Three functional polymorphisms in CCDC170 were associated with osteoporosis phenotype

Xinhong Liu1,2,3,4,*, Yu-Gang Li3,*, Fang Tan6,†, Jia Liu1,2,3, Ruokun Yi1,2,3 and Xin Zhao1,2,3,‡

ABSTRACT

MicroRNAs (miRNAs) play essential roles in regulating bone formation and homeostasis. Genomic variations within miRNA target sites may therefore be important sources of genetic differences in osteoporosis risk. The function of CCDC170 in bone biology is still unclear. To verify the function of CCDC170, we knocked down CCDC170 in cells and mice and searched for miRNA recognition sites within CCDC170 using the TargetScan, miRNAsNP, and miRBase databases. In this study, our results demonstrated that CCDC170 plays an important role in the positive regulation of bone formation. MiR-153-3p, miR-374b-3p, miR-4274, miR-572 and miR-2964a-5p inhibited CCDC170 expression in an allele-specific manner by binding GWAS lead SNPs rs6932603, rs3757322 and rs3734806. These findings may improve our understanding of the association between CCDC170, miRNAs, GWAS lead SNPs, and osteoporosis pathogenesis and may provide a potential therapeutic target for osteoporosis therapy.

KEY WORDS: CCDC170, miRNA, GWAS lead SNP, Osteoporosis risk

INTRODUCTION

Osteoporosis is a common complex disease defined by bone mineral density (BMD) that is highly heritable, with heritability estimates of 0.5–0.85 (Ralston and Uitterlinden, 2010). With increased age and hormonal changes, the body’s bone mass decreases. When bone mass decreases and bone fragility increases, fractures and osteoporosis will eventually occur (Albargh and Ralston, 2003). Fractures due to osteoporosis are a major public health burden (Cauley, 2013). This has led to an increased effort in developing more effective means of treating and preventing bone disease (Sabik and Farber, 2017). Discovering genetically variable loci and clarifying their biological functions in BMD variations are important to understanding the etiology of osteoporosis and developing new approaches to screen, prevent, and treat osteoporosis (Mei et al., 2019).

Single-nucleotide polymorphism (SNP)-based genome-wide association studies (GWASs) have already identified large number differences in the allele expression of three GWAS lead SNPs

and exploring the relationship between miRNA, SNPs and CCDC170 in osteoporosis, we performed a series of gene overexpression and knockdown experiments in cells and mice vivo. In the present study, we found that CCDC170 could positively regulate bone formation via three GWAS lead SNPs [rs6932603 (C–T), rs3757322 (G/T) and rs3734806 (A/G)]. In addition, we found that miR-153-3p, miR-374b-3p, miR-4274, hsa-miR-572 and hsa-miR-2964a-5p differentially bound to CCDC170 3′-UTR to inhibit osteogenesis. In summary, we found a new function of CCDC170 in bone biology and five new miRNAs that promote the development of osteoporosis, providing new options and targets for the treatment of osteoporosis.

RESULTS

Differences in the allele expression of three GWAS lead SNPs

To determine whether the rs6932603-C and rs6932603-T, rs3757322-G and rs3757322-T, rs3734806-A and rs3734806-G
allele expression differed, U2OS and 293T cells were transfected with the rs6932603-C, rs6932603-T, rs3757322-G, rs3757322-T, rs3734806-A and rs3734806-G allele psiCHECK-2 vectors. The luciferase reporter assay showed that the rs6932603-T allele expression level was significantly lower than that of the rs6932603-C allele, the rs3757322-T allele expression level was significantly lower than that of the rs3757322-G allele, and the rs3734806-G allele expression level was significantly lower than that of the rs3734806-A allele in the U2OS cells and 293T cells (Fig. 1A, B).

miRNAs differentially regulated expression of the allele variants of GWAS lead SNPs

