Experimental and Molecular Pathology 107 (2019) 158–164

Contents lists available at ScienceDirect

Experimental and Molecular Pathology

journal homepage: www.elsevier.com/locate/yexmp

miR-26b modulates OA induced BMSC osteogenesis through regulating GSK3β/β-catenin pathway

He Hu[a], Chuanlong Zhao[b], Peiguang Zhang[c], Yalong Liu[d], Yulian Jiang[c], Enquan Wu[a], Hao Xue[c], Caiyun Liu[e], Zhehai Li[f,g,*]

[a] Department of Orthopedics, The Inner Mongolia People's Hospital, Hohhot 010017, Inner Mongolia, PR.China.
[b] Department of Orthopedics, Ordos Central Hospital, Ordos 017000, Inner Mongolia, PR.China.
[c] Department of Orthopedics, The Third Affiliated Hospital, Inner Mongolia Medical University, Baotou, 014010, Inner Mongolia, PR.China.
[d] Department of Orthopedics, Yangling, Demonstration Zone hospital District Hospital Xianyang 712100, Xian, PR.China.
[e] Hunan Youcheng Biotechnology Co. Ltd, Changsha, 410000, China
[f] Department of Orthopedics, Beijing Northern Hospital, China North Industries, Beijing 100089, PR.China.
[g] Inner Mongolia Medical University, Hohhot 014010, Inner Mongolia, PR.China.

ARTICLE INFO

Keywords:
BMSC
miR-26b
GSK3-β
Osteogenesis
OA
DEX

ABSTRACT

Backgrounds: Osteoactivin (OA) is a key regulator promoting bone marrow stromal cells osteogenesis progress, while Dexamethasone (Dex) could inhibit OA induced osteogenesis and lead to osteoporosis. miR-26b increased during BMSC osteogenesis but whether it participates in this progress is enigma. Osteogenesis is under regulation of canonical Wnt signaling pathway which could serve as potential target for miR-26b. It bears therapeutic potential if miR-26b could regulate osteogenesis and antagonize Dex induced Osteoporosis (OP).

Methods: BMSC were isolated from bone marrow of rats and induced for osteogenesis by OA administration. We detected miR-26b mRNA level together with osteogenesis related genes or Wnt signal pathway related genes by qRT-PCR. BMSC cells with miR-26b inhibitor or mimics revealed the effect of miR-26b on osteogenesis. The osteogenesis efficiency was detected by Alizarin Red staining and ALP activity. Protein level of canonical Wnt signal pathway and other proteins were detected by Western blot. The interaction between miR-26b and GSK3β was detected by dual luciferase reporter assay.

Results: We found that miR-26b was increased during OA induced BMSC osteogenesis. Inhibiting miR-26b could lead to osteogenesis inhibition while miR-26b mimics could promote this progress. The key regulator of Wnt signal pathway GSK3β is down-regulated when miR-26b was overexpressed, resulting in β-catenin activation. Since Dex could promote GSK3β expression and inhibit Wnt signal, miR-26b could also alleviate Dex induced osteogenesis inhibition.

Conclusion: Our findings indicate that miR-26b promoted BMSC osteogenesis by directly targeting GSK3β and activating canonical Wnt signal pathway, suggesting miR-26b might be serve as potential therapeutic candidate of osteoporosis.

Introduction

Osteoporosis (OP) is a systemic skeletal disease. Its main features are: decreased bone mass, bone microarchitecture damage , and increased bone fragility which could lead to more frequent bone fractures. Up to 28 million individuals suffers from OP around the world. OP falls into two major categories: primary OP and secondary OP (Armas & Recker, 2012; Coughlan & Dockery, 2014). Prolonged hormone administration, especially glucocorticoid (GC) and dexamethasone (Dex) administration, is the leading cause of secondary osteoporosis(Kang et al., 2016; Yuasa et al., 2015; Zhou et al., 2014). This is largely because that GC could inhibit bone marrow stromal cell (BMSC) osteogenesis and proliferation (Kang et al., 2016). Dex is an immune inhibitor and is widely used clinically for autoimmune diseases, immunological rejection and inflammations. One of the most sever side effect of Dex administration is induction of OP(Yuasa et al., 2015; Florine et al., 2013; Li et al., 2013a). It was noticed that OA could induce BMSC osteogenesis and antagonize Dex induced OP due to

* **Corresponding author at:**Department of Orthopedics, Beijing Northern Hospital, China North Industries, No.10 courtyard, Che Dao Gou, Beijing 100089, P.R. China.
E-mail address: lizhehai0472@126.com (Z. Li).

https://doi.org/10.1016/j.yexmp.2019.02.003
Received 10 July 2018; Received in revised form 29 January 2019; Accepted 11 February 2019
Available online 13 February 2019

unknown mechanism (Moussa et al., 2014; Sondag et al., 2014). Study about this mechanism may help developing new clinical strategies for treatment of Dex associated OP.

MicroRNA (miRNA) is small non-coding RNA molecule around 22–24 nt. Its major function is regulating gene expression on post-transcriptional level through targeting mRNA. MicroRNA could bind with 3'UTR of mRNA, interact with RNA induced silencing complex (RISC) and lead to mRNA degradation (Mobergslien & Sioud, 2014; Squadrito et al., 2014). Recent studies of miR-365, miR-21, miR-23a/b and miR-106b indicated involvement of miRNA during osteogenesis of BMSC. miR-21 could reverse OP through modulating RECK and miR-34 could target Tgif2 and inhibit OP via antagonize osteoclastogenesis (Fang et al., 2016; Li et al., 2013b; Liu et al., 2017; Xu et al., 2017; Krzeszinski et al., 2014). These findings clearly reveal the importance of miRNA in BMSC osteogenesis. It is also noteworthy that miR-26b is up regulated during BMSC osteogenesis(Trompeter et al., 2013), however whether it is required for osteogenesis and the regulatory mechanism remains enigma.

