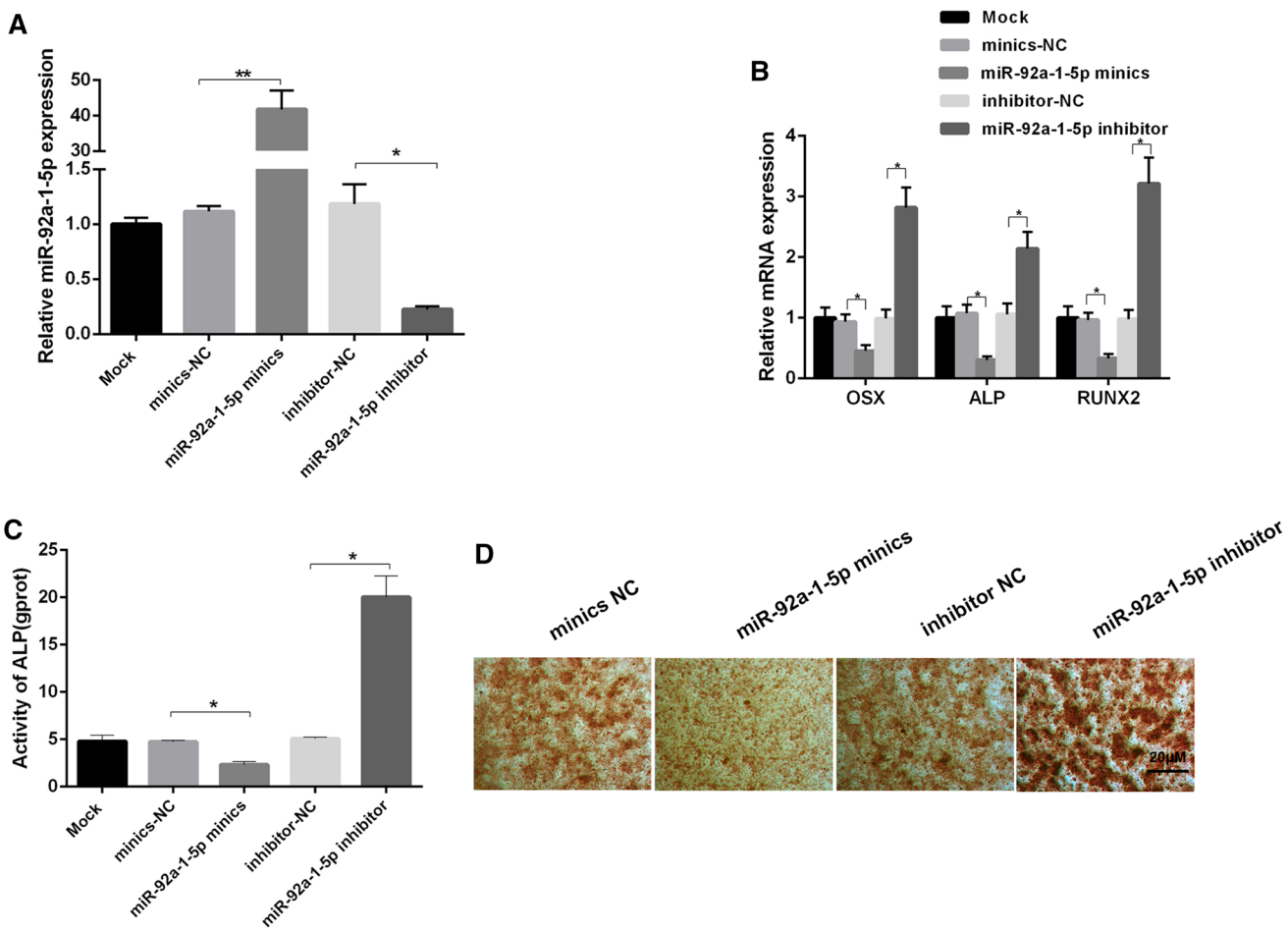


Fig. 2 MiR-92a-1-5p inhibits the osteogenic differentiation and mineralization. **a** Expression of miR-92a-1-5p was validated in MC3T3-E1 cells transfected with miR-92a-1-5p mimics or inhibitors and their corresponding controls by qRT-PCR. **b** qRT-PCR analysis of osteogenic-related genes, OSX, ALP, and RUNX2, in MC3T3-E1 cells. **c** Activity of ALP at 14 days after BMP2 treatment. **d** Effect of miR-92a-1-5p according to ARS of MC3T3-E1 cells. Results are derived from at least three independent experiments. $^{*}p < 0.05$, $^{**}p < 0.01$

but enhanced by transfection with miR-92a-1-5p inhibitors (Fig. 2c). In addition, the mineralization was analyzed, and several mineralization nodules were observed in the cells with miR-92a-1-5p inhibitors (Fig. 2d).

Mir-92a-1-5p downregulated the β-catenin expression at both transcription and protein levels

The Wnt/β-catenin signaling pathways played important roles in osteogenic differentiation. We explored whether miR-92a-1-5p regulated the osteogenic differentiation by suppressing β-catenin expression. The results of qRT-PCR and western blot revealed a notable downregulation of β-catenin after miR-92a-1-5p mimics were transfected into MC3T3-E1 cells, whereas β-catenin was upregulated in cells transfected with miR-92a-1-5p inhibitors (Fig. 3a, b). These results suggested that miR-92a-1-5p possibly regulated β-catenin gene expression in osteogenic differentiation.

β-Catenin was negatively regulated by miR-92a-1-5p involved in osteogenic differentiation

To further confirm whether the Wnt/β-catenin signaling pathway was involved in osteoblast differentiation by miR-92a-1-5p, we co-transfected GSK3-β siRNA and miR-92a-1-5p mimics into MC3T3-E1 cells. As shown in Fig. 4a–c, silencing GSK3-β could reverse the expression of β-catenin and ossification-related genes attenuated by miR-92a-1-5p mimics, as confirmed by qRT-PCR and western blot. Meanwhile, the Wnt signaling activator, Licl, was also used. The suppressive effects of miR-92a-1-5p on the expression of β-catenin and ossification-related genes were markedly impeded after Licl treatment (Fig. 4d–f). ARS showed the same tendency (Fig. 4g). Overall, these findings strongly suggested that β-catenin was negatively regulated by miR-92a-1-5p in the osteogenic differentiation.