Next, we tested a biological model in which miR-153-3p differentially regulated the C/T allele variants of rs6932603 in CCDC170. In this model, rs6932603-T decreased the CCDC170 transcription levels, leading to an increased predicted osteoporosis risk (http://mirdsnp.ccr.buffalo.edu/) (Fig. 2A). To test our model, we co-transfected the rs6932603-C/T allele psiCHECK-2 vector and miR-153-3p mimics or NC mimics in U2OS cells and 293T cells. The luciferase reporter assay results showed that the miR-153-3p mimics significantly downregulated rs6932603-T expression in both cell lines (Fig. 2F). As an additional test of our model, we co-transfected the rs932603-C/T allele psiCHECK-2 vector and miR-153-3p inhibitors or NC inhibitors were co-transfected into 293T cells and U2OS cells. Expression of rs6932603-T was significantly upregulated in both cell lines (Fig. 2K). Using the same prediction method, we found that miR-374b-3p, miR-4274, miR-572 and miR-2964a-5p mimics significantly downregulated rs6932603-T expression in the cell variants of rs3757322 G/T and rs3734806 A/T in CCDC170 (Fig. 2B-E). Then, we confirmed that miR-374b-3p mimics and miR-4274 mimics significantly downregulated rs3757322-T expression (Fig. 2G,H), miR-572 mimics and miR-2964a-5p mimics significantly downregulated rs3734806-G (Fig. 2J), miR-374b-3p inhibitors and miR-4274 inhibitors significantly upregulated rs3757322-T expression (Fig. 2L,M), and miR-572 inhibitors and miR-2964a-5p inhibitors significantly upregulated rs3734806-G (Fig. 2N,O). Western blot analysis showed that overexpression of these five miRNAs can reduce the expression of CCDC170 at the protein level, while inhibition of these five miRNAs can increase the expression of CCDC170 (Fig. 2P). These results indicated that miR-153-3p, miR-374b-3p, miR-4274, miR-572 and miR-2964a-5p inhibited CCDC170 expression by differentially binding three GWAS lead SNPs.

miRNA overexpression repressed the expression of osteogenesis marker genes and increased the expression of osteoclastogenesis marker genes

To explore the functional role of these five miRNAs in osteosarcoma and osteoporosis, miR-153-3p, miR-374b-3p, miR-4274, miR-572 and miR-2964a-5p mimics were transfected into U2OS cells carrying different alleles of GWAS lead SNPs with NC mimics (Table 1). qRT-PCR results showed that miR-153-3p, miR-374b-3p, miR-4274 and miR-2964a-5p overexpression significantly downregulated the expression of early-stage osteogenesis marker genes Runx2 and Osterix, medium-stage osteogenesis marker genes ALP and Col1a1, and late-stage osteogenesis marker genes OPG and OCN (Fig. 3A–C,E), and bone formation-related genes WNT4 and WNT16 (Fig. 3F–H,J). We also found that when overexpressing miR-153-3p, miR-374b-3p, miR-4274 and miR-2964a-5p osteoclastogenesis inhibitor factor OPG was significantly downregulated and that of osteoclastogenesis marker genes TRACP and CTSK was significantly upregulated (Fig. 3K–M,O). However, miR-572 overexpression significantly upregulated the expression of Runx2, Osterix, ALP, Col1a1, OPG, TRACP and CTSK and significantly downregulated the
Fig. 2. MiRNAs differentially regulated the allele variants of GWAS lead SNPs. (A–E) Schematic diagram of the miR-153-3p, miR-374b-3p, miR-4274, miR-572 and miR-2964a-5p binding sites on CCDC170. (F) The overexpression of miR-153-3p significantly suppressed rs6932603-T allele levels in U2OS cells and 293T cells. (G,H) The overexpression of miR-374b-3p and miR-4274 significantly suppressed rs3757322-T allele levels in U2OS cells and 293T cells. (I,J) The overexpression of miR-572 and miR-2964a-5p significantly suppressed rs3748806-G allele levels in U2OS cells and 293T cells. (K) Knockdown of miR-153-3p significantly upregulated rs6932603-T allele levels in U2OS cells and 293T cells. (L,M) Knockdown of miR-374b-3p and miR-4274 significantly upregulated rs3757322-T allele levels in U2OS cells and 293T cells. (N,O) Knockdown of miR-572 and miR-2964a-5p significantly upregulated rs3748806-G allele levels in U2OS cells and 293T cells. (P) Overexpression of these five miRNAs can reduce the expression of CCDC170 at the protein level, while inhibition of these five miRNAs can increase the expression of CCDC170. The data are from three independent transfection experiments, with assays performed in triplicate (n=6). Firefly luciferase activity was normalized to Renilla luciferase activity. Error bars show the standard deviation for six technical replicates of a representative experiment. P-values were calculated using a two-tailed Student’s t-test. **P<0.01, ***P<0.001.
Fig. 3. MiRNA overexpression repressed the expression of osteogenesis marker genes and increased the expression of osteoclastogenesis marker genes. (A–C) The overexpression of miR-153-3p, miR-374b-3p and miR-4274 significantly suppressed the expression of Runx2, Osterix, ALP, Col1a1, OPN and OCN. (D) The overexpression of miR-572 significantly increased the expression of Runx2, ALP, Col1a1, OPN and OCN. (E) The overexpression of miR-2964a-5p significantly suppressed the expression of Osterix, ALP, Col1a1, OPN and OCN. (F,G,I) The overexpression of miR-153-3p, miR-374b-3p and miR-572 significantly suppressed the expression of WNT4 and WNT16. (H) The overexpression of miR-4274 significantly suppressed the expression of WNT4, and the expression level of WNT16 was decreased, but the difference was not significant. (J) The overexpression of miR-2964a-3p significantly suppressed the expression of WNT16, but the expression level of WNT4 did not change. (K) The overexpression of miR-153-3p significantly suppressed the expression of OPG and significantly increased the expression of TRACP. (L–N) The overexpression of miR-374b-3p, miR-4274 and miR-572 significantly suppressed the expression of OPG and significantly increased the expression of TRACP and CTSK. (O) The overexpression of miR-2964a-5p significantly suppressed the expression of OPG and significantly increased the expression of CTSK. The expression level of TRACP was increased, but the difference was not significant. The data are from three independent transfection experiments, with assays performed in triplicate ($n=8$). The error bars show the standard deviation for four technical replicates of a representative experiment. $P$-values were calculated using a two-tailed Student’s t-test. NS>0.05, *$P<0.05$, **$P<0.01$, ***$P<0.001$. 

