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Long non-coding RNA MIR22HG inhibits the adipogenesis of human bone marrow mesenchymal stem cells with the involvement of Wnt/β-catenin pathway

Chanyuan Jin[1,4,#]; Ziyao Zhuang[2,4,#]; Lingfei Jia[3,4,*]; Yunfei Zheng[2,4,*]

[1] Second Clinical Division, Peking University School and Hospital of Stomatology, Beijing, 100081, China
[2] Department of Orthodontics, Peking University School and Hospital of Stomatology, Beijing, 100081, China
[3] Central Laboratory, Peking University School and Hospital of Stomatology, Beijing, 100081, China
[4] National Center of Stomatology; National Clinical Research Center for Oral Diseases; National Engineering Laboratory for Digital and Material Technology of Stomatology, Beijing, 100081, China

Abstract: Osteoporosis is a frequently occurring bone remodeling disorder worldwide with one characteristic being decreasing bone mineral density and a predisposition to bone fracture, which diminishes patients' quality of life. Several studies showed that imbalance between the osteogenesis and adipogenesis of bone marrow mesenchymal stem cells (BMSCs) took part in the development of osteoporosis. In previous study, we found MIR22HG regulated the osteogenesis of human BMSCs positively. In this study, we found that MIR22HG was decreased during the adipogenesis of human BMSCs and exerted negative effects on adipogenesis with the involvement of Wnt/β-catenin signaling pathway both *in vitro* and *in vivo*. Nitazoxanide could inhibit Wnt signaling and relieve MIR22HG's suppression on adipogenesis. These findings indicated that MIR22HG had great potential in clinical application for osteoporosis treatment and prevention.

Introduction

Osteoporosis is a metabolic bone disease with one characteristic being increasing adipocyte tissue volume in bone marrow, which results in lower bone strength and fragility (Justesen *et al.*, 2001; Meunier *et al.*, 1971). It has been discussed for centuries and still remains a medical challenge worldwide (Rachner *et al.*, 2011). The regulatory mechanism of osteoporosis is multifactorial, such as inflammation, angiogenesis, imbalance between osteoblasts and adipocytes (Lacativa and Farias, 2010; Rodríguez *et al.*, 2009; Tong *et al.*, 2019). In age-related osteoporosis, bone marrow mesenchymal stem cells (BMSCs) are inclined to differentiate into adipocytes rather than osteoblasts, generating an abnormal number of adipocytes and leading to osteoporosis. Wnt/β-catenin signaling is involved in osteoporosis as it takes an important part in regulating the balance between osteogenic and adipogenic differentiation of BMSCs (Huang *et al.*, 2019; Takada *et al.*, 2009). Activating the classical Wnt/β-catenin signaling pathway can stabilize β-catenin and promote osteogenesis (Canalis, 2013). Besides, Wnt signaling restrains adipogenesis via inhibiting the adipogenic transcription factors CCAAT enhancer-binding proteins (C/EBPα) and peroxisome proliferator-activated receptor γ (PPARγ) (Ross *et al.*, 2000). Nitazoxanide (NTZ) is a clinically used antiparasitic drug and has been proved to efficiently inhibit Wnt signaling (Qu *et al.*, 2018). Our work used NTZ as an inhibitor of Wnt signaling.

Long non-coding RNAs (lncRNAs) are non-protein-coding RNA molecules with an average length longer than 200 nucleotides, possessing multiple cellular functions such as chromatin organization, transcriptional regulation, and post-translational modification (West and Lagos, 2019). Many researchers have revealed lncRNAs' important roles in adipogenesis (Chen *et al.*, 2020; Pan *et al.*, 2020; Shapira *et al.*, 2017; Zhang *et al.*, 2020). MIR22HG takes its name as it is the host gene of MiR-22 and has been validated to take effects in various cancers such as glioma, colorectal cancer, lung cancer, etc. Our group reported that MIR22HG was differentially expressed in the osteogenesis of human periodontal ligament stem cells (PDLSCs) and promoted the

osteogenic differentiation of PDLSCs (Zheng *et al.,* 2018). Moreover, MIR22HG was considerably decreased in BMSCs from osteoporotic mice compared with normal mice and promoted the osteogenesis of human BMSCs via PTEN/AKT pathway (Jin *et al.,* 2020). However, the relationship between MIR22HG and adipogenesis of stem cells remains unknown. As a product of MIR22HG, MiR-22 promoted the osteogenesis and inhibited the adipogenesis of mesenchymal stem cells by targeting HDAC6 (Huang *et al.,* 2012). To investigate whether MIR22HG itself directly influences the adipogenic differentiation of BMSCs, we decided to investigate the role of MIR22HG in the adipogenesis of BMSCs. Our research showed that MIR22HG could inhibit the adipogenesis of human BMSCs with the involvement of Wnt/β-catenin signaling. These findings suggest that MIR22HG might be a feasible target for osteoporosis treatment.