Wnt signal pathway, especially canonical Wnt pathway controls many biological processes, including cell proliferation, bone development and stem cell maintenance. The key regulatory factor within Wnt pathway is GSK3(Wu & Pan, 2010). By phosphorylating β-catenin, GSK3 promotes β-catenin degradation within a so-called destruction complex, which eventually lead to Wnt signal inhibition. It is reported that dysregulation of Wnt signal pathway will lead to bone diseases. Growing evidence shows that Alkaline phosphatase (ALP), osteopontin (OPN), bone gamma-carboxyglutamate protein (BGLAP, also called OCN), collagen type1A1 (COL1A1) are all bone specific matrix proteins, and are related with bone formation (Li et al., 2013a; Xu et al., 2017). Runt related transcription factor 2 (RUNX2) is a critical transcriptional regulator of osteoblast diferentiation and enhances the transcription of these osteoblast-related genes: ALP, OPN, OCN, COL1A1(Xiao et al., 2005). Moreover, activation of canonical Wnt signaling could activated the expression of RUNX2 and the downstream osteoblast-related genes and lead to increased osteogenesis (Banerjee et al., 2016; Isacsson et al., 2005). Interestingly, it was reported that Wnt signal pathway is involved in Dex induced OP and its activation could rescue osteoblast/adipocyte balance of BMSC(Wu et al., 2009).

Thus, we proposed a hypothesis that miR-26b could regulate BMSC osteogenesis through regulating Wnt signal pathway. miR-26b targets GSK3, down-regulates its expression and activated Wnt signal pathway through up regulating β-catenin. This could promote BMSC osteogenesis, which alleviates the inhibitory effect of Dex on BMSC osteogenesis. Our study could reveal the mechanism behind miR-26b in regulating BMSC osteogenesis and bears therapeutic potential for Dex induced OP.

Material and methods

1.BMSC isolation and cell culture. Animal experiments were conveyed following local regulations and policies for the care and use of laboratory animals. Sprague Dawley rats were anesthetized with 10% chloral hydrate (1 ml/300 g, i.p.) and then dipped in 75% ethanol for 20 min. Under aseptic conditions, the bilateral lower limb femurs of rats were obtained followed by the removal of attached fatty tissues, connective tissues, and periosteum. The femurs were placed in a small aseptic beaker and then moved on a super clean bench. Thereafter, the femurs were stored on a sterile culture dish. After washing with PBS, the bilateral mummification ends in the femur were resected. A total of 5 ml DMEM (Gibco) containing 10% FBS (Gibco) was mixed with 0.5 ml of heparin. The marrow cavities of the femurs were rinsed by the mixed solution for three to four times. The flushing fluid was fully beaten followed by cell resuspension with DMEM containing 10% FBS. The cells (density was adjusted to 1×10^6 cells/ml) were inoculated in a 25 cm^2 culture bottle and were then cultured (37 °C, 5% CO_2, saturated humidity). The medium was changed every three days. After the adherent cells reached 80% to 90% fusion, the culture medium was abandoned followed by washing with PBS for three times. The pre-heated (37 °C) digestion liquid containing 0.25% trypsin and 0.02% EDTA was used for digestion at room temperature followed by passage with a ratio of 1:2.

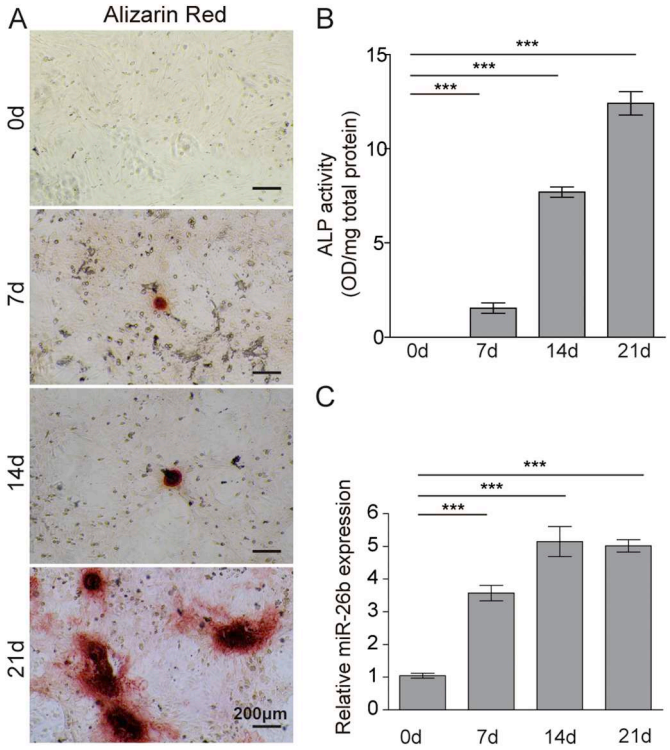

Fig. 1. Up-regulating of miR-26b during OA induced BMSC osteogenesis.

A. BMSC osteogenesis was induced by OA as indicated and detected via Alizarin Red staining.
B. ALP activity of cells described in A.
C. miR-26b expression level in cells described in A was determined through qRT-PCR. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

2.BMSC osteogenesis. Rat BMSC were cultured by Osteogenesis Induction Medium for BMSC (Cyagen). For Dex administration, cells were cultured with 4 uM Dex; for rOA administration, cells were cultured with 100 ng/ml rOA.

3.miR-26b inhibitor and mimics infection. The miR-26b inhibitors and mimics were purchase from GenePharm (China, Shanghai). Stable cell lines were prepared as following procedures: cells cultured to 80% confluence in a 6-well plate (roughly 1×10^6 cells cells) were infected with miRNA inhibitor or mimics via pLKO.1(addgene) lentivirus vector system following manufactures' instructions. After infection, stable cell lines were selected with 1μg/ml puromycin for 6 days, then expanded for further experiments.