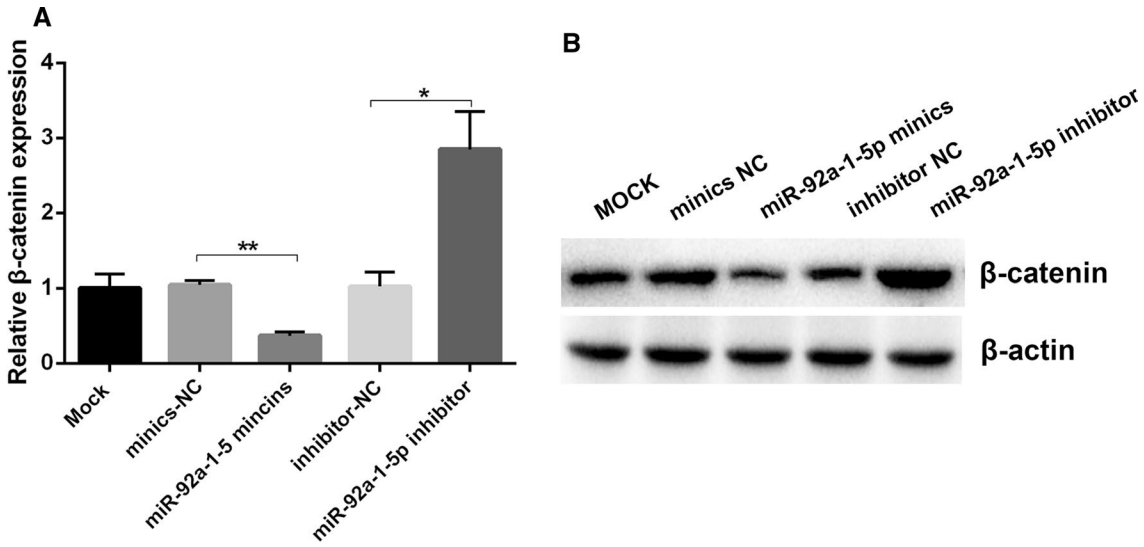


Fig. 3 Mir-92a-1-5p downregulates β-catenin expression at both the transcription and protein levels. The expression of β-catenin was detected in MC3T3-E1 cells transfected with miR-92a-1-5p mimics or inhibitors and their corresponding controls by qRT-PCR (**a**) and western blot (**b**). Results are derived from at least three independent experiments. *$p < 0.05$, **$p < 0.01$

Discussion

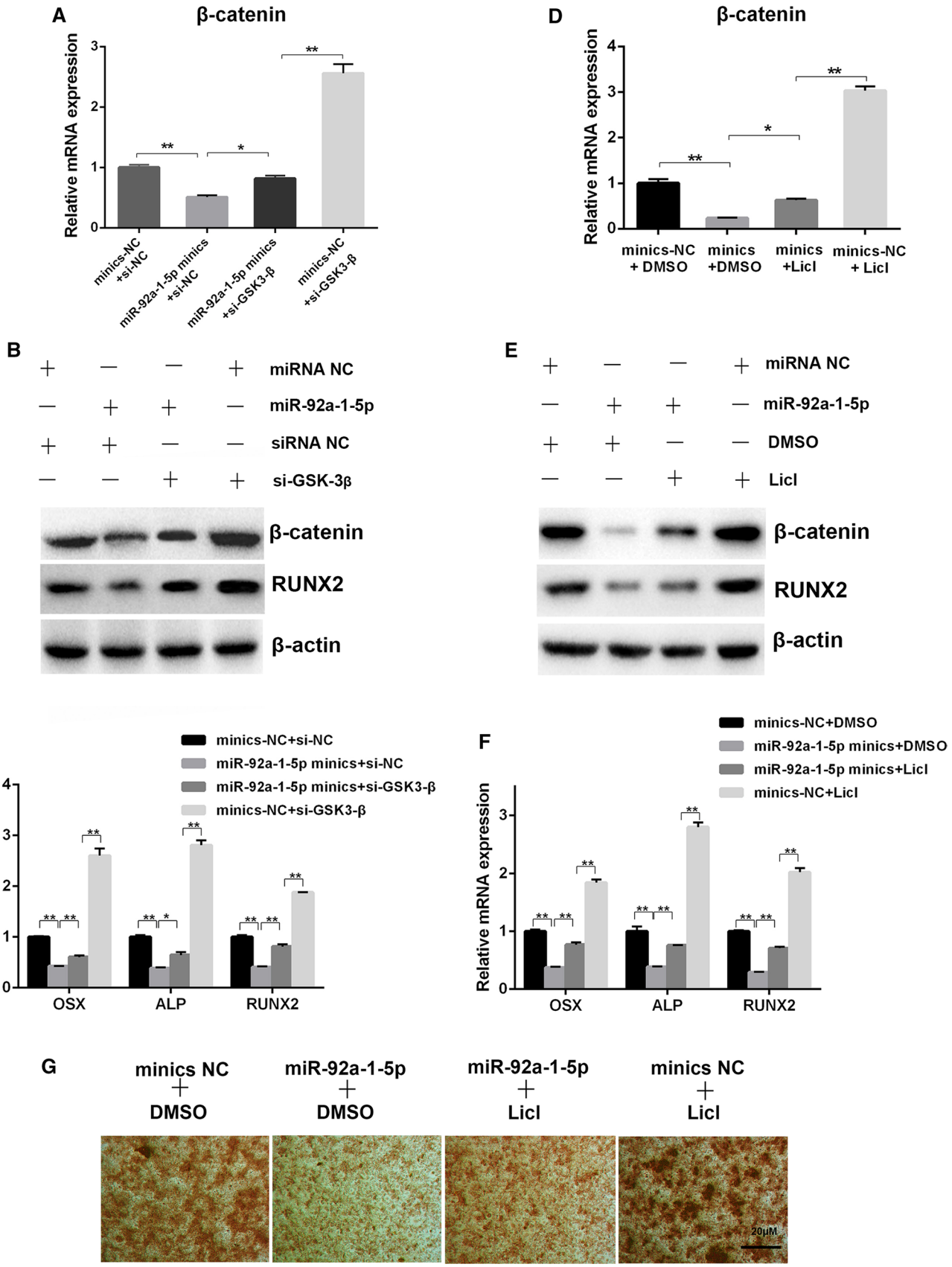
In this study, we revealed for the first time that miR-92a1-5p was obviously downregulated during osteogenic differentiation. Furthermore, we demonstrated that miR-92a-1-5p was a negative regulator of osteogenic differentiation because its overexpression reduced osteogenic differentiation, whereas its silencing led to the opposite effect. Finally, we demonstrated that miR-92a-1-5p blocked osteogenic differentiation partially by suppressing β-catenin expression.