Materials and Methods

Cell culture
Human BMSCs were obtained from ScienCell Research Laboratories (San Diego, CA, USA) and cultured in growth medium made up of DMEM supplemented with 10% fetal bovine serum and 1% antibiotics. Cells were kept in a humidified incubator with 5% CO_2 at 37°C. Adipogenesis was induced in standard growth medium supplemented with 50 nM insulin (Sigma-Aldrich, Saint Louis, MO, USA), 100 nM dexamethasone (Sigma-Aldrich), 0.5 mM 3-isobutyl-1-methylxanthine (Sigma-Aldrich), and 200 μM indomethacin (Sigma-Aldrich) as described (Jin *et al.,* 2017). The adipogenic medium was changed every 2 days, and cells were harvested at the indicated times.

Transfection of lentiviruses and small-interfering RNAs
The recombinant lentiviruses containing full-length MIR22HG and the scramble control were purchased from Cyagen Biosciences (Guangzhou, China) as described (Jin *et al.,* 2020). Small interfering RNAs targeting MIR22HG and the scramble control were bought from Integrated Biotech Solutions Co. (Shanghai, China). The sequences are listed in Table 1.

RNA extraction and quantitative real-time PCR
Total RNA was extracted using TRIzol (Invitrogen) and reverse-transcribed into cDNA using a cDNA Reverse Transcription Kit (Takara). qRT-PCR was carried out using SYBR Green Master Mix on the ABI Prism 7500 Real-Time PCR System (Applied Biosystems). Relative expression was calculated by the $2-\Delta\Delta Ct$ method and normalized to the expression of GAPDH. Primers are shown in Table 1.

Protein extraction and Western blotting
The cells were lysed in Radio immunoprecipitation assay (RIPA) lysis buffer to extract total protein. Quantification of protein was performed using the BCA protein assay reagent (Bio-Rad Laboratories, Hercules, CA, USA). Thirty microgram proteins were resolved by 10% SDS–polyacrylamide gels and transferred to PVDF membranes (Millipore, Billerica, MA, USA). Primary antibodies against β-catenin and GAPDH (HuaxingBio Science, Beijing, China) were diluted 1:1000. The membranes were first incubated with primary antibodies overnight at 4°C, and then with corresponding secondary antibodies (1:10,000, Cell Signaling Technology). The band intensity was quantified by the Image J software (http://rsb.info.nih.gov/ij/) and normalized to the GAPDH band.

Oil red O staining
After 14 days adipogenic induction, cells were fixed with 4% formalin in PBS for 30 min, and then reacted with 60% saturated Oil Red O dye (Sigma-Aldrich) for 20 min. The Oil Red O-stained lipid droplets were photographed by LSM microscopy (Carl Zeiss, Oberkochen, Germany). To quantify the amount of lipid droplet formation, the stained cells were incubated with isopropanol to extract the dye from the lipid droplets, and the absorbance of reaction solution was then examined at 510 nM with a microplate reader (Bio-Rad).

Heterotopic bone formation assay in vivo
Human BMSCs were cultured in adipogenic medium for 1 week before the animal experiment. Then, cells were gathered, laden onto collagen sponge (8 mm × 8 mm × 2 mm) and then incubated at 37°C for 3 h, followed by centrifugation at 150×g for 5 min and implanted subcutaneously on the dorsal space of 5-week-old, BALB/c homozygous nude (nu/nu) mice (10 mice per group) as described previously (Huang *et al.,* 2017; Jin *et al.,* 2020). All animal experiments were approved by the Institutional Animal Care and Use Committee of the Peking University