4.Alizarin Red staining. Osteogenesis was examined by staining mineralized nodules with Alizarin Red S as described previously (Florine et al., 2013). Briefly, cells were washed with PBS and soaked in 40 mM Alizarin Red (pH 4.2) for 30 min at 37 °C, then washed with PBS and imaged.

5.Alkaline phosphatase (ALP) activity. We measured ALP activity in the cell supernatants using colorimetric assay kit (Jiancheng, Nanjing). ALP activity was measured at 405 nm in a micro-plate reader (Gen5), and calculated with pNP standard curve.

6. qRT-PCR. qRT-PCR was done as previously described (Kang et al., 2016). Total cellular RNA was isolated from cultured cells using the RNeasy Mini Kit (Qiagen, Valencia, CA, USA) according to the manufacturer's protocol. Real-time PCR was carried out on a 96-well plate

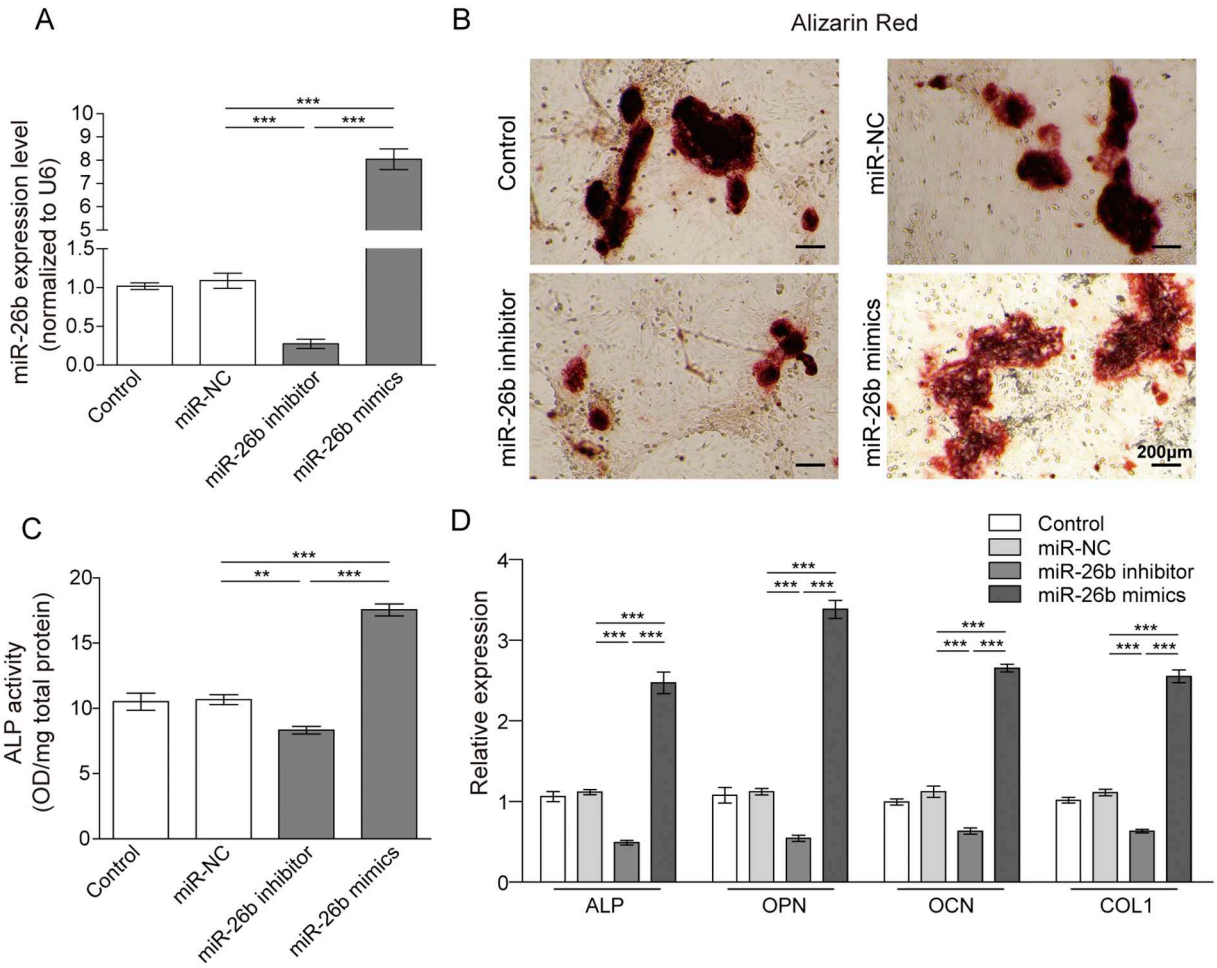

Fig. 2. miR-26b promotes OA induced BMSC osteogenesis.

D. miR26-b level was determined by qRT-PCR in BMSCs with miR-NC, miR-26b inhibitor and mimics as indicated.
E. Cells described in A were treated with OA and stained with Alizarin Red.
F. Osteogenesis were determined by ALP activity assay in cells described in A
G. Osteogenesis related genes ALP, OPN, OCN, COL1A1were detected in cells described in A by qRT-PCR. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

ABI Prism 7500 Sequence Detection system (Applied BioSystems, Foster City, CA, USA) using SYBR Green PCR Master Mix (Takara Bio Inc., Otsu, Japan). Cycling conditions were as follows: 40 cycles of 94 °C for 5 s, 60 °C for 34 s, and 72 °C for 30 s. e comparative $2^{-\Delta\Delta Ct}$ method was used to calculate the relative expression of each target gene; the expression levels of all genes were normalized to β-actin or U6. The sequence of primer was as shown: ALP, forward: 5′- CCTTCTTCCGTCA GTACCGT-3′, reverse: 5'-AGCTGGTTCATCCCGATTGT-3′; OPN, forward: 5′- GAGGAGAAGGCGCATTACAG-3′, reverse: 5′- ACAGAATCCT CGCTCTCTGC-3′; Ocn, forward: 5'-CAAGTCCCACACAGCAACTC-3′, reverse: 5'-GTCCATTGTTGAGGTAGCGC-3′; Col1a1, forward: 5′- AAC AAGGGAGGAGAGAGTGC-3′, reverse: 5′- AGTCTCTTGCTTCCTCC CAC-3′; MiR-26b, forward: 5′- CGCCCGTTCAAGTAATTCAGG-3′, reverse: 5′- CGGCCCAGTGTTCAGACTAC-3′.

