Glucocorticoid inhibits cell proliferation in differentiating osteoblasts by microRNA-199a targeting of WNT signaling

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Abstract

The inhibition of osteoblast proliferation by glucocorticoids (GCs) is very important in the etiology of GC-induced osteoporosis. The mechanisms of this process are still not fully understood. The results of recent studies have indicated an important role for microRNAs in GC-mediated responses in various cellular processes, including cell proliferation and apoptosis. Therefore, we developed the hypothesis that these regulatory molecules might be involved in GC-decreased osteoblast proliferation. Western blotting, quantitative real-time PCR, cell proliferation assays, and luciferase assays were employed to investigate the role of miRNAs in GC-inhibited osteoblast proliferation. microRNA-199a-5p was significantly increased in osteoblasts treated with dexamethasone (Dex). To delineate the role of microRNA-199a-5p, we silenced and overexpressed microRNA-199a-5p in osteoblasts. We found that overexpressing microRNA-199a-5p remarkably increased the inhibition effect of Dex on osteoblast proliferation, and depleting microRNA-199a-5p significantly attenuated Dex-inhibited osteoblast proliferation. Results of mechanistic studies indicated that microRNA-199a-5p inhibited FZD4 and WNT2 expression through a microRNA-199a-5p binding site within the 3'-UTR of FZD4 and WNT2. The post-transcriptional repression of FZD4 and WNT2 were further confirmed by luciferase reporter assay. These results indicated that microRNA-199a-5p may play a significant role in GC-inhibited osteoblast proliferation by regulating the WNT signaling pathway.

Key Words

- ▶ microRNA-199a-5p
- ▶ glucocorticoids
- ▶ WNT signaling
- ▶ osteoblasts
- ▶ proliferation

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Introduction

Glucocorticoids (GCs) are widely used for the treatment of inflammatory and immune diseases, including asthma, rheumatoid arthritis, Crohn's disease, and so on (Tait

et al. 2008, Shi *et al.* 2014a,b). Although GCs are used extensively to relieve these diseases, increased bone fragility attributed to osteopenia is a serious side effect of prolonged

GC administration (Kondo *et al.* 2008). Indeed, GC-induced osteoporosis (GIO) is currently the third leading cause of osteoporosis, following sex-steroid deficiency and old age (Weinstein *et al.* 1998). GC-treated patients are at a twofold higher risk of suffering from a fracture, irrespective of their bone mineral density (Weinstein 2001). Limited information is available on the pathogenesis of GIO, because the clinical picture of GIO mostly reflects the combined effects of the underlying systemic disease and the secondary effects induced by GC treatment.

The elucidation of the cellular and molecular mechanisms that lead to GIO and the development of improved means of identifying those at risk remain important challenges (Hong *et al.* 2008). It is generally accepted that reduced bone formation is the predominant effect of GCs on bone turnover (Canalis *et al.* 2004, Seibel *et al.* 2013). Results of previous studies have indicated that the induction of osteoblast apoptosis and the inhibition of osteoblast proliferation and differentiation eventually lead to a reduction in bone formation (Weinstein *et al.* 1998, Pereira *et al.* 2001, Hong *et al.* 2011). It has been well established from the results of both *in vivo* and *in vitro* studies that GCs regulate osteoblast apoptosis and differentiation. However, the precise molecular events that underlie the effect of GCs on proliferation pathways in osteoblasts are still not known.

MicroRNAs (miRNAs) are endogenous, non-coding RNAs that are 19–25 nucleotides in length; they regulate various biological processes, including cell proliferation, apoptosis, development, hematopoiesis, organogenesis, and tumorigenesis. miRNAs bind to matched sequences in the 3'-UTR of target mRNAs and either repress the translation or degrade the transcript of their target mRNAs (Zamore & Haley 2005, Couzin 2007). miRNAs may also promote the translation of selected mRNAs in a cell-cycle-phase-dependent way (Zeng 2006). Results of recent studies have indicated that some miRNAs, such as miR-27a, miR-29a, miR-34a, miR-125b, miR-133, miR-138, miR-199a, miR-206, miR-338, miR-335, and miR-378, play a crucial role in bone formation (Taipaleenmaki *et al.* 2012). Furthermore, results of previous studies have indicated that many of these miRNAs, including miR-27a, miR-29a, miR-34a, miR-125b, miR-199a, and miR-574, could mediate cell proliferation (Huang *et al.* 2012, Ma *et al.* 2012, Wei *et al.* 2012, Xu *et al.* 2012a,b, Chiyomaru *et al.* 2013, Wu *et al.* 2013). Moreover, these six miRNAs have been reported to regulate the WNT signaling pathway (Chiyomaru *et al.* 2013, Liu *et al.* 2013, Nagano *et al.* 2013, Guo *et al.* 2014, Rathod *et al.* 2014, Hashemi *et al.* 2015), which is a crucial regulator

of GC-mediated bone acquisition and remodeling activities. Thus, we proposed the hypothesis that these molecules might be involved in GC-repressed osteoblast proliferation.