TABLE 1

Sequence of RNA and DNA oligonucleotides

Name	Sense Strand/Sense Primer (5'–3')	Antisense Strand/Antisense Primer (5'–3')
Primers for qRT-PCR		
MIR22HG	AGCGGACGCAGTGATTTGCT	TGGCAGCTTTAGCTGGGTCA
PPARγ	GAGGAGCCTAAGGTAAGGAG	GTCATTTCGTTAAAGGCTGA
FABP4	AGCACCATAACCTTAGATGGGG	CGTGGAAGTGACGCCTTTCA
AdiQ	CTTGCAAGAACCGGCTCAGATCCTCCC	GAGCTGTTCTACTGCTATTAGCTCTGC
GAPDH	ATGGGGAAGGTGAAGGTCG	GGGGTCATTGATGGCAACAATA
siRNA		
si-MIR22HG	GGAUUUAGUUAGUGAUAAGUU	CUUAUCACUAACUAAAUCCUU
si-NC	UUCUCCGAACGUGUCACGUU	ACGUGACACGUUCGGAGAAUU

Health Science Center (LA2014233) and were carried out in line with the Institutional Animal Guidelines.

Statistical analysis
All results are presented as mean ± SD. Statistical analysis was accomplished by Student's *t*-test using IBM SPSS statistical software (version 21). Differences with $P < 0.05$ were treated statistically significant. All experiments were done for three repeats (N = 3).

RESULTS

MIR22HG was decreased notably at the initial stage of the adipogenesis of BMSCs
We measured the expression of MIR22HG in the adipogenesis of human BMSCs to evaluate if MIR22HG is involved in this adipogenesis. As expected, the mRNA expressions of adipogenesis-associated genes fatty acid binding protein 4 (FABP4), adiponectin (AdiQ) and PPARγ were significantly increased (Figs. 1A–1C). qRT-PCR results showed that the expression of MIR22HG was greatly downregulated at the initial stage of the adipogenesis of BMSCs (Fig. 1D).

MIR22HG inhibited the adipogenic differentiation in vitro
To examine the role of MIR22HG in regulating the adipogenesis of human BMSCs, we transfected human BMSCs with recombinant lentiviruses containing full-length MIR22HG (MIR22HG group) and the scramble control (NC group), small interfering RNAs targeting MIR22HG (si-MIR22HG group) and the scramble control (si-NC group). The expression of MIR22HG was obviously decreased in si-MIR22HG transfected group compared to si-NC group and increased in MIR22HG group compared to NC group (Figs. 2A and 2D). The mRNA

levels of adipogenic markers PPARγ and FABP4 were upregulated in si-MIR22HG group compared to si-NC group and downregulated in MIR22HG group compared to NC group both in proliferation medium (PM) and adipogenic medium (AM) (Figs. 2B, 2C, 2E and 2F). Oil Red O staining exposed a significant increase of lipid droplets in si- MIR22HG group compared to si-NC group and a decrease in MIR22HG group compared to NC group (Figs. 2G and 2H). All these results suggested MIR22HG took part in the adipogenesis of BMSCs.

MIR22HG inhibited newly formed adipose tissue in vivo
Human BMSCs expressing si-MIR22HG, MIR22HG and the control were cultured in AM for 7 days. Then, cells were loaded onto Bio-Oss Collagen scaffolds and implanted in the dorsum of nude mice subcutaneously (Fig. 3A). After 8 weeks, the formation of adipose tissue was further characterized by Oil red O staining, showing intracellular lipid gathering (Fig. 3B). Quantification of the newly generated adipose tissue consistently showed a greater ratio of adipogenesis in the si-MIR22HG group (45.9%) compared to the si-NC group (12.6%) and a lower ratio in the MIR22HG group (4.1%) compared to the NC group (16.7%) (Fig. 3C). Those data *in vivo* provided further validation for our hypothesis.