7.Western Blot. The protein was extracted with RIPA, quantified, and separated on SDS-PAGE. The membranes were blocked in 5% BSA for 1 h at room temperature, and incubated with primary antibodies at 4C overnight. Membranes were washed by TBST 3 times (7 min each time) and incubated with HRP-conjugated secondary antibodies for another 1 h at room temperature. The blots were analyzed by enhanced chemiluminescence (ECL) western blotting detection system (GE HealthCare Bio-Sciences, Piscataway, NJ, USA). Actin, β-catenin, GAPDH antibodies were obtained from Santa Cruz; GSK3β , RUNX2 antibodies were obtained from CST.

8.Luciferase reporter assay. Luciferase constructs were constructed by cloning WT and mutated GSK3β 3'UTR sequences into pGL3-luc system. The corresponding mutant constructs (MUT- GSK3β) with ten mutated residues in the region of seeding sequence were generated by site-directed mutagenesis (Fig. 3). Control vector was TK-Rellina–pGL3. Cells were transiently transfected with the corresponding cDNA using Xfect™Polymer. The luciferase reporter activity was assayed with the Dual-Luciferase reporter assay system (Promega, E1910) by following the manufacturer's instructions. All the samples were tested in triplicates, and the results were normalized relative to control rellina activity.

9.Statistical analysis. All data values were shown as mean ± standard deviation (SD). Comparison of all other results was performed by one-way analysis of variance (ANOVA) with Tukey's comparison analysis and the statistical significance was analyzed using Student *t*-test and analysis of variance. Data was considered significant when $*p < .05$, $**p < .01$, $***p < .001$. The Graphpad Prism 5 (Graphpad Software Inc.,) was used to compare groups.

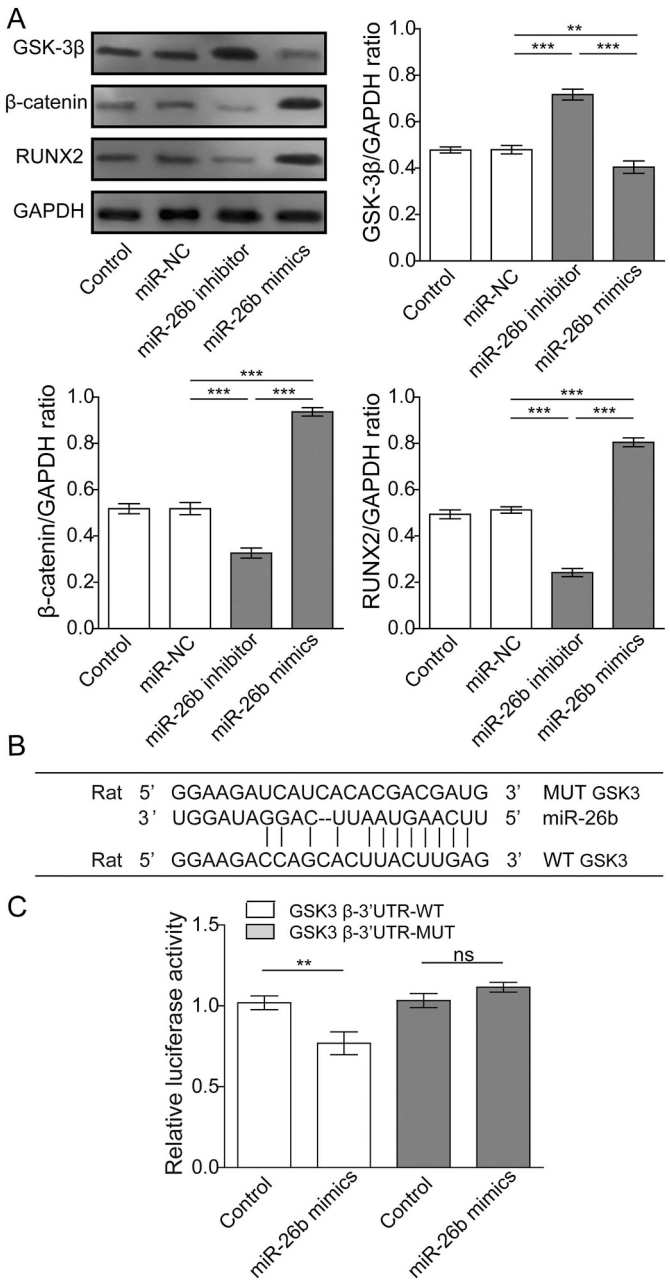

Fig. 3. miR-26b modulates RUNX2 expression through regulating GSK3/β-catenin pathway.

H. BMSC with miR-NC, miR-26b inhibitor and miR-26b mimics were subjected to SDS-PAGE and detected for GSK3β, β-catenin , RUNX2 expression. GAPDH served as loading control. Statistical analysis of protein levels was shown in right panel.

I. Luciferase activity were detected in BMSC containing WT or mutated GSK3-β 3'UTR and transfected with miR-26b mimics or miR-NC.

J. Schematic picture of miR26b and GSK3-β 3'-UTR in different species as indicated.

Results

Up-regulating of miR-26b during OA induced BMSC osteogenesis.