Biology Open (2021) 10, bio050930. doi:10.1242/bio.050930
expression of WNT4 and WNT16 (Fig. 3D,I,N). ELISA results showed that miR-153-3p, miR-374b-3p, miR-4274 and miR-572 overexpression significantly downregulated the protein content of Runx2 and significantly upregulated the protein content of TRACP (Table 2). These results indicated that miR-153-3p, miR-374b-3p, miR-4274 and miR-2964a-5p overexpression inhibited osteogenesis and promoted osteoclastogenesis.

miRNA knockdown increased the expression of osteogenesis marker genes and repressed the expression of osteoclastogenesis marker genes

To further verify the role of these five miRNAs in osteogenesis and osteoclastogenesis, miR-153-3p, miR-374b-3p, miR-4274, miR-572 and miR-2964a-5p inhibitors and non-complementary (NC) inhibitors were transfected into U2OS cells. By qRT-PCR, we found that knockdown of miR-153-3p, miR-374b-3p, miR-4274 and miR-2964a-5p, the expression of Runx2, Osterix, ALP, Col1α1, OPG and OCN was significantly upregulated (Fig. 4A–C,E), that of WNT4 and WNT16 was significantly upregulated (Fig. 4F–H,J), OPG expression was significantly upregulated and TRACP and CTSK expression was significantly downregulated (Fig. 4K–M,O). When miR-572 was knocked down, the expression of Runx2, Osterix, ALP, Col1α1, OPG and OCN was significantly downregulated (Fig. 4I), and the expression of OPG, TRACP and CTSK was significantly upregulated (Fig. 4N). ELISA results showed that miR-153-3p, miR-374b-3p, miR-4274, miR-572 and miR-2964a-5p knockdown significantly upregulated the protein content of Runx2 and significantly downregulated the protein content of TRACP and CTSK (Table 2). These results indicated that knockdown of miR-153-3p, miR-374b-3p, miR-4274 and miR-2964a-5p promoted osteogenesis and inhibited osteoclastogenesis.

CCDC170 overexpression increased the expression of osteogenesis marker genes and repressed the expression of osteoclastogenesis marker genes in U2OS and K7M2wt cells

To verify whether the changes in the expression levels of osteogenic and osteoclast markers upon overexpression or inhibition of these five miRNAs are directly caused by changes in CCDC170, we overexpressed CCDC170 in human U2OS cells and mouse K7M2wt cells. By qRT-PCR, we found that the expression of Runx2, Osterix, ALP, Col1α1, OPG and OCN (BGL) was significantly upregulated (Fig. 5A,B), the expression of WNT4 and WNT16 was significantly upregulated (Fig. 5C,D), the expression of OPG was significantly upregulated and the expression of TRACP and CTSK was significantly downregulated (Fig. 5E,F). ELISA results showed that the protein content of Runx2 was significantly upregulated and the protein content of TRACP was significantly downregulated (Table 2). These results indicated that overexpression of CCDC170 promoted osteogenesis and inhibited osteoclastogenesis.