In the present study, we examined the role of miR-199a-5p in the repression of osteoblast proliferation by GCs. We detected some expression of miRNA, which has been reported to be related to bone formation and to mediate WNT signaling, in osteoblasts exposed to GCs. Several miRNAs were found to be altered, and miR-199a-5p was identified as a strong candidate for being the miRNA that was responsible for the decreased osteoblast proliferation in response to GC. Further studies confirmed that miR-199a-5p regulated osteoblast proliferation by targeting WNT signaling. Therefore, miRNAs and miRNA-regulated gene silencing may contribute to the inhibitory effects of GCs on osteoblast proliferation.

Materials and methods

In vivo treatment of mice

Seven-day-old neonatal C57 female mice were used for the present study. Stock solutions of 1 mg/ml dexamethasone (Dex) were prepared in ethanol as previously described by Gohel *et al.* (1999). Dosing solutions were prepared by diluting the stock solution with normal saline. After measurement of their weight, mice were given daily s.c. injections of Dex (1.0 mg/kg BW). At 72 h, mice were weighed and killed. The entire calvarium was removed for real-time quantitative reverse transcription polymerase chain reaction (real-time qRT-PCR).

Cell culture

The MC3T3-E1 cell line was supplied by the Shanghai Institute of Orthopaedics and Traumatology. Lines of less than 20 passages were used in the present study. Primary osteoblasts were obtained from neonatal murine calvaria using methods described previously (Shi *et al.* 2014a,b). Cells were cultured with alpha-minimal essential media (α -MEM) (Invitrogen) supplemented with 10% fetal bovine serum (FBS) and 100 μ g/ml penicillin/streptomycin. All experiments were performed under differentiation conditions, that is, in the presence of 50 μ g/ml ascorbic acid or 4 mmol/l β -glycerophosphate (Sigma-Aldrich). HEK293 cells were cultured in DMEM (Invitrogen). The cultures were supplemented with 10% FBS and 100 μ g/ml penicillin/streptomycin.

Cell proliferation assay

A cell counting kit (CCK-8) assay (Dojindo Laboratories, Kumamoto, Japan) was used to measure cell proliferation according to the manufacturer's guidelines. Briefly, cells were resuspended in 200 μ l cell culture medium, seeded at a density of 1×10^3 cells per well in 96-well microtiter plates, and incubated overnight for cell attachment. A 10 μ l volume of CCK-8 reagents was added to each well 1 h before the end of incubation. The optical density value of each sample was measured at a wavelength of 450 nm on a microplate reader to determine the viability of the cells. The proliferation rate was normalized to the value at time point 0.

5-ethynyl-2'-deoxyuridine assay

Exponential-growth-phase cells were seeded into 24-well plates and incubated with serum-free α -MEM for 24 h. In brief, 5-ethynyl-2'-deoxyuridine (EdU) (Sigma-Aldrich) solution was added to cell culture medium to give a final concentration of 1:1000 and then incubated for 2 h. Cell fixative (containing 4% paraformaldehyde in PBS) was added before incubation at room temperature for 30 min. After the cells were washed with PBS two times, click reaction buffer (100 mM Tris-HCl (pH 8.5), 1 mM CuSO₄, 100 μ M Apollo 550 fluorescent azide, 100 mM ascorbic acid) was added for 10–30 min while the cells were protected from light. Then, cells were washed three times with 0.5% Triton X-100 and subsequently stained with Hoechst (5 μ g/ml) for 30 min at room temperature. Samples were stored in the dark at 4 °C until they were examined using a fluorescence microscope (Olympus). EdU-positive cells were calculated by the following formula: (EdU add-in cells/Hoechst stained cells) $\times$ 100%.

5-(and-6)-carboxyfluorescein diacetate, succinimidyl ester assay

Cell proliferation was measured using 5-(and-6)-carboxyfluorescein diacetate, succinimidyl ester (CFSE) staining and flow cytometry (Xu *et al.* 2006). Cells were incubated with CFSE (Invitrogen) at a concentration of 5 μ M in PBS for 15 min at 37 °C. The reaction was stopped by adding FCS. Cells were replated at a density of 48 000 cells/well in six-well dishes and incubated for 3–5 days. After preparation by trypsinization and washing, fluorescence intensity was measured by flow cytometry, using excitation at 488 nm at the FL1 detection channel, and analyzed with CellQuest Software Becton Dickinson, Franklin Lakes, NJ, USA.