MIR22HG inhibited adipogenesis via β-catenin pathway
To figure out whether MIR22HG regulates adipogenesis via Wnt/β-catenin pathway in BMSCs, we performed western blot analysis and the results showed that MIR22HG overexpression promoted the protein level of β-catenin (Figs. 4A and 4B). NTZ was used as a β-catenin inhibitor dissolved in dimethyl sulfoxide (DMSO) and was added into PM/AM with the final concentration of 10 μM. With the treatment of NTZ, β-catenin was decreased, and the increase
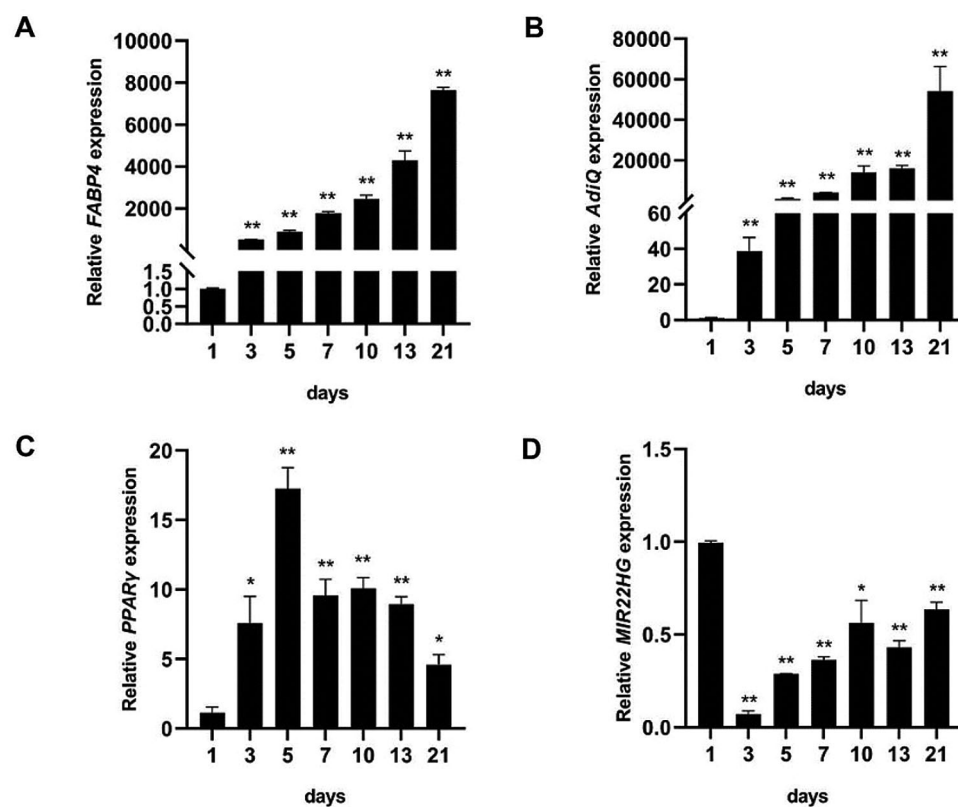