Bone remodeling plays a key role in the maintenance and regeneration of bone tissues [26–28]. The imbalance of bone remodeling leads to bone abnormalities, of which the most common one is osteoporosis [29–31]. Efficacious bone regeneration could influence the management of bone and musculoskeletal-related disorders [4, 5]. Osteoblast differentiation requires numerous genes to control coordinated processes, including cell proliferation, cell differentiation, bone matrix production, and mineralization [32–34]. An increasing number of studies have identified several miRNAs that play important regulatory roles in bone biology [35–37]. Some miRNAs have been recently discovered as crucial negative or positive regulators of posttranscriptional gene expression and are considered important for osteogenesis. For example, miR-590-5p promotes osteoblast differentiation [20], whereas miR-145 inhibits osteoblast differentiation [18]. Nonetheless, there may be more miRNAs related to osteoblast differentiation that are yet to be discovered.

In this study, we showed that the gene expression of miR-92a-1-5p was markedly decreased in induced MC3T3-E1 cells during osteogenic differentiation by BMP2. The expression levels of osteogenic-related genes, including OSX, ALP, and RUNX2, were significantly upregulated. These data suggested that miR-92a was involved in the expression of regulators of osteogenic differentiation by influencing the expression of osteogenic differentiation markers [38, 39]. We found that transfection with miR-92a-1-5p mimics markedly reduced the expression of ossification-related genes, and the knockdown of miR-92a-1-5p expression by miRNA inhibitors considerably increased the ossification-related gene levels in MC3T3-E1 cells, further supporting the hypothesis that miR-92a-1-5p is involved in osteogenic differentiation.

BMP2 stimulated osteoblast differentiation along with Wnt signaling. BMP-2 activated Wnt signaling by inducing the expression of Wnt3a, Wnt1, and Lrp5, while inhibiting the expression of β-TrCP [40]. When exposed to both BMP and Wnt signaling, osteoblastogenesis was maximized [41]. Moreover, BMP signaling was promoted when the Wnt signaling was activated by Wnt3a or the overexpression of

◂**Fig. 4** β-catenin may be negatively regulated by miR-92a-1-5p involved in osteogenic differentiation. MC3T3-E1 cells were transfected with miR-92a-1-5p mimics and/or GSK3-β siRNA: **a** mRNA level of β-catenin was analyzed by qRT-PCR. **b** β-Catenin and RUNX2 were detected by western blot. **c** Osteogenic-related genes, OSX, ALP, and RUNX2, were detected by qRT-PCR analysis. MC3T3-E1 cells were treated with Licl and/or miR-92a-1-5p. **d** mRNA level of β-catenin was analyzed by qRT-PCR. **e** β-Catenin and RUNX2 were detected by western blot. **f** Osteogenic-related genes, OSX, ALP, and RUNX2, were detected by qRT-PCR analysis. **g** Effect of miR-92a-1-5p on the mineralization of differentiated MC3T3-E1 cells. Results are derived from at least three independent experiments. *$p < 0.05$, **$p < 0.01$