MiR-199a-5p target gene prediction

We used a computation and bioinformatics-based approach to predict the putative targets of miR-199a-5p through TargetScan, which is hosted by the Wellcome Trust Sanger Institute. FZD4 and WNT2 were predicted to be potential target genes of miR-199a-5p by the TargetScan program.

Western blot analysis

The protein samples were extracted from osteoblasts via procedures that have previously been described in detail (Yang *et al.* 2007). Protein samples (approximately 50 μ g) were fractionated by SDS-PAGE (7.5–10% polyacrylamide gels). Separated proteins were blot transferred onto a nitrocellulose membrane. After blocking with 0.1% Tween 20 and 5% nonfat dry milk in Tris-buffered saline at room temperature for 1 h, the membrane was incubated overnight at 4 °C in one of the following primary antibodies as an internal control: FZD4 (1:400; Peprotech, Rocky Hill, NJ, USA), WNT2 (1:400; Santa Cruz Biotechnology, Santa Cruz, CA, USA), β -catenin (1:200; Santa Cruz Biotechnology), Runx2 (1:200; Santa Cruz Biotechnology), Osterix (1:400; Santa Cruz Biotechnology), and β -actin (1:1000; Santa Cruz Biotechnology). The membrane was incubated with HRP-conjugated secondary antibody (1:2000) for 1 h and detected using the ECL Western Blot System (Amersham Biosciences).

Synthesis of miRNAs and sequences of miR-199a-5p inhibitors

MiR-199a-5p (sense: 5'-CCCAGUGUUCAGACUACCU-GUUC-3', antisense: 5'-ACAGGUAGUCUGAACACUGG-GUU-3') and its antisense oligonucleotides (AMOs: 5'-GAACAGGTAGTCTGAACACTGGG-3') were synthesized by Integrated DNA Technologies. Additionally, a scrambled RNA was used as a negative control (NC) (sense: 5'-UUCUCCGAACGUGUCACGUTT-3', antisense: 5'-ACGUGACACGUUCGGAGAATT-3'). DNA fragments of the 3'-UTRs of WNT2 and FZD4 mRNA containing the putative miR-199a-5p binding sequence were synthesized by Invitrogen. These fragments were then cloned into the multiple cloning sites downstream of the luciferase gene (HindIII and SpeI sites) in the pMIR-REPORT luciferase miRNA expression reporter vector (Ambion Inc, Austin, TX, USA), as described previously (Yang *et al.* 2007).

Figure 1

The inhibition of differentiating osteoblast proliferation by Dex. (A) The proliferation of MC3T3-E1 cells was measured by CCK8 after the cells were treated with 10^{-9} , 10^{-8} , or 10^{-7} M Dex from day 1 to day 5. The results indicated that Dex decreased the viability of MC3T3-E1 cells in a dose- and time-dependent manner. $n=3$, $**P<0.01$. (B) Representative photomicrographs ($\times 400$) of EdU staining (top panels) and corresponding total cell photomicrographs (middle panels). Blue: Hoechst labeling of cell nuclei;

red: EdU labeling of nuclei of proliferative cells. (C) Quantitative data showing the percentage of EdU-positive cells in different treatment groups (number of red vs number of blue nuclei). $n=3$, $**P<0.01$. (D) MC3T3-E1 cells were stained with CFSE before plating, cultured for 5 days, and analyzed by flow cytometry as described in the Materials and methods section. The peak CFSE fluorescence intensity on flow cytometry was shifted by Dex. $n=3$. Con, control; Dex, dexamethasone.

Transfection of miR-199a-5p/AMO-199a-5p in osteoblasts

The MC3T3-E1 cells/primary osteoblasts were cultured with differentiation medium for 24 h. Osteoblasts cultured in six-well plates were divided into different groups. Osteoblasts in the control group were cultured in

differentiation culture medium. Those in the miRNA group were transfected with miR-199a-5p and/or inhibitors for 48 h under differentiation conditions, with X-tremeGENE siRNA Transfection Reagent (Roche), according to the manufacturer's instructions. Those in the Dex (Sigma-Aldrich) group were treated with 10^{-7} M Dex for

Figure 2

The upregulation of miR-199a-5p expression by Dex in differentiating osteoblasts. (A) Real-time qRT-PCR analysis of miR-574-3p, miR-27a, miR-29a, miR-34a, miR-125b, and miR-199a-5p expression in MC3T3-E1 cells treated with 10^{-7} M Dex for 5 days. $n=3$, $**P<0.01$. (B) Real-time qRT-PCR analysis of miR-199a-5p expression in MC3T3-E1 cells treated with 10^{-7} M

Dex from day 1 to day 5. $n=3$, $**P<0.01$. (C) Real-time qRT-PCR analysis of miR-199a-5p expression in primary osteoblasts treated with 10^{-7} M Dex for 5 days. $n=3$, $**P<0.01$. (D) Real-time qRT-PCR analysis of miR-199a-5p expression in the calvarias from mice treated with Dex for 3 days. $n=3$, $**P<0.01$. Con, control; Dex, dexamethasone; miR, microRNA.