FIGURE 1. The expression pattern of adipogenic markers and MIR22HG in the adipogenesis of human BMSCs. (A–C) relative mRNA expression levels of FABP4, AdiQ, PPARγ were measured by qRT-PCR on days 1, 3, 5, 7, 10, 13, 21 of adipogenic induction. (D) qRT-PCR analysis of MIR22HG during the adipogenesis of human BMSCs. Results are presented as the mean ± SD, * $P < 0.05$, **$P < 0.01$, compared with day 1. Statistical analysis was accomplished by Student's *t*-test, N = 3.
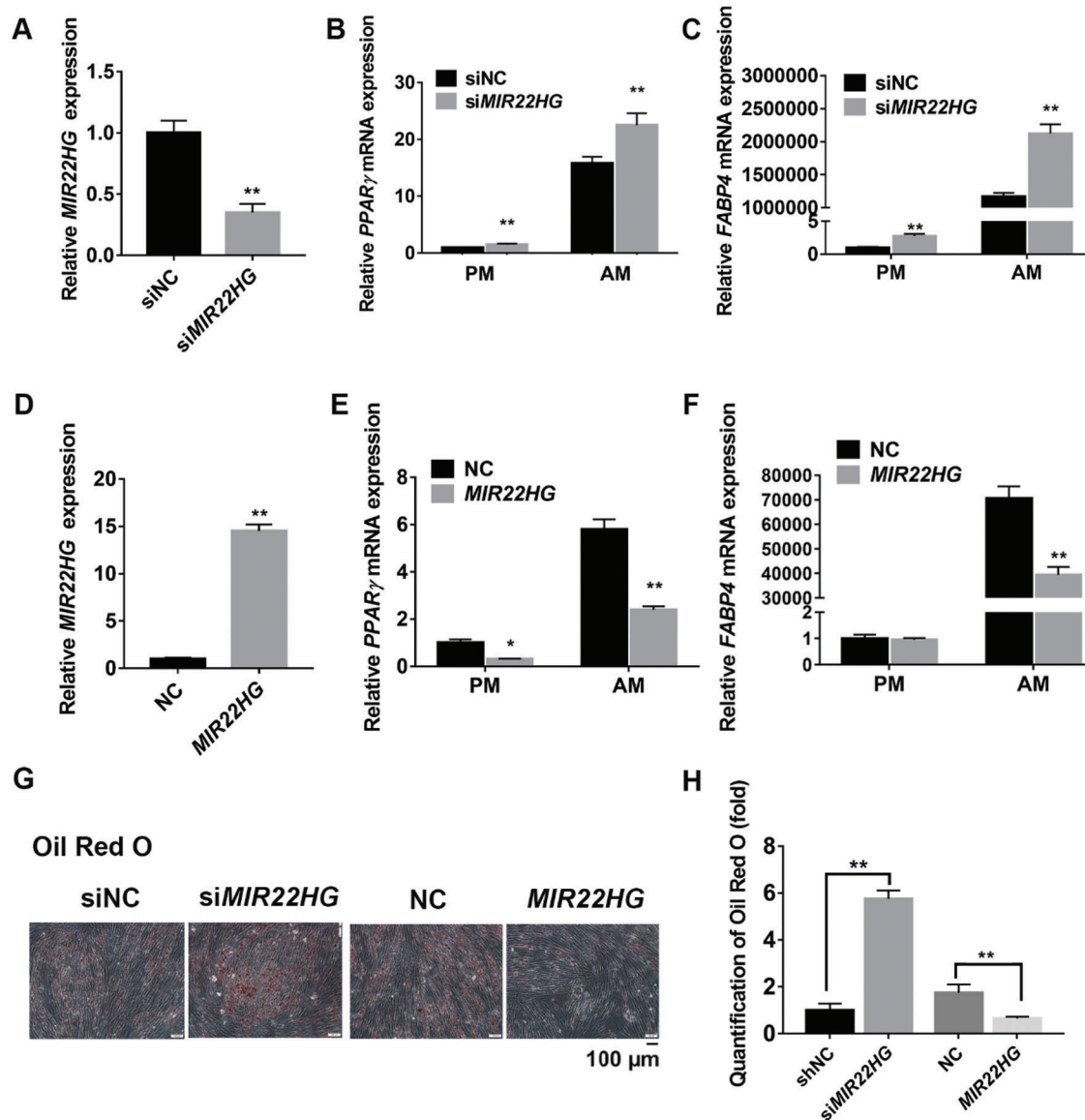
FIGURE 2. MIR22HG inhibited the adipogenesis of human BMSCs. The human BMSCs were transfected with small interfering RNA negative control (si-NC group), small interfering RNAs targeting MIR22HG (si-MIR22HG group), lentivirus overexpressing MIR22HG (MIR22HG group) or the scrambled vector (NC group). (A, D) the efficiency of transfection was measured by qRT-PCR. (B–C) and (E–F) cells were cultured in proliferation medium (PM) or adipogenic medium (AM) for 7 days. qRT-PCR analysis shows PPARγ and FABP4 mRNA expression. (G–H) images of Oil red O staining in the si-NC, si-MIR22HG, NC and MIR22HG groups (top row) after 14 days' adipogenic induction. Histograms show quantification of Oil red O staining by spectrophotometry. Results are presented as the mean ± SD, *$P < 0.05$, **$P < 0.01$, compared with the si-NC and NC groups, relatively. Statistical analysis was accomplished by Student's t-test, N = 3.

of β-catenin induced by MIR22HG was relieved compared to the control group (Figs. 4C and 4D). Furthermore, after 7 days of adipogenic induction under the treatment of NTZ in NC (NC +NTZ) and MIR22HG overexpression group (MIR22HG +NTZ), PPARγ and FABP4 were increased compared to NC group and MIR22HG group which were cultured without NTZ (Figs. 4E and 4F). Oil Red O staining disclosed that NTZ could relieve MIR22HG's suppression on the adipogenesis (Figs. 4G and 4H). These data indicated that MIR22HG improved the expression of β-catenin and inhibited the adipogenesis, while NTZ showed an antagonistic effect.