CCDC170 knockdown repressed the expression of osteogenesis marker genes and increased the expression of osteoclastogenesis marker genes in U2OS and K7M2wt cells

To further verify the role of CCDC170 in promoting osteogenesis and inhibiting osteoclastogenesis, CCDC170 siRNA and siRNA NC were transfected into U2OS cells and K7M2wt cells. By qRT-PCR, we found that human CCDC170 siRNA1# and siRNA2# and mouse CCDC170 siRNA1# and siRNA2# could specifically knock down the expression of CCDC170 (Fig. 6A,B). In follow-up experiments using human CCDC170 siRNA1# and mouse CCDC170 siRNA1#, we found that the expression of Runx2, Osterix, ALP, Col1α1, OPG and OCN was significantly downregulated (Fig. 6C,D), the expression of WNT4 and WNT16 was significantly downregulated (Fig. 6E,F), the expression of OPG was significantly downregulated and the expression of TRACP and CTSK was significantly upregulated (Fig. 6G,H). ELISA results showed that the protein content of Runx2 was significantly downregulated and the protein content of TRACP was significantly upregulated (Table 2). These results indicated that knockdown of CCDC170 expression inhibited osteogenesis and promoted osteoclastogenesis.

CCDC170 knockdown repressed the expression of osteogenesis marker genes and increased the expression of osteoclastogenesis marker genes in mice vivo

To further verify the role of CCDC170 in promoting osteogenesis and inhibiting osteoclastogenesis in mice vivo, CCDC170 2′-Ome siRNA and siRNA NC were tail vein injected into mice. By qRT-PCR, we found that the expression of CCDC170, Runx2, Osterix, ALP, Col1α1, OPG and OCN was significantly downregulated (Fig. 7A), the expression of WNT4 and WNT16 was significantly downregulated (Fig. 7B), the expression of OPG was significantly downregulated and the expression of TRACP and CTSK was significantly upregulated (Fig. 7C). Serum ELISA results showed that the protein content of Runx2, OPG, WNT4 and WNT16 was significantly downregulated and the protein content of TRACP and CTSK was significantly upregulated (Table 2). These results indicated that knockdown of CCDC170 expression inhibited osteogenesis and promoted osteoclastogenesis in mice vivo.

| Group                          | Runx2 (pg/ml) | TRACP (pg/ml) |
|-------------------------------|---------------|---------------|
| mimics NC                     | 252.62±16.35  | 206.34±10.28  |
| miR-153-3p                    | 187.96±12.78**| 283.02±10.01***|
| miR-374b-3p                   | 164.56±14.99***| 261.73±11.96***|
| miR-4274                      | 208.42±30.27* | 276.42±13.62***|
| miR-572                       | 166.05±20.19***| 259.84±13.89***|
| miR-2964                      | 185.74±24.11* | 214.99±20.46**|
| vector control (U2OS)         | 241.02±14.11  | 192.17±10.44  |
| CCDC170 (U2OS)                | 484.53±27.93***| 66.02±8.43***|
| vector control (K7M2 wt)      | 243.25±13.09  | 191.07±10.69  |
| CCDC170 (K7M2 wt)             | 406.95±15.40***| 135.65±10.86***|

Table 2. The levels of Runx2, TRACP (n=8) (miRNAs overexpression and knockdown in U2OS, CCDC170 overexpression and knockdown in U2OS and K7M2 wild-type cells)
**CCDC170 knockdown repressed bone formation in mice**

To directly evaluate the effect of *CCDC170* in promoting osteogenesis and inhibiting osteoclastogenesis in mice vivo, *CCDC170* 2′-Ome siRNA and siRNA NC were tail vein injected into mice. Ten mice were selected from each group for tibia and femur TRAP staining and osteoclasts count, results showed that...
CCDC170 knockdown significantly increased the number of osteoclasts (Fig. 8A,B). Four mice were randomly selected from each group for tibia and femur μCT, all images presented are representative of the respective groups (Fig. 8C,D). The tibia and femur μCT results showed that CCDC170 knockdown decreased the BV/TV, Tb.N, Tb.Th (Fig. 8E–G) and BMD (Fig. 8I), and increased the Tb.Sp (Fig. 8H).