5 days. The miRNA and Dex group cells were transfected with miR-199a-5p and/or inhibitors for 48 h under 10^{-7} M Dex and then treated with Dex for 3 days.

Luciferase activity assay

After 24 h of starvation in serum-free medium, HEK293 cells (1×10^5 per well) were transfected with 1 μ g miR-199a-5p/AMO-199a-5p or 1 μ g PGL3 target DNA (firefly luciferase vector) and 0.1 μ g PRL-TK (TK-driven Renilla luciferase expression vector) with lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. Luciferase activity was measured 48 h after transfection with a dual luciferase reporter assay kit (Promega) on a luminometer (Lumat LB9507).

Quantification of miRNA levels

The mirVana real-time qRT-PCR miRNA Detection kit (Ambion) was used in conjunction with real-time PCR with SYBR Green I for quantification of miR-199a-5p transcript as described previously (Yang *et al.* 2007).

Statistical analysis

All data were analyzed using SPSS version 19.0 (SPSS, Inc.). The composite data are expressed as means $\pm$ S.E.M. Statistical analysis was performed with one-way ANOVA followed by Dunnett's test where appropriate. Differences were considered to be significant for $P \leq 0.05$.

Figure 3

The repression of MC3T3-E1 cell proliferation by miR-199a-5p. (A) The proliferation of MC3T3-E1 cells was measured by CCK8 assay after cells were transfected with miR-199a-5p and/or AMO-199a-5p from day 1 to day 5. $n=3$, $**P<0.01$. (B) Representative photomicrographs ($\times 400$) of EdU staining (top panels) and corresponding total cell photomicrographs (middle panels). Blue: Hoechst labeling of cell nuclei; red: EdU labeling of nuclei of proliferative cells. (C) Quantitative data showing the percentages of EdU-positive cells in different treatment groups (number of red

versus number of blue nuclei). $n=3$, $**P<0.01$. (D) MC3T3-E1 cells transfected with miR-199a-5p and/or AMO-199a-5p were stained with CFSE and analyzed by flow cytometry as described in the Materials and methods section. $n=3$. (E) Western blot analysis of Runx2 and Osterix expression in MC3T3-E1 cells transfected with miR-199a-5p and/or AMO-199a-5p. $n=3$, $**P<0.01$, $*P<0.05$. Con, control; NC, negative control; miR, microRNA.

Results

The effect of Dex on differentiating osteoblast proliferation

To determine whether Dex regulated the proliferation of differentiating osteoblasts, we first used the CCK-8 assay to monitor cell proliferation. MC3T3-E1 cells under differentiation conditions were treated with Dex at different concentrations (0 , 10^{-9} , 10^{-8} , and 10^{-7} M) from day 1 to day 5. The results indicated that Dex decreased the proliferation of MC3T3-E1 cells in a dose- and time-dependent manner (Fig. 1A).

Furthermore, MC3T3-E1 cells were stimulated with 10^{-7} M Dex for 5 days before the EdU assay. EdU-stained photomicrographs and corresponding photomicrographs of all cells are shown in Fig. 1B. The proportion of cells with EdU-positive nuclei is shown in Fig. 1C. EdU incorporation was decreased in the Dex group, which indicates that Dex could inhibit MC3T3-E1 cell proliferation.

To further examine proliferation, MC3T3-E1 cells were cultured with 10^{-7} M Dex for 5 days and stained with CFSE. CFSE irreversibly couples to cellular proteins. When cells divide, CFSE labeling is distributed equally between daughter cells, which are half as fluorescent as

their parents are. The peak CFSE fluorescence intensity on flow cytometry was shifted by Dex, which indicates that cells treated with Dex had fewer cycles of cell replication as compared with the control group (Fig. 1D).