Discussion

In our previous study, osteoporosis mice models were set up successfully and our group found MIR22HG was considerably

decreased in BMSCs from osteoporotic mice compared with normal mice. Because imbalance between the adipogenesis and osteogenesis of BMSCs is one factor of osteoporosis, we decided to explore the roles of MIR22HG in the osteogenic and adipogenic differentiation of BMSCs. Our previous study showed MIR22HG reinforced the osteogenesis of human BMSCs via lowering phosphatase and tensin homolog (PTEN) and activating AKT signaling (Jin et al., 2020). In this study, we found that MIR22HG was decreased during the adipogenesis of human BMSCs and regulated adipogenesis negatively both in vitro and in vivo with the involvement of Wnt/β-catenin signaling. Wnt/β-catenin pathway could not only inhibit adipogenesis but also stimulate the osteogenesis of BMSCs (Cawthorn et al., 2012; Si et al., 2006). AKT signaling was validated to involve in the adipogenesis of BMSCs
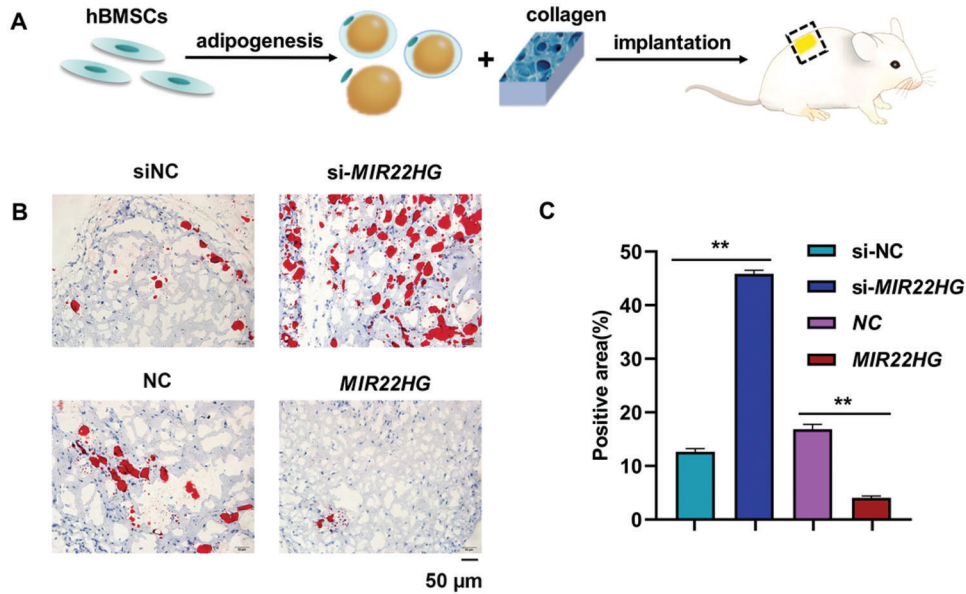
FIGURE 3. MIR22HG inhibited adipose tissue formation *in vivo*. (A) Schematic diagram illustrating the experimental setup. (B) Oil red O staining of the specimens implanted with adipogenic-induced human BMSC/collagen complex in si-NC group, si-MIR22HG group, NC group and MIR22HG overexpressed group. Scale bar, 50 μm. (C) quantitative analysis of the Oil red O-stained areas of newly generated tissue at 8 weeks after implantation. The areas were expressed as percentages of the total areas measured by Image J. Results are presented as the mean ± SD, *P < 0.05, **P < 0.01. Statistical analysis was accomplished by Student's *t*-test, N = 3.

(Song *et al.*, 2017; Wang *et al.*, 2017) and PTEN was a negative regulator of AKT/GSK3β/β-catenin signaling (Chen *et al.*, 2019). Thus, our findings suggested that MIR22HG regulated the osteogenesis and adipogenesis through a complicated network with β-catenin being a core node.