**DISCUSSION**

Identifying target genes is pivotal to understanding the roles of miRNAs in various diseases including osteoporosis (Saetrom et al., 2009; Liu et al., 2015; Juzénas et al., 2015). The TargetScan, miRNAsNP, and miRbase databases were used to identify direct targets of miRNAs. CCDC170 was predicted to be a target of miR-153-3p, miR-374b-3p, miR-4274, miR-572 and miR-2964a-5p. In our study, we found that these five miRNAs could differentially regulate the allele variants of three GWAS lead SNPs in the CCDC170 3′-UTR as demonstrated by the luciferase reporter assay. Our study provides a possible mechanism for this finding, in that upregulation of miR-153-3p, miR-374b-3p, miR-4274, miR-572 and miR-2964a-5p resulted in decreased CCDC170 expression in osteoporosis.

Multiple studies have found that CCDC170 plays an important role in breast cancer, and multiple genome-wide linkage analyses have shown that CCDC170 is one of the genes most strongly linked to BMD (Jiang et al., 2007; Fimereli et al., 2018). However, no in vivo or in vitro experiments have directly verified the role of CCDC170. Our experimental results directly demonstrate that CCDC170 plays a positive role in bone formation in vitro and in vivo. CCDC170 knockdown significantly increased the number of osteoclasts, BMD and other osteogenesis indicators were decreased significantly.

MicroRNAs mediate posttranscriptional regulation of various genes, and some have been shown to influence osteoporosis (Jones et al., 2012; Lian et al., 2015). MiR-153-3p has been shown to play an important role in many diseases, such as lung cancer, breast cancer, and osteosarcoma cell proliferation (Chen et al., 2015; Niu et al., 2015; Zuo et al., 2019). MiR-374b has been shown to play an important role in the proliferation and differentiation of various cell lines (Wu et al., 2018; Lee et al., 2017; Feng et al., 2011). An association analysis of SNPs located in pri-miRNA sequences with BMD found that miR-4274 overexpression could contribute to the osteoporotic phenotype (De et al., 2017). MiR-572 has been shown to play an important role in renal cell carcinoma, nasopharyngeal carcinoma, and human ovarian carcinoma (Pan et al., 2018; Yan et al., 2017; Zhang et al., 2015). To date, no reports have directly demonstrated the role of miR-153-3p, miR-374b-3p, miR-2964a-5p and miR-2964a-5p in the pathogenesis of osteoporosis, while miR-2964a-5p is a new miRNA with no functional reports. Here, we showed that miR-153-3p, miR-374b-3p, miR-4274 and miR-2964a-5p suppressed osteogenesis and promoted osteoclastogenesis by regulating the expression of CCDC170. It has been reported that miRNAs have dual functions, and gene expression can be inhibited when the miRNA is located in the cytosol, while gene transcription can be activated when the miRNA is located in the nucleus (Suzuki et al., 2017; Xiao et al., 2017). In our experimental results, miR-572 promoted osteogenesis and suppressed osteoclastogenesis, and we suspect that it activated the expression of another positively regulated bone-forming gene.

**Conclusion**

In conclusion, our study demonstrated that CCDC170 plays an important role in the positive regulation of bone formation and annotated new functions for this gene in vitro and in vivo. MiR-153-3p, miR-374b-3p, miR-4274, miR-572 and miR-2964a-5p inhibit CCDC170 expression in an allele-specific manner by binding
GWAS lead SNPs rs6932603, rs3757322 and rs3734806. These findings may improve our understanding of the association between CCDC170, miRNAs, GWAS lead SNPs, and osteoporosis pathogenesis and may provide a potential therapeutic target for osteoporosis therapy.

**MATERIALS AND METHODS**

**Cell cultures**
The human embryonic kidney cell line HEK293T was obtained from the Cell Bank of Wuhan University (Wuhan, China). The human osteosarcoma cell line U2OS and the mouse osteosarcoma cell line K7M2wt were purchased from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China). HEK293T cells and K7M2wt cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM) (HyClone, Waltham, MA, USA) and U2OS cells were grown in McCoy’s 5A medium (HyClone). Media were supplemented with 10% fetal bovine serum (Invitrogen, Carlsbad, CA, USA), 100 U/ml penicillin, and 100 μg/ml streptomycin (Invitrogen). All cells were maintained at 37°C in a humidified incubator at 5% CO2.