Upregulation of miR-199a-5p expression by Dex in differentiating osteoblasts

To examine the role of miRNAs in the repression of osteoblast proliferation by Dex, we first detected some miRNA expression, which has been reported to be related to bone formation and to mediate WNT signaling, in MC3T3-E1 cells exposed to 10^{-7} M Dex for 5 days. Several miRNAs were found to be altered, and miR-199a-5p was identified as a strong candidate for being the miRNA that was responsible for the GC-decreased osteoblast proliferation. We found that miR-574-3p and miR-27a were weakly affected during this time frame. However, the expression of miR-199a-5p was significantly upregulated by Dex (Fig. 2A). The results of studies indicated that Dex increased the expression of miR-199a-5p in a time-dependent manner (Fig. 2B). Similarly, the upregulation of miR-199a-5p expression was also observed in primary osteoblasts treated with

Figure 4

The repression of primary osteoblast proliferation by miR-199a-5p. (A) Proliferation of primary osteoblasts was measured by CCK8 assay after cells were transfected with miR-199a-5p and/or AMO-199a-5p from day 1 to day 5. $n=3$, $**P<0.01$. (B) Quantitative data showing the percentages of EdU-positive cells in different treatment groups (number of red versus

number of blue nuclei). $n=3$, $**P<0.01$, $*P<0.05$. (C) Primary osteoblasts transfected with miR-199a-5p and/or AMO-199a-5p were stained with CFSE and analyzed by flow cytometry as described in the Materials and methods section. $n=3$. Con, control; NC, negative control; miR, microRNA.

10^{-7} M Dex for 5 days (Fig. 2C). Furthermore, we observed the effects of Dex on miR-199a-5p expression *in vivo*. We found that the expression of miR-199a-5p was significantly upregulated in calvarias from mice treated with Dex (Fig. 2D). Thus, we proposed the hypothesis that miR-199a-5p might be involved in Dex-decreased osteoblast proliferation.

Repression of differentiating osteoblast proliferation by miR-199a-5p

To delineate the role of miR-199a-5p in osteoblast proliferation, we performed loss-of-function and gain-of-function experiments in which we decreased and increased the quantities of miR-199a-5p with a miR-199a-5p inhibitor and a miR-199a-5p mimic respectively. MC3T3-E1 cells were transfected with miR-199a-5p and/or AMO-199a-5p from day 1 to day 5. Then we assessed cell proliferation with CCK-8 assays. We found that overexpression of miR-199a-5p decreased MC3T3-E1 cell proliferation. However, the depletion of miR-199a-5p with AMO-199a-5p resulted in increased MC3T3-E1 cell proliferation.

Furthermore, the results of EdU assays by fluorescence microscopy further indicated that miR-199a-5p mimic clearly decreased MC3T3-E1 cell proliferation (Fig. 3B and C), and miR-199a-5p inhibitor significantly increased osteoblast proliferation. Similar results were further confirmed by the results of CFSE fluorescence intensity assays (Fig. 3D). In addition, the effect of miR-199a-5p on osteoblast differentiation was examined using western blotting. The levels of expression of osteogenic marker genes, including *Runx2* and *Osterix*, were significantly affected in MC3T3-E1 cells that had been transfected with miR-199a-5p and/or AMO-199a-5p for 48 h, which indicates that miR-199a-5p is also involved in osteoblast differentiation (Fig. 3E).

Furthermore, primary osteoblasts were also used to test the effects of miR-199a-5p on cell proliferation. The proliferation of primary osteoblasts transfected with miR-199a-5p and/or AMO-199a-5p was measured by CCK8, EdU assay, and CFSE fluorescence intensity assay. Similarly, we found that miR-199a-5p significantly decreased primary osteoblast proliferation, and AMO-199a-5p increased it (Fig. 4A, B and C). Taken together, these results indicate that miR-199a-5p could regulate osteoblast proliferation.

Figure 5

The involvement of miR-199a-5p in Dex-reduced MC3T3-E1 cell proliferation. (A) The proliferation of MC3T3-E1 cells was measured by CCK8 assay after cells were transfected with miR-199a-5p and/or AMO-199a-5p under Dex from day 1 to day 5. $n=3$, $**P<0.01$. (B) Quantitative data showing the percentage of EdU-positive cells in different treatment groups (number of

red versus number of blue nuclei). $n=3$, $**P<0.01$. (C) MC3T3-E1 cells transfected with miR-199a-5p and/or AMO-199a-5p under Dex were stained with CFSE and analyzed by flow cytometry as described in the Materials and methods section. $n=3$. Con, control; Dex, dexamethasone; NC, negative control; miR, microRNA.