Consistent to our study, MIR22HG was proved to be an inducer of the Wnt/β-catenin signaling pathway via its product, miR-22. MiR-22 targeted two inhibitors of β-catenin, SFRP2 and PCDH15 and exerted its effect on glioblastoma progression (Han *et al.*, 2020). SFRP2 exerted inhibition through competing with Frizzled for binding Wnt (Kawano and Kypta, 2003). PCDH15 could decrease the activity of Wnt signaling. But the detailed mechanisms still remain unsolved (Han *et al.*, 2020). Contrary to our findings, MIR22HG was reported to negatively regulate the Wnt/β-catenin pathway and inhibit cell proliferation and migration in cholangiocarcinoma (Hu *et al.*, 2019). It seems that MIR22HG regulates Wnt/β-catenin pathway differently in special situations. In the present study, MIR22HG may also exert its function in the adipogenesis via miR22/SFRP2 and miR22/PCDH15 axes. Detailed mechanisms between MIR22HG and Wnt/β-catenin signaling need to be researched in the future. Additionally, NTZ was used as a single approach to inhibit Wnt/β-catenin pathway in our study. As a chemical compound, it was proved to influence autophagy differently in various cell lines (Shou *et al.*, 2020; Sun *et al.*, 2021; Wang *et al.*, 2018). NTZ also significantly inhibited proliferation and promoted apoptosis in cardiomyocytes (Gong *et al.*, 2021). Although effects of NTZ on BMSCs stay unidentified, it is possible that NTZ can influence many aspects of BMSCs such as proliferation,

autophagy, and apoptosis. To better understand the mechanism, further study could focus on the effects of NTZ on BMSCs other than suppressing Wnt/β-catenin pathway.