A
β-catenin
Relative mRNA expression
minics-NC +si-NC
miR-92a-1-5p minics +si-NC
miR-92a-1-5p minics +si-GSK3-β
minics-NC +si-GSK3-β
D
β-catenin
Relative mRNA expression
minics-NC + DMSO
minics +DMSO
minics +LiCl
minics-NC + LiCl
B
miRNA NC
miR-92a-1-5p
siRNA NC
si-GSK-3β
β-catenin
RUNX2
β-actin
E
miRNA NC
miR-92a-1-5p
DMSO
LiCl
β-catenin
RUNX2
β-actin
C
minics-NC+si-NC
miR-92a-1-5p minics+si-NC
miR-92a-1-5p minics+si-GSK3-β
minics-NC+si-GSK3-β
Relative mRNA expression
OSX
ALP
RUNX2
F
minics-NC+DMSO
miR-92a-1-5p minics+DMSO
miR-92a-1-5p minics+LiCl
minics-NC+LiCl
Relative mRNA expression
OSX
ALP
RUNX2
G
minics NC + DMSO
miR-92a-1-5p + DMSO
miR-92a-1-5p + LiCl
minics NC + LiCl
20μM

β-catenin/TCF4 [42]. Here, we confirmed that miR-92a-1-5p negatively regulated the expression of β-catenin, thereby suppressing osteogenic differentiation. The Wnt/β-catenin pathway is considered as the most attractive target for skeletal homeostasis and bone repair therapeutic intervention [43–46]. The activating mutations of the Wnt function in humans cause high bone mass phenotypes, such as van Buchem disease, whereas inactivating mutations cause osteopenic diseases, such as osteoporosis–pseudoglioma syndrome [47, 48]. In our investigation, we found that β-catenin was notably downregulated after miR-92a-1-5p mimics were transfected into MC3T3-E1 cells, whereas it was upregulated in the cells transfected with miR-92a-1-5p inhibitors. Either the addition of the Wnt signaling activator Licl or the knockdown of GSK3-β attenuated the suppression effect of β-catenin by miR-92a-1-5p. This finding was supported by the results of qRT-PCR and western blot. Thus, we hypothesized that miR-92a-1-5p regulated the translational and transcriptional levels of β-catenin, underlying its role in osteogenesis.

In conclusion, MiR-92a-1-5p was identified as a novel regulator in osteogenic differentiation by negatively regulating β-catenin. Therefore, this study provided new insights into the possibility of miR-92a-1-5p being a potential therapeutic target in the management of bone regeneration-related diseases.

Compliance with ethical standards

Conflict of interest All authors declare that they have no conflict of interest.