Figure 6

The involvement of miR-199a-5p in Dex-reduced primary osteoblast proliferation. (A) The proliferation of primary osteoblasts was measured by CCK8 assay after cells were transfected with miR-199a-5p and/or AMO-199a-5p under Dex from day 1 to day 5. $n=3$, $**P<0.01$. (B) Quantitative data showing the percentage of Edu-positive cells in different treatment

groups (number of red versus number of blue nuclei). $n=3$, $**P<0.01$, $*P<0.05$. (C) Primary osteoblasts transfected with miR-199a-5p and/or AMO-199a-5p under Dex were stained with CFSE and analyzed by flow cytometry as described in the Materials and methods section. $n=3$. Con, control; Dex, dexamethasone; NC, negative control; miR, microRNA.

Involvement of miR-199a-5p in the reduction of osteoblast proliferation in response to Dex

We consequently investigated whether miR-199a-5p was involved in the decreased osteoblast proliferation in response to Dex. To this end, we observed the effect of Dex on proliferation in MC3T3-E1 cells transfected with miR-199a-5p and/or AMO-199a-5p for 5 days. The results of CCK-8 assays indicated that miR-199a-5p markedly increased the reduction in MC3T3-E1 cell proliferation in response to Dex. Furthermore, the co-application of miR-199a-5p and AMO-199a-5p almost completely abolished the effect of miR-199a-5p. Moreover, the inhibitory effect of Dex on MC3T3-E1 cell proliferation was significantly alleviated when MC3T3-E1 cells alone were transfected with AMO-199a-5p, which indicates that miR-199a-5p is involved in Dex-decreased osteoblast proliferation (Fig. 5A). Similar results were further confirmed by the results of Edu assay and flow cytometric analysis of CFSE intensity (Fig. 5B and C). All of these results were further verified in primary osteoblasts (Fig. 6A, B and C).

Repression of WNT signaling by miR-199a-5p transfection

The observations presented in the previous sections indicate that miR-199a-5p is involved in osteoblast proliferation. It is possible that miR-199a-5p targets several regulatory factors related to osteoblast proliferation. To address this issue, we used a computational and bioinformatics-based approach to predict the putative targets related to proliferation through TargetScan. These explorations led to the identification of candidate targets of miR-199a-5p: WNT signaling, including FZD4 and WNT2 (Fig. 7A and B). Results of western blot analysis of FZD4 and WNT2 expression in the MC3T3-E1 cells treated with 10^{-7} M Dex for 5 days are shown in Fig. 7C and D. We found that Dex decreased FZD4 and WNT2 expression in MC3T3-E1 cells. To verify that FZD4 and WNT2 are indeed repressed post-transcriptionally by miR-199a-5p, we determined the effect of miR-199a-5p on protein expression. The results of western blot analysis indicated that miR-199a-5p markedly lowered the levels of FZD4 and WNT2 proteins in MC3T3-E1 (Fig. 7E and F). Co-application of miR-199a-5p and AMO-199a-5p almost completely

Figure 7

The repression of WNT signaling by miR-199a-5p transfection. (A) The sequences showing the unique sites of miRNA::mRNA complementarity between miR-199a-5p and FZD4. (B) The sequences showing the unique sites of miRNA::mRNA complementarity between miR-199a-5p and WNT2. (C) Western blot analysis of FZD4 expression in MC3T3-E1 cells treated with 10^{-7} M Dex for 5 days. $n=3$, $**P<0.01$. (D) Western blot analysis of WNT2 expression in MC3T3-E1 cells treated with 10^{-7} M Dex for 5 days. $n=3$,

$**P<0.01$. (E) Western blot analysis of FZD4 expression in MC3T3-E1 cells transfected with miR-199a-5p and/or AMO-199a-5p for 48 h. $n=3$, $**P<0.01$. (F) Western blot analysis of WNT2 expression in MC3T3-E1 cells transfected with miR-199a-5p and/or AMO-199a-5p for 48 h. $n=3$, $**P<0.01$. Con, control; Dex, dexamethasone; NC, negative control; miR, microRNA.

abolished the effect of miR-199a-5p. Moreover, application of AMO-199a-5p alone increased the levels of FZD4 and WNT2 in MC3T3-E1, which indicates that there is a basal level of miR-199a-5p activity in osteoblasts (Fig. 7E and F). Similarly, we also tested the effects of miR-199a-5p on the levels of FZD4 and WNT2 proteins in primary osteoblasts. The results indicated that miR-199a-5p significantly decreased FZD4 and WNT2 protein expression, and AMO-199a-5p increased it (Fig. 8A and B). In addition, we observed the effect of miR-199a-5p on the expression of β -catenin, a key mediator of the WNT signaling pathway, in primary osteoblasts. We found that miR-199a-5p

decreased β -catenin expression, which indicates that miR-199a-5p could inhibit WNT signaling (Fig. 8B).