To confirm the relationship between OA induced BMSC osteogenesis and miR-26b expression level, we first isolated BMSC from rats and cultured these cells in osteogenesis induction medium with OA. The osteogenesis efficiency is analyzed by Alizarin Red staining, which is a widely used method for osteogenesis identification (Glynn et al., 2013). In BMSC induced for osteogenesis differentiation for 0–3 weeks as indicated, Alizarin Red staining validated successful osteogenesis (Fig. 1A). Moreover, ALP activity was increased in a time dependent manner (Fig. 1B). To evaluate the association of miR-26b and BMSC osteogenesis, we next detected miR-26b level by qRT-PCR in these cells, and clearly its expression is enhanced during OA induced osteogenesis progress. The miRNA level increased to almost 4-fold after 3 weeks' induction versus day 0 (Fig. 1C). This finding indicates potential connection between miR-26b expression level OA induced osteogenesis of BMSC.

miR-26b promotes OA induced BMSC osteogenesis.

For further validation of this connection between miR-26b and osteogenesis, BMSC were stably expressed with miR-NC as negative control, and miR-26b inhibitor or mimics respectively via lentivirus system. Stable cell lines were selected and validated by qRT-PCR detection of corresponding RNA levels of miR-26b (Fig. 2A). Osteogenesis were induced by OA in these stable cell lines, and osteogenesis efficiency were detected by Alizarin Red staining. As shown in Fig. 2B, miR-26b overexpression dramatically promoted calcium accumulation, while inhibition of miR-26b exerted opposing effects. ALP activity assay exerted similar result (Fig. 2C). We also detected mRNA level of osteogenesis related genes ALP, OPN, OCN, COL1A1 (Li et al., 2013a; Xu et al., 2017) with qRT-PCR. Results indicated that these genes were significantly enhanced in miR-26b mimics group. All these genes were suppressed by miR-26b inhibitor administration, indicating miR-26b as a key regulatory factor in promoting osteogenesis genes expression (Fig. 2D). These findings taken together demonstrated the importance of miR-26b in OA induced osteogenesis.

miR-26b modulates RUNX2 expression through regulating GSK3β/β-catenin pathway.

The mechanism between miR-26b and osteogenesis remains enigma, so we next aimed to reveal the hidden linkage between miR-26b and osteogenesis related genes. Canonical Wnt signal pathway, or GSK3β/β-catenin pathway plays crucial role in bone differentiation. It's not clear whether this pathway is affected by miR-26b. In BMSC stably expressing miR-26b inhibitor, mimics or NC control, we detected the protein levels of GSK3β, β-catenin and RUNX2 (Fig. 3A). From the result we could clearly see that the protein level of GSK3β were decreased by miR-26b mimics, which were enhanced to about twice the intensity in miR-26b inhibitor group versus control and NC groups. Moreover, the downstream protein β-catenin was activated in BMSC cells with miR-26b mimics, while decreased in BMSC cells with miR-26b inhibitor. To detect the relationship between osteogenesis and Wnt pathway, we also detected RUNX2 protein level, a protein closely related with BMSC osteogenesis. The expression pattern of RUNX2 is much similar with β-catenin. MiR-26b activation increased RUNX2 expression and inhibition of miR-26b lead to RUNX2 (Fig. 3A).

The potential binding site of miR-26b on the 3'-UTR of GSK3 was predicted using Starbase 2.0 software (http://starbase. sysu.edu.cn/starbase2/index.php) and the TargetScan (www. targetscan.org/vert_71/) database(Fig. 3B). We then generated luciferase reporter plasmids with mutated or wild-type (WT) 3'UTR of GSKβ. Stable miR-26b BMSC cells lines were transfected with these luciferase reporter systems. In cells with mutated GSK3β 3'UTR luciferase reporter, miR-26b exerted no effect on luciferase activity. However, the luciferase activity of WT-GSK3–3'UTR luciferase reporter is dramatically inhibited by miR-26b mimics (Fig. 3C).

Taken together, miR-26b could directly target GSK3β and modulate Wnt signal pathway, up regulating bone formation associated genes including RUNX2 and promote BMSC osteogenesis.

Dexamethasone induced BMSC osteogenesis inhibition is alleviated by miR-26b over expression.

It's widely accepted that dexamethasone (Dex) administration could inhibit osteogenesis differentiation of BMSC(Florine et al., 2013; Li et al., 2013a; Xu et al., 2017). In this study, we aimed to evaluate