References

1. Zhang J, Guan J, Qi X, Ding H, Yuan H, Xie Z, Chen C, Li X, Zhang C, Huang Y (2016) Dimethyloxaloylglycine promotes the angiogenic activity of mesenchymal stem cells derived from iPSCs via activation of the PI3K/Akt pathway for bone regeneration. Int J Biol Sci 12:639–652. https://doi.org/10.7150/ijbs.14025
2. Ortega-Oller I, Padial-Molina M, Galindo-Moreno P, O'Valle F, Jodar-Reyes AB, Peula-Garcia JM (2015) Bone regeneration from PLGA micro-nanoparticles. Biomed Res Int 2015:415289. https://doi.org/10.1155/2015/415289
3. Serrano J, Romo E, Bermudez M, Narayanan AS, Zeichner-David M, Santos L, Arzate H (2013) Bone regeneration in rat cranium critical-size defects induced by Cementum Protein 1 (CEMP1). PLoS One 8:e78807. https://doi.org/10.1371/journal.pone.0078807
4. Thor A, Palmquist A, Hirsch JM, Rannar LE, Derand P, Omar O (2016) Clinical, morphological, and molecular evaluations of bone regeneration with an additive manufactured osteosynthesis plate. J Craniofacial Surg 27:1899–1904. https://doi.org/10.1097/SCS.0000000000002939
5. D'Mello S, Atluri K, Geary SM, Hong L, Elangovan S, Salem AK (2017) Bone regeneration using gene-activated matrices. AAPS J 19:43–53. https://doi.org/10.1208/s12248-016-9982-2
6. Liu X, McKenzie JA, Maschhoff CW, Gardner MJ, Silva MJ (2017) Exogenous hedgehog antagonist delays but does not prevent fracture healing in young mice. Bone 103:241–251. https://doi.org/10.1016/j.bone.2017.07.017
7. Wang Y, Newman MR, Ackun-Farmmer M, Baranello MP, Sheu TJ, Puzas JE, Benoit DSW (2017) Fracture-targeted delivery of beta-catenin agonists via peptide-functionalized nanoparticles augments fracture healing. ACS Nano 11:9445–9458. https://doi.org/10.1021/acsnano.7b05103
8. Wang J, He M, Wang G, Fu Q (2017) Organic gallium treatment improves osteoporotic fracture healing through affecting the OPG/RANKL ratio and expression of serum inflammatory cytokines in ovariectomized rats. Biol Trace Elem Res. https://doi.org/10.1007/s12011-017-1123-y
9. Simpson A (2017) The forgotten phase of fracture healing: the need to predict nonunion. Bone Joint Res 6:610–611. https://doi.org/10.1302/2046-3758.610.BJR-2017-0301
10. Bushati N, Cohen SM (2007) microRNA functions. Annu Rev Cell Dev Biol 23:175–205. https://doi.org/10.1146/annurev.cellbio.23.090506.123406
11. Henshall DC (2014) MicroRNA and epilepsy: profiling, functions and potential clinical applications. Curr Opin Neurol 27:199–205. https://doi.org/10.1097/WCO.0000000000000079
12. Foshay KM, Gallicano GI (2007) Small RNAs, big potential: the role of MicroRNAs in stem cell function. Curr Stem Cell Res Ther 2:264–271
13. Hobert O (2008) Gene regulation by transcription factors and microRNAs. Science 319:1785–1786. https://doi.org/10.1126/science.1151651
14. Taipaleenmaki H, Bjerre Hokland L, Chen L, Kauppinen S, Kassem M (2012) Mechanisms in endocrinology: micro-RNAs: targets for enhancing osteoblast differentiation and bone formation. Eur J Endocrinol 166:359–371. https://doi.org/10.1530/EJE-11-0646
15. Chen L, Holmstrom K, Qiu W, Ditzel N, Shi K, Hokland L, Kassem M (2014) MicroRNA-34a inhibits osteoblast differentiation and in vivo bone formation of human stromal stem cells. Stem Cells 32:902–912. https://doi.org/10.1002/stem.1615
16. Bae Y, Yang T, Zeng HC, Campeau PM, Chen Y, Bertin T, Dawson BC, Munivez E, Tao J, Lee BH (2012) miRNA-34c regulates Notch signaling during bone development. Hum Mol Genet 21:2991–3000. https://doi.org/10.1093/hmg/dds129
17. Jia J, Tian Q, Ling S, Liu Y, Yang S, Shao Z (2013) miR-145 suppresses osteogenic differentiation by targeting Sp7. FEBS Lett 587:3027–3031. https://doi.org/10.1016/j.febslet.2013.07.030
18. Fukuda T, Ochi H, Sunamura S, Haiden A, Bando W, Inose H, Okawa A, Asou Y, Takeda S (2015) MicroRNA-145 regulates osteoblastic differentiation by targeting the transcription factor Cbfb. FEBS Lett 589:3302–3308. https://doi.org/10.1016/j.febslet.2015.09.024
19. Wang H, Cui Y, Luan J, Zhou X, Li C, Li H, Shi L, Han J (2017) MiR-5100 promotes osteogenic differentiation by targeting Tob2. J Bone Miner Metab 35:608–615. https://doi.org/10.1007/s00774-016-0799-y
20. Vishal M, Vimalraj S, Ajeetha R, Gokulnath M, Keerthana R, He Z, Partridge NC, Selvamurugan N (2017) MicroRNA-590-5p stabilizes Runx2 by targeting Smad7 during osteoblast differentiation. J Cell Physiol 232:371–380. https://doi.org/10.1002/jcp.25434
21. Seeliger C, Karpinski K, Haug AT, Vester H, Schmitt A, Bauer JS, van Griensven M (2014) Five freely circulating miRNAs and bone tissue miRNAs are associated with osteoporotic fractures. J Bone Miner Res 29:1718–1728. https://doi.org/10.1002/jbmr.2175
22. Lai L, Song Y, Liu Y, Chen Q, Han Q, Chen W, Pan T, Zhang Y, Cao X, Wang Q (2013) MicroRNA-92a negatively regulates Toll-like receptor (TLR)-triggered inflammatory response in