Verification of interactions between miR-199a-5p and its target genes

We constructed chimeric vectors by placing the 3'-UTRs of FZD4 and WNT2 into the 3'-UTR of a luciferase reporter plasmid. We performed luciferase reporter assays using HEK293 cells that do not express miR-199a-5p (data not shown). Compared with the NC, transfection of miR-199a-5p with the luciferase reporter gene linked to

Figure 8

The repression of WNT signaling by transfection with miR-199a-5p.

(A) Western blot analysis of FZD4 and WNT2 expression in primary osteoblasts treated with 10^{-7} M Dex for 5 days. $n=3$, $**P<0.01$.

(B) Western blot analysis of FZD4, WNT2, and β -catenin expression in

primary osteoblasts transfected with miR-199a-5p and/or AMO-199a-5p for 48 h. $n=3$, $**P<0.01$. Con, control; Dex, dexamethasone; NC, negative control; miR, microRNA.

the 3'-UTR of FZD4 or WNT2 resulted in a significant decrease in luciferase activity, and co-application of miR-199a-5p with AMO-199a-5p alleviated the decrease in luciferase activity, whereas AMO-199a-5p alone had no effect (Fig. 9A and B). These results indicate that FZD4 and WNT2 are the targets of miR-199a-5p.

Successful delivery of miR-199a-5p, AMO-199a-5p, and NC to the cells was further verified by comparing the miR-199a-5p levels 48 h after transfection of the constructs in MC3T3-E1 cells and primary osteoblasts. Transfection resulted in a significant increase in miR-199a-5p levels (Fig. 9C). It is worth noting that miR-199a-5p levels were dynamic after transfection. The data were collected 48 h after transfection because the plateau level was reached at that time. These results proved the feasibility of all of the experiments.

Discussion

Increased concentrations of GCs could cause the development of Cushing's syndrome with severe osteoporosis. Results of previous studies have indicated that GCs have potent inhibitory effects on osteoblast proliferation (Walsh *et al.* 2001, Hong *et al.* 2011). However, the molecular mechanisms involved in GC-inhibited osteoblast proliferation are still poorly understood. In the present study, we showed that miR-199a-5p plays a significant role in GC-decreased osteoblast proliferation by regulating the WNT signaling pathway.

Results described in several recent reports have indicated that GCs exert post-transcriptional control through the regulation of miRNA processing and expression (De Iudicibus *et al.* 2013). Xing *et al.* (2014) reported that GCs induced apoptosis by inhibiting miRNA cluster miR-17-92 expression in chondrocytic cells. Furthermore, miR-29b and miR-29c have been reported to be involved in the Toll-like receptor control of GC-induced apoptosis in human plasmacytoid dendritic cells (Hong *et al.* 2013). In addition, miRNAs could also negatively regulate the GC receptor (GR) transcriptional response by directly targeting the 3'-UTR of GR α mRNA (Lv *et al.* 2012). It is also known that osteoblasts are critical target cells of GCs. Results of our previous studies indicated that miR-17-92a downregulation by GCs leads to Bim targeting and to the induction of osteoblast apoptosis (Guo *et al.* 2013). Furthermore, GCs could also increase receptor activator of nuclear factor B ligand expression by downregulating miR-17/20a in osteoblasts, which indirectly enhances osteoclastogenesis and bone resorption (Shi *et al.* 2014a,b). However, to our knowledge, there have been no reports on whether miRNA expression could be regulated by GCs in osteoblast proliferation. The present study is the first to observe the effect of GCs on miRNAs in osteoblast proliferation.

We detected some miRNA expression in osteoblasts that were exposed to GCs. These miRNAs have been reported to be related to cell proliferation and bone formation in previous studies (Huang *et al.* 2012,

Figure 9

Verification of the interactions between miR-199a-5p and their target genes. (A) Verification of FZD4 as a cognate target of miR-199a-5p. Data on luciferase reporter activity show the interaction between miR-199a-5p and FZD4 3'-UTRs. $n=3$, $**P<0.01$. (B) Verification of WNT2 as a cognate target of miR-199a-5p. Data on luciferase reporter activity shows the interaction between miR-199a-5p and WNT2 3'-UTRs. $n=3$, $**P<0.01$, $*P<0.05$. (C) MiR-199a-5p levels in MC3T3-E1 cells and primary osteoblasts with transfection of sequence determined by real-time qRT-PCR. $n=3$, $**P<0.01$. Con, control; NC, negative control; miR, microRNA.