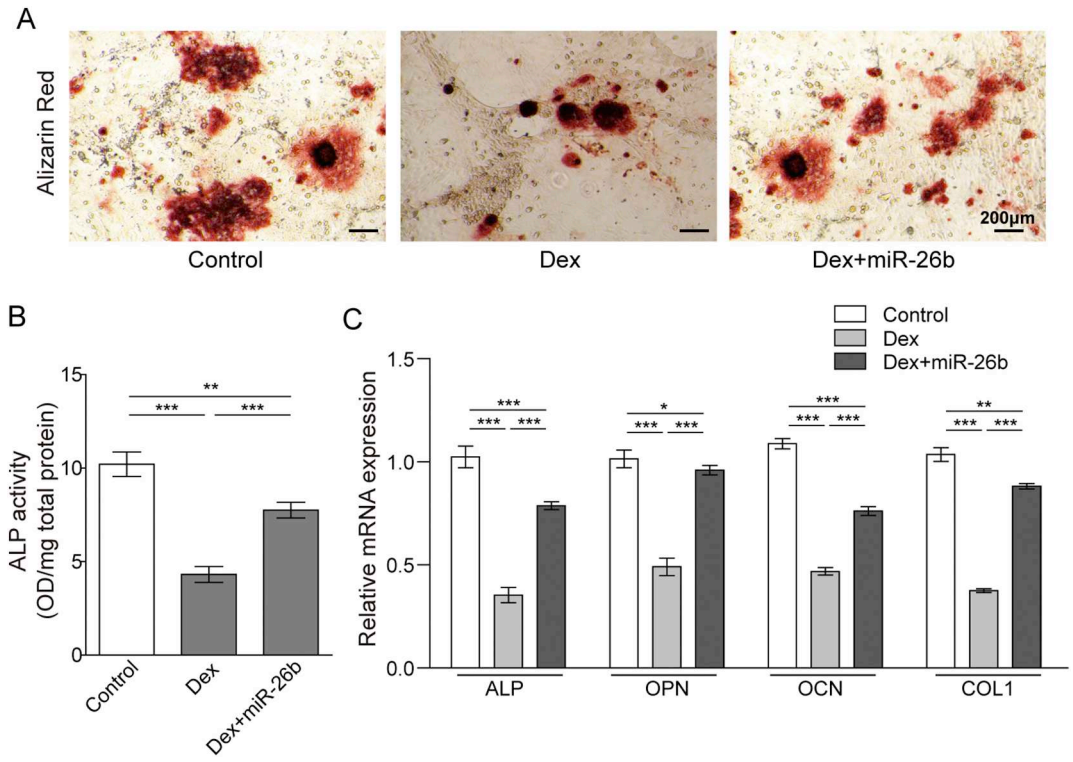

Fig. 4. Dex inhibited BMSC osteogenesis is alleviated by miR-26b over expression.

K. BMSC treated with Dex, transfected with miR-26b or control as indicated during OA induced osteogenesis were subjected to Alizarin Red staining.
L. ALP activity was analyzed in cells described in A.
M. Relative expression of ALP, OPN, OCN, COL1A1were detected in cells as described in A. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

whether miR-26b could alleviated Dex induced osteogenesis inhibition. Control or stable overexpression of miR-26b BMSC cell lines were treated with Dex Alizarin Red signal were dramatically decreased by Dex administration comparing to control group. But overexpression of miR-26b could partly alleviate this effect (Fig. 4A). To validate this finding we conveyed ALP activity analysis in cells describe above, and the result is consistent: Dex administration decreased BMSC ALP activity and miR-26b could inhibit such effect of Dex (Fig. 4B). The mRNA level of osteogenesis related genes was also detected by qRT-PCR. Cells were treated as described above and detected for ALP, OPN, OCN, COL1A1 expression. In consistent with previous findings, the inhibition effect of Dex is alleviated by miR-26b (Fig. 4C). Thus, miR-26b could antagonize Dex and promotes osteogenesis.

miR-26b antagonize Dex inhibited BMSC osteogenesis through modulating GSK3/β-catenin pathway.

Now that we have proven the controversial effect between miR-26b and Dex, we next aimed to uncover the molecular mechanism behind this phenomenon. GSK3β is targeted by miR-26b, but it's not clear whether Dex could promote GSK3β expression and inhibit Wnt signal. As shown in figure 5GSK3β is obviously up regulated by Dex treatment, and the protein levels of β-catenin and RUNX2 were decreased. Whereas overexpression of miR-26b nearly abolished the effect of Dex on GSK3β, β-catenin and RUNX2(Fig. 5).

Discussion

Osteoactivin (OA), also known as DC-HIL or GPNMB, is an essential factor in bone marrow stromal cell differentiation. Besides BMSC osteogenesis, it is also involved in multiple biological processes including proliferation, cell adhesion, extra cellular matrix synthesis and cell differentiation. Its most important function is to modulate bone marrow stromal cell osteogenesis(Moussa et al., 2014; Sondag et al., 2014; Abdelmagid et al., 2008). A series of miRNA participated in BMSC osteogenesis, for example, miR-29c-3p could negatively regulate the osteogenesis differentiation of BMSC by targets on dishevelled 2(Wang et al., 2018). Moreover, Camp E et.al found that miR-376c-3p reduces IGF1R/Akt signaling in BMSC and inhibited osteogenesis (Camp et al., 2018). Noteworthy, miR-26b is also reported as up regulated during BMSC osteogenesis(Trompeter et al., 2013), so its highly possible that this miRNA is closely related with BMSC osteogenesis.

In this study we induced BMSC osteogenesis by OA and detected miRNA-26b expression during this process. Indeed, miR-26b expression dramatically increased and is closely related with BMSC osteogenesis (Fig. 1C). Ectopic miR-26b dramatically promoted the osteogenesis progress induced by OA (FIG. 2). This finding strongly validated our speculation that miR-26b is closely related with BMSC osteogenesis. It was reported that miR-26bcould accelerate osteogenic differentiation of somatic stem cell, which supported our finding(Trompeter et al., 2013). Since osteogenesis is decreased in OP patients, miR-26b could thus probably alleviate osteoporosis by promoting osteogenic differentiation. Given that OA administration enhanced miR-26b, we could thus speculate that OA could enhance miR-26b and promote BMSC osteogenesis, which finally inhibit OP. The remaining question is that through which molecular mechanism could miR-26b regulate osteogenesis.