macrophages by targeting MKK4 kinase. J Biol Chem 288:7956–7967. https://doi.org/10.1074/jbc.M112.445429
23. Bonauer A, Carmona G, Iwasaki M, Mione M, Koyanagi M, Fischer A, Burchfield J, Fox H, Doebele C, Ohtani K, Chavakis E, Potente M, Tjwa M, Urbich C, Zeiher AM, Dimmeler S (2009) MicroRNA-92a controls angiogenesis and functional recovery of ischemic tissues in mice. Science 324:1710–1713. https://doi.org/10.1126/science.1174381
24. Liu Y, Liu W, Hu C, Xue Z, Wang G, Ding B, Luo H, Tang L, Kong X, Chen X, Liu N, Ding Y, Jin Y (2011) MiR-17 modulates osteogenic differentiation through a coherent feed-forward loop in mesenchymal stem cells isolated from periodontal ligaments of patients with periodontitis. Stem Cells 29:1804–1816. https://doi.org/10.1002/stem.728
25. Zhou M, Ma J, Chen S, Chen X, Yu X (2014) MicroRNA-17-92 cluster regulates osteoblast proliferation and differentiation. Endocrine 45:302–310. https://doi.org/10.1007/s12020-013-9986-y
26. Notomi T, Kuno M, Hiyama A, Nozaki T, Ohura K, Ezura Y, Noda M (2017) Role of lysosomal channel protein TPC2 in osteoclast differentiation and bone remodeling under normal and low-magnesium conditions. J Biol Chem 292:20998–21010. https://doi.org/10.1074/jbc.M117.780072
27. Hasegawa T, Amizuka N (2017) Bone remodeling and modeling/mini-modeling. Clin Calcium 27:1713–1722
28. Jimi E (2017) The role of osteoclastic bone resorption on bone remodeling. Clin Calcium 27:1689–1695
29. Wang N, Xue P, Wu X, Ma J, Wang Y, Li Y (2018) Role of sclerostin and dkk1 in bone remodeling in type 2 diabetic patients. Endocr Res 43:29–38. https://doi.org/10.1080/07435800.2017.1373662
30. Yamamoto N (2017) Basic concept of bone remodeling. Clin Calcium 27:1683–1688
31. Inoue D (2017) Regulation of bone remodeling by anti-resorptives. Clin Calcium 27:1775–1783
32. Beck GR Jr, Moran E, Knecht N (2003) Inorganic phosphate regulates multiple genes during osteoblast differentiation, including Nrf2. Exp Cell Res 288:288–300
33. Pico MJ, Hashemi S, Xu F, Nguyen KH, Donnelly R, Moran E, Flowers S (2016) Glucocorticoid receptor-mediated *cis*-repression of osteogenic genes requires BRM-SWI/SNF. Bone Rep 5:222–227. https://doi.org/10.1016/j.bonr.2016.07.006
34. Swinehart IT, Schlientz AJ, Quintanilla CA, Mortlock DP, Wellik DM (2013) Hox11 genes are required for regional patterning and integration of muscle, tendon and bone. Development 140:4574–4582. https://doi.org/10.1242/dev.096693
35. Marini F, Cianferotti L, Brandi ML (2016) Epigenetic mechanisms in bone biology and osteoporosis: can they drive therapeutic choices? Int J Mol Sci. https://doi.org/10.3390/ijms17081329
36. Komori T (2016) Glucocorticoid signaling and bone biology. Horm Metab Res 48:755–763. https://doi.org/10.1055/s-0042-110571