Ma *et al.* 2012, Taipaleenmaki *et al.* 2012, Wei *et al.* 2012, Xu *et al.* 2012a,b, Chiyomaru *et al.* 2013, Wu *et al.* 2013). Furthermore, these miRNAs could mediate WNT signaling (Chiyomaru *et al.* 2013, Liu *et al.* 2013, Nagano *et al.* 2013, Guo *et al.* 2014, Rathod *et al.* 2014, Hashemi *et al.* 2015), a crucial factor in decreased bone formation in response to GCs. Therefore, we first examined whether these six

miRNAs were involved in the regulation of osteoblast proliferation by GCs. The results indicated that only miR-199a-5p was a strong candidate for being responsible for the decreased osteoblast proliferation in response to GCs. We speculated that miRNAs might play different roles in different cells. The present study did not apply miRNA chips to screen miRNA expression in response to the decreased osteoblast proliferation due to GCs. Therefore, several differentially expressed miRNAs may have been missed. Future studies are required to identify other miRNAs involved in the inhibition of osteoblast proliferation in response to GCs.

The present results indicated that miR-199a-5p was significantly increased during the decreased osteoblast proliferation in response to Dex. Furthermore, overexpression of miR-199a-5p decreased osteoblast proliferation. However, the depletion of miR-199a-5p with AMO-199a-5p resulted in an increased proliferation of osteoblasts. Results of previous studies have indicated that miR-199a-5p is a ubiquitously expressed miRNA whose expression is modulated at key time points during development, growth, and regeneration in cancers (Xu *et al.* 2012a,b, Alexander *et al.* 2013, Shi *et al.* 2014a,b). Shi *et al.* (2014a,b) showed that miR-199a-5p affects porcine preadipocyte proliferation and differentiation. Alexander *et al.* (2013) found that miR-199a-5p affects WNT signaling, cell proliferation, and myogenic differentiation. Furthermore, lentivirus-mediated overexpression of miR-199a-5p has been shown to inhibit cell proliferation in human hepatocellular carcinoma (Xu *et al.* 2012a,b). Although the role of miR-199a-5p in the pathogenesis of several diseases, including breast cancer, lymphoma, and pulmonary hypertension, was reported as part of these studies, the present study is the first, to our knowledge, to uncover the role of miR-199a-5p in metabolic bone diseases and osteoblast proliferation.

GCs, which regulate diverse physiological effects, have both genomic and nongenomic mechanisms (Stahn & Buttgerit 2008). The steroid's nongenomic effects occur within seconds to minutes and are mediated by the GR or by another means, such as G-protein-coupled receptors (Stahn & Buttgerit 2008). It has been demonstrated that GCs are unable to suppress bone formation in the absence of GR expression in osteoblasts because their proliferation, differentiation, and apoptosis become immune to GCs (Rauch *et al.* 2010). Therefore, the GC-inhibited proliferation of osteoblasts is triggered via GR-dependent transcriptional regulation. Results from a previous study indicated that GCs could regulate miRNA expression via a GR-mediated direct DNA binding mechanism

(De Iudicibus *et al.* 2013). In the present study, we did not explore whether GR was involved in the regulation of miR-199a-5p by GCs in osteoblasts. Therefore, further studies need to be performed to uncover the role of GC-regulated miR-199a-5p in osteoblasts. WNT/ β -catenin signaling plays an important role in bone development and metabolism by controlling both bone formation and resorption (Wang *et al.* 2014). Wnt ligands are secreted molecules that bind to cell-surface receptors that are encoded by the Frizzled and LDL-receptor-related proteins. Once bound, the ligands initiate a cascade of intracellular events that activate β -catenin activity and the DNA binding protein TCF, which eventually results in the transcription of target genes (Wang *et al.* 2014). It has been confirmed that GCs could inhibit canonical WNT signaling to suppress bone formation (Guanabens *et al.* 2014). In the present study, miR-199a-5p was involved in GC-inhibited osteoblast proliferation insofar as it targeted WNT signaling. The results of previous studies have also indicated that miR-199a-5p could target several factors that regulate cell proliferation within the WNT signaling pathway, including FZD4 and WNT2 (Alexander *et al.* 2013).

Our data provide new evidence that miR-199a-5p plays a dominant role in GC-inhibited osteoblast proliferation. The inhibitory effect of miR-199a-5p on osteoblast proliferation involves the disruption of WNT signaling. The present study is an effort to better understand the molecular mechanism of GIO and to provide new insights into the potential contribution of miRNAs to GC-mediated osteoblast proliferation and bone formation.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Author contribution statement

L G and C G S were involved in the conception and hypothesis delineation; L G and C G S designed the experiments, conducted the luciferase and quantitative real-time PCR experiments, and wrote the manuscript; J Q, B H, H B Z, and H K performed the EdU staining and CFSE assay; P H modified the manuscript; M J and P H performed part of the luciferase and western blot analyses; and L F D designed and conducted the animal studies.

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