One of the most important pathways during bone formation is canonical Wnt signaling pathway. This pathway signals through regulating β-catenin protein level. In resting cells β-catenin is constantly degraded by a so-called destruction complex. In this complex β-catenin get phosphorylated by CK1 and GSK3β. Phosphorylated β-catenin could then be ubiquitinated by E-3 ligase and subjected to proteasomal degradation(Wu & Pan, 2010; Amit et al., 2002). It was reported that

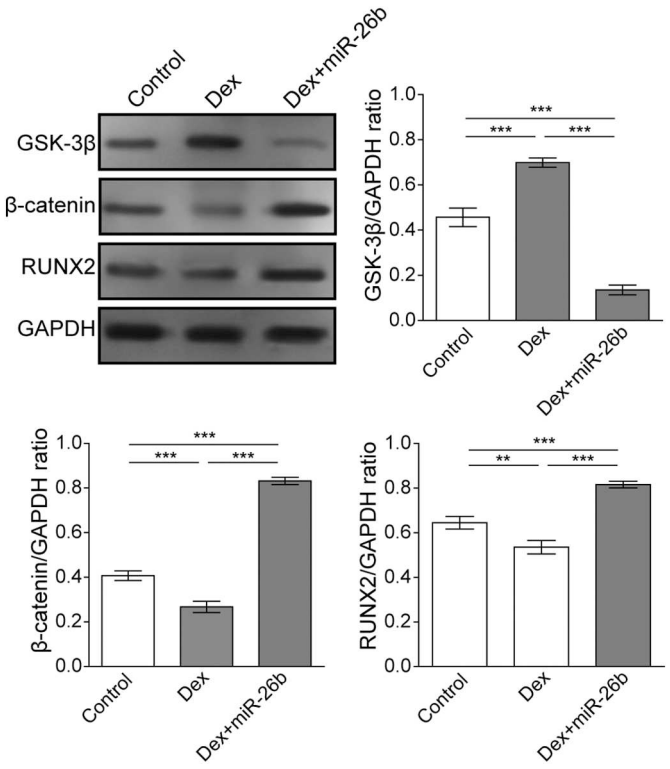

Fig. 5. miR-26b antagonize Dex inhibited BMSC osteogenesis through modulating GSK3/β-catenin pathway. BMSC were treated with Dex and transfected with miR-26b as indicated and detected for GSK3, β-catenin , RUNX2 expression. GAPDH served as loading control.

multiple chemicals could regulate the osteogenic differentiation of stem cells through activation of GSK3β(Kong et al., 2013). We speculated whether this pathway is affected by miR-26b, and detected the key regulator protein GSK3β and β-catenin together with a downstream protein RUNX2 (which is also related with osteogenesis) (FIG. 3). Our data suggested that miRNA-26b could directly interact with 3'UTR of mRNA of GSK3β as proven by luciferase reporter, and Western blot analysis, and regulate canonical Wnt signaling pathway, which finally promoted BMSC osteogenesis. Similar regulatory axis was identified in rheumatoid arthritis synovial fibroblasts where miR-26b inhibited GSK-3β expression and alters the Wnt/GSK-3β/β-catenin pathway, which could support our findings(Sun et al., 2015).

Dexamethasone is an immune inhibitor widely used in clinical treatment for autoimmune diseases, immunological rejection and inflammations (Yuasa et al., 2015). However, previous studys have demonstrated that prolonged administration of Dex could induce serious OP through inhibiting osteogenesis(Li et al., 2013a; Hu et al., 2018). Interestingly, Xu et al. have shown that miR-365 ameliorates DEX-induced suppression of cell viability and osteogenesis by regulating the expression of HDAC4 in osteoblasts(Xu et al., 2017). Shi et al. have shown that miR-17/20a may play a significant role in Dex-induced osteoclast differentiation and function by targeting the RANKL expression in osteoblast cells(Shi et al., 2014). In addition, it was report that miR-216a rescues dexamethasone suppression of osteogenesis via regulating PI3K/AKT pathway(Li et al., 2015). Thus, in this study we attempted to determine the regulatory role of miR-26b in Dex induced BMSC osteogenesis inhibition. We found that miR-26b could antagonize Dex and promotes osteogenesis through modulating GSK3β/β-catenin pathway.

In summary, we are the first to confirm that miR-26b is upregulated by OA-induced and can promote osteogenic differentiation of BMSCs in vitro, but also can effectively antagonize the suppression of Dex on osteoblast differentiation, through targeting GSK3β, which, in turn, results in the activation of the β-catenin signaling pathway. This study enrich the understanding of molecular mechanism of OA-induced osteogenic differentiation and reveal miR-26b/GSK3β/β-catenin may be a potent therapeutic agent for the prevention and treatment of Dex-induced osteoporosis. It would be difficult to overexpress miR-26b in patients. Thus, for future work, we will be using commercially available GSK3β inhibitors to block GSK3β/β-catenin pathway directly. Moreover, exploring the upstream regulatory factors of miR-26b is also critical for a better understanding of the OA-miR-26b axis, and may provide more potential therapeutic targets.

Conclusions

In conclusion, our study revealed the pro-osteogenesis effect of miR-26b and uncovered the molecular mechanism. MiR-26b directly targets GSK3β and activates bone-formation related canonical Wnt signaling pathway. We also proved that miR-26b could antagonize Dex induced BMSC osteogenesis. These findings may bear therapeutic potential for Dex induced OP, and could provide support for future researches.

Acknowledgements

We would like to give our sincere gratitude to the reviewers for their constructive comments.

Funding

This work was supported by the National Natural Science Foundation Project (No. 81260141) and The Inner Mongolia People's Hospital Funding Project (No.201816).

Conflict of interest

The authors declare they have no conflict of interest.