In terms of clinical application, BMSCs are relatively easy to obtain and less likely to form tumor after implantation (Griffin *et al.*, 2011). Advancement of BMSC-based treatments for the restoration of bone tissue is demonstrated by various human clinical studies (Gómez-Barrena *et al.*, 2018; Gómez-Barrena *et al.*, 2020; Rojewski *et al.*, 2019). For instance, one Phase I clinical trial was completed in 2018. Osteoporosis patients were injected autologous fucosylated BMSCs collected 30 days before injection and BMSCs were proved to be feasible with no observed short-term adverse events (ClinicalTrials.gov identifier: NCT02566655) (Hu *et al.*, 2019). However, BMSCs still have some shortcomings as potential seed cells for cell therapy in the treatment of osteoporosis such as inefficiency of osteogenic differentiation. Gene modification of BMSCs will make the BMSC-based treatments safer and more effective in the future. We suggested that MIR22HG might be a gene target to modify the ability of BMSCs to differentiate. Besides, Wnt/β-catenin signaling has been a target of several agents for osteoporosis. Romozumab is a newly developed anti-osteoporotic drug which binds a secreted protein sclerostin and reverses its inhibition on Wnt signaling. It was approved by Food and Drug Administration (FDA) in April 2019 (Mcclung *et al.*, 2014). Our results showed that MIR22HG could regulate the expression of β-catenin and influence the adipogenesis. We hypothesized that MIR22HG might serve as a nucleic drug targeting Wnt signaling in the future.
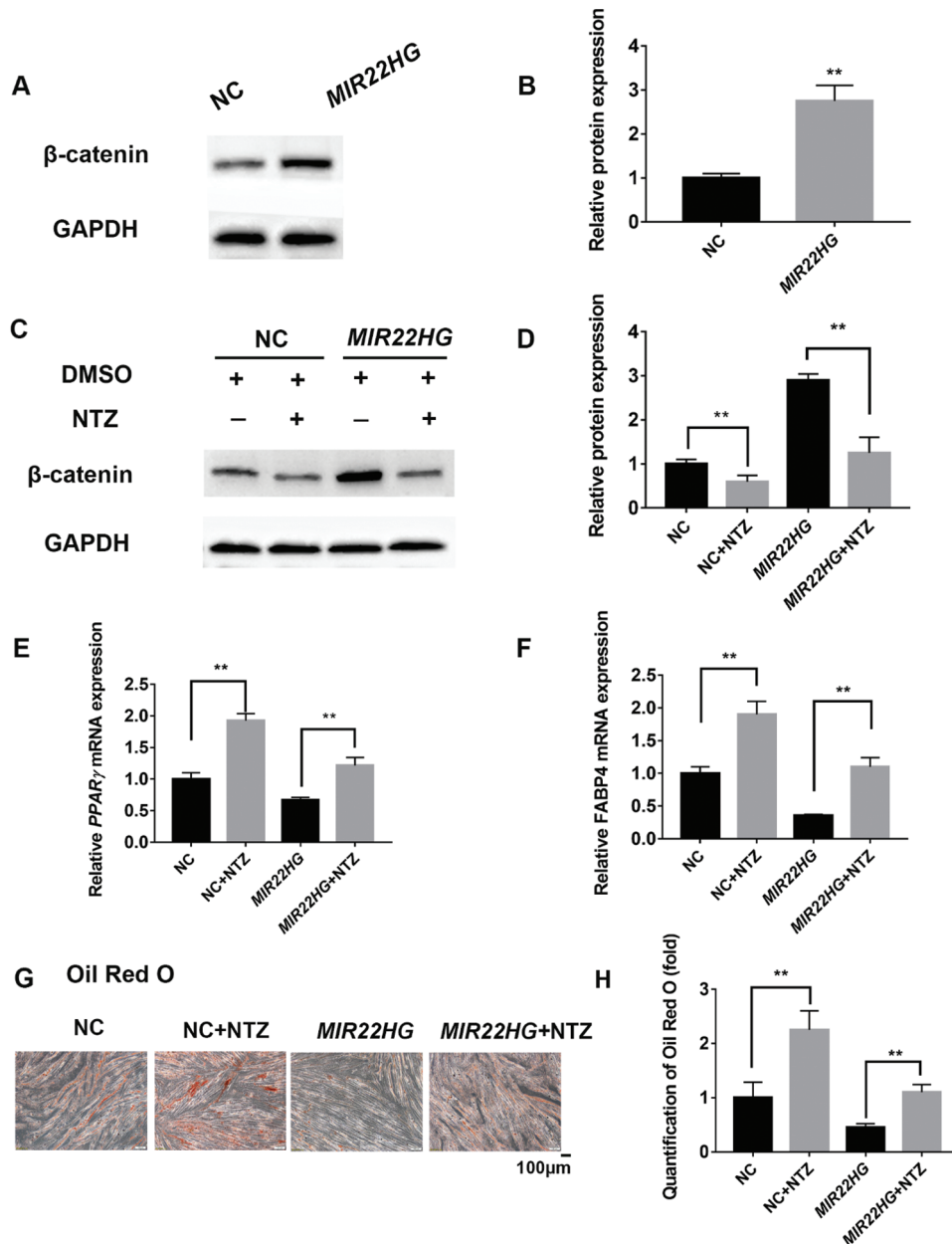
FIGURE 4. MIR22HG inhibited the adipogenesis of human BMSCs via Wnt/β-catenin pathway. (A–B) western blot analysis and quantification of protein expression of β-catenin and GAPDH in NC and MIR22HG groups. (C–D) NTZ and DMSO were added into PM respectively to treat cells for 96 h. Western blot analysis and quantification of protein expression of β-catenin and GAPDH in NC+DMSO, NC+NTZ, MIR22HG+DMSO and MIR22HG+NTZ groups. (E–F) relative mRNA expression levels of PPARγ and FABP4 were measured by qRT-PCR after 7 days adipogenic induction in NC and MIR22HG groups with or without NTZ treatment, relative to NC groups. (G–H), images and quantification of Oil Red O staining for lipid droplet in the NC, NC+NTZ, MIR22HG and MIR22HG+ NTZ groups. Results are presented as the mean ± SD, *$P < 0.05$, **$P < 0.01$. Statistical analysis was accomplished by Student's *t*-test, N = 3.

Conclusion

Overall, this is the first study to recognize the negative effect of MIR22HG on the adipogenesis of human BMSCs with the involvement of Wnt/β-catenin signaling pathway. Our research suggested the potential of MIR22HG to become a therapeutic target of osteoporosis.

Availability of Data and Materials: The data that support the finding of this study are available from the corresponding author upon reasonable request.

Authors' Contribution: The authors confirm contribution to the paper as follows: study conception and design: Yunfei Zheng, Lingfei Jia; data collection: Chanyuan Jin; analysis and interpretation of results: Chanyuan Jin, Ziyao Zhuang; draft manuscript preparation: Ziyao Zhuang. All authors reviewed the results and approved the final version of the manuscript.

Conflicts of Interest: The authors declare that they have no conflicts of interest to report regarding the present study.

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