37. Del Carpio-Cano FE, Dela Cadena RA, Sawaya BE (2013) HIV and bone disease: a perspective of the role of microRNAs in bone biology upon HIV infection. J Osteoporos 2013:571418. https://doi.org/10.1155/2013/571418
38. Guerrero J, Oliveira H, Aid R, Bareille R, Siadous R, Letourneur D, Mao Y, Kohn J, Amedee J (2017) Influence of the three-dimensional culture of human bone marrow mesenchymal stromal cells within a macroporous polysaccharides scaffold on Pannexin 1 and Pannexin 3. J Tissue Eng Regen Med. https://doi.org/10.1002/term.2625
39. Xu SJ, Qiu ZY, Wu JJ, Kong XD, Weng XS, Cui FZ, Wang XM (2016) Osteogenic differentiation gene expression profiling of hMSCs on hydroxyapatite and mineralized collagen. Tissue Eng Part A 22:170–181. https://doi.org/10.1089/ten.TEA.2015.0237
40. Rawadi G, Vayssiere B, Dunn F, Baron R, Roman-Roman S (2003) BMP-2 controls alkaline phosphatase expression and osteoblast mineralization by a Wnt autocrine loop. J Bone Miner Res 18:1842–1853. https://doi.org/10.1359/jbmr.2003.18.10.1842
41. Tang N, Song WX, Luo J, Luo X, Chen J et al (2009) BMP-9-induced osteogenic differentiation of mesenchymal progenitors requires functional canonical Wnt/beta-catenin signalling. J Cell Mol Med 13:2448–2464. https://doi.org/10.1111/j.1582-4934.2008.00569.x
42. Zhang R, Oyajobi BO, Harris SE, Chen D, Tsao C, Deng HW, Zhao M (2013) Wnt/beta-catenin signaling activates bone morphogenetic protein 2 expression in osteoblasts. Bone 52:145–156. https://doi.org/10.1016/j.bone.2012.09.029
43. Huang C, Ogawa R (2010) Mechanotransduction in bone repair and regeneration. FASEB J 24:3625–3632. https://doi.org/10.1096/fj.10-157370
44. Ghadakzadeh S, Kannu P, Whetstone H, Howard A, Alman BA (2016) beta-Catenin modulation in neurofibromatosis type 1 bone repair: therapeutic implications. FASEB J 30:3227–3237. https://doi.org/10.1096/fj.201500190RR
45. Baht GS, Silkstone D, Vi L, Nadesan P, Amani Y, Whetstone H, Wei Q, Alman BA (2015) Exposure to a youthful circulation rejuvenates bone repair through modulation of beta-catenin. Nat Commun 6:7131. https://doi.org/10.1038/ncomms8131
46. Minear S, Leucht P, Miller S, Helms JA (2010) rBMP represses Wnt signaling and influences skeletal progenitor cell fate specification during bone repair. J Bone Miner Res 25:1196–1207. https://doi.org/10.1002/jbmr.29
47. Shi GX, Zheng XF, Zhu C, Li B, Wang YR, Jiang SD, Jiang LS (2017) Evidence of the role of R-spondin 1 and its receptor Lgr4 in the transmission of mechanical stimuli to biological signals for bone formation. Int J Mol Sci. https://doi.org/10.3390/ijms18030564
48. Leucht P, Helms JA (2015) Wnt signaling: an emerging target for bone regeneration. J Am Acad Orthop Surg 23:67–68. https://doi.org/10.5435/JAAOS-23-01-67