References

Abdelmagid, S.M., et al., 2008. Osteoactivin, an anabolic factor that regulates osteoblast differentiation and function. Exp. Cell Res. 314 (13), 2334–2351.
Amit, S., et al., 2002. Axin-mediated CKI phosphorylation of beta-catenin at Ser 45: a molecular switch for the Wnt pathway. Genes Dev. 16 (9), 1066–1076.
Armas, L.A., Recker, R.R., 2012. Pathophysiology of osteoporosis: new mechanistic insights. Endocrinol. Metab. Clin. N. Am. 41 (3), 475–486.
Banerjee, P., Dutta, S., Pal, R., 2016. Dysregulation of Wnt-Signaling and a candidate set of miRNAs underlie the effect of metformin on neural crest cell development. Stem Cells 34 (2), 334–345.
Camp, E., et al., 2018. miRNA-376c-3p mediates TWIST-1 inhibition of bone marrow-derived stromal cell Osteogenesis and can reduce aberrant bone formation of TWIST-1 Haploinsufficient Calvarial cells. Stem Cells Dev. 27 (23), 1621–1633.
Coughlan, T., Dockery, F., 2014. Osteoporosis and fracture risk in older people. Clin Med (Lond) 14 (2), 187–191.
Fang, T., et al., 2016. miR-106b-5p and miR-17-5p suppress osteogenic differentiation by targeting Smad5 and inhibit bone formation. Exp. Cell Res. 347 (1), 74–82.
Florine, E.M., et al., 2013. Effects of dexamethasone on Mesenchymal stromal cell Chondrogenesis and Aggrecanase activity: comparison of Agarose and self-assembling peptide scaffolds. Cartilage 4 (1), 63–74.
Glynn, E.R., et al., 2013. Culture conditions for equine bone marrow mesenchymal stem cells and expression of key transcription factors during their differentiation into osteoblasts. J Anim Sci Biotechnol 4 (1), 40.
Hu, H., et al., 2018. Osteoactivin inhibits dexamethasone-induced osteoporosis through up-regulating integrin beta1 and activate ERK pathway. Biomed. Pharmacother. 105, 66–72.
Isacsson, A., Kanje, M., Dahlin, L.B., 2005. Induction of activating transcription factor 3 (ATF3) by peripheral nerve compression. Scand. J. Plast. Reconstr. Surg. Hand Surg. 39 (2), 65–72.
Kang, H., et al., 2016. Glucocorticoids impair bone formation of bone marrow stromal stem cells by reciprocally regulating microRNA-34a-5p. Osteoporos. Int. 27 (4), 1493–1505.
Kong, X., et al., 2013. GSK3beta is a checkpoint for TNF-alpha-mediated impaired osteogenic differentiation of mesenchymal stem cells in inflammatory microenvironments. Biochim. Biophys. Acta 1830 (11), 5119–5129.
Krzeszinski, J.Y., et al., 2014. miR-34a blocks osteoporosis and bone metastasis by inhibiting osteoclastogenesis and Tgif2. Nature 512 (7515), 431–435.
Li, J., et al., 2013a. Dexamethasone shifts bone marrow stromal cells from osteoblasts to

adipocytes by C/EBPalpha promoter methylation. Cell Death Dis. 4, e832.
Li, Q., et al., 2013b. MiR-21/Smad 7 signaling determines TGF-beta1-induced CAF formation. Sci. Rep. (3), 2038.
Li, H., et al., 2015. miR-216a rescues dexamethasone suppression of osteogenesis, promotes osteoblast differentiation and enhances bone formation, by regulating c-Cbl-mediated PI3K/AKT pathway. Cell Death Differ. 22 (12), 1935–1945.
Liu, K., et al., 2017. Silencing miR-106b accelerates osteogenesis of mesenchymal stem cells and rescues against glucocorticoid-induced osteoporosis by targeting BMP2. Bone 97, 130–138.
Mobergslien, A., Sioud, M., 2014. Exosome-derived miRNAs and cellular miRNAs activate innate immunity. J Innate Immun 6 (1), 105–110.
Moussa, F.M., et al., 2014. Osteoactivin promotes osteoblast adhesion through HSPG and alphavbeta1 integrin. J. Cell. Biochem. 115 (7), 1243–1253.
Shi, C., et al., 2014. MicroRNA-17/20a inhibits glucocorticoid-induced osteoclast differentiation and function through targeting RANKL expression in osteoblast cells. Bone 68, 67–75.
Sondag, G.R., et al., 2014. Osteoactivin induces transdifferentiation of C2C12 myoblasts into osteoblasts. J. Cell. Physiol. 229 (7), 955–966.
Squadrito, M.L., et al., 2014. Endogenous RNAs modulate microRNA sorting to exosomes and transfer to acceptor cells. Cell Rep. 8 (5), 1432–1446.
Sun, J., et al., 2015. MicroRNA-26b inhibits cell proliferation and cytokine secretion in human RASF cells via the Wnt/GSK-3beta/beta-catenin pathway. Diagn. Pathol. 10, p. 72.
Trompeter, H.I., et al., 2013. MicroRNAs miR-26a, miR-26b, and miR-29b accelerate osteogenic differentiation of unrestricted somatic stem cells from human cord blood. BMC Genomics 14, 111.
Wang, F.L., et al., 2018. miR-29c-3p targeted dishevelled 2 on osteogenesis differentiation of rat bone marrow mesenchymal stem cells in high-fat environment. Zhonghua Kou Qiang Yi Xue Za Zhi 53 (10), 694–700.
Wu, D., Pan, W., 2010. GSK3: a multifaceted kinase in Wnt signaling. Trends Biochem. Sci. 35 (3), 161–168.
Wu, G., et al., 2009. Inhibition of GSK3 phosphorylation of beta-catenin via phosphorylated PPPSPXS motifs of Wnt coreceptor LRP6. PLoS One 4 (3), e4926.
Xiao, G., et al., 2005. Cooperative interactions between activating transcription factor 4 and Runx2/Cbfa1 stimulate osteoblast-specific osteocalcin gene expression. J. Biol. Chem. 280 (35), 30689–30696.
Xu, D., et al., 2017. miR-365 ameliorates dexamethasone-induced suppression of Osteogenesis in MC3T3-E1 cells by targeting HDAC4. Int. J. Mol. Sci. 18 (5).
Yuasa, M., et al., 2015. Dexamethasone enhances osteogenic differentiation of bone marrow- and muscle-derived stromal cells and augments ectopic bone formation induced by bone morphogenetic protein-2. PLoS One 10 (2), e0116462.
Zhou, D.A., et al., 2014. Influence of glucocorticoids on the osteogenic differentiation of rat bone marrow-derived mesenchymal stem cells. BMC Musculoskelet. Disord. 15, p. 239.