**Oligonucleotide synthesis and plasmid constructs**
MiR-153-3p mimics, miR-153-3p inhibitors, miR-374b-3p mimics, miR-374b-3p inhibitors, miR-572 mimics, miR-572 inhibitors, miR-2964a-5p mimics, miR-2964a-5p inhibitors, miRNA NC, inhibitor NC, human CCDC170 siRNA, mouse CCDC170 siRNA, siRNA NC and SNP genotyping oligonucleotide primers were chemically synthesized (GenePharma, Shanghai, China). The human CCDC170 and mouse CCDC170 coding sequences were amplified by whole gene synthesis, and the products were inserted into the pIRES2-EGFP vector (Tsingke, Beijing, China). The inserted sequences were verified by sequencing. Complementary oligonucleotides containing rs6932603-C, rs6932603-T, rs3757322-G, rs3757322-T, rs3734806-G and rs3734806-G were annealed and subsequently cloned into the luciferase reporter vector pSicHECK-2 (Promega, Madison, WI, USA). Vector constructed primers and oligonucleotide sequences are presented in Table S1.

**Genotyping of GWAS SNPs in two cell lines**
The PCR-seq method was used to obtain the genotypes of rs6932603, rs3757322 and rs3734806 in U2OS cells and HEK293T cells. A 600-bp

---

**Fig. 6.** CCDC170 knockdown repressed the expression of osteogenesis marker genes and increased the expression of osteoclastogenesis marker genes. (A) Human CCDC170 siRNA1#, siRNA2# can specifically knockdown the expression of CCDC170. (B) Mouse CCDC170 siRNA1# and siRNA2# can specifically knockdown the expression of CCDC170. (C) Knockdown of CCDC170 significantly suppressed the expression of Runx2, Osterix, ALP, Cal1a1, OPN and OCN (BGL) in U2OS cells and K7M2wt cells. (E,F) Knockdown of CCDC170 significantly suppressed the expression of WNT4 and WNT16 in U2OS cells and K7M2wt cells. (G,H) Knockdown of CCDC170 significantly suppressed the expression of OPG and significantly increased the expression of TRACP and CTAK. The error bars show the standard deviation for four technical replicates of a representative experiment. P-values were calculated using a two-tailed Student’s t-test. *P<0.05, **P<0.01, ***P<0.001.
sequence centered on rs6932603 (C-T) and a 1000-bp sequence centered on rs3757322 (G/T) and rs3734806 (A/G) were PCR amplified from genomic DNA from the two cell lines using the primers. SNPs rs6932603, rs3757322 and rs3734806 were subsequently genotyped by sequencing the amplified products.

**Cell transfection and luciferase reporter assay**

The rs6932603-G; rs6932603-T; rs3757322-G; rs3757322-T; rs3734806-A, and rs3734806-G alleles were amplified and cloned into the psiCHECK-2 vector by inserting them between the Sgf/I/Nol sites. Cells were singly transfected with the SNP allele psiCHECK-2 vector constructs or co-transfected with the SNP allele psiCHECK-2 constructs and miRNA mimics, NC mimics, miRNA inhibitors, or NC inhibitors at 20 nM using Lipofectamine 3000 transfection reagent (Invitrogen) with serum-free media for 48 h per the manufacturer’s instructions (the amount of plasmids is 2 μg/well). The luciferase reporter assay was performed using the Dual-Luciferase Reporter Assay (Promega), and the relative luciferase activity was calculated after transfection for 48 h by normalizing the firefly luminescence to that of Renilla. Experiments were performed in triplicate, and all experiments were repeated three times.

**RNA extraction and qPCR**

Total RNA from spinal cord, U2OS cells and K7M2wt cells was isolated by TRIzol reagent (Invitrogen, Carlsbad, CA, USA). One microliter of oligo(dT)18 primer (500 ng) and 1.0 μl of total RNA (1.0 μg) were added to 10.0 μl of nuclease-free water and heated on a gradient PCR instrument for 5 min at 65°C according to the manufacturer’s recommendations (RevertAid First-Strand cDNA Synthesis Kit; Thermo Fisher Scientific, Inc., Waltham, MA, USA). 2 μl of cDNA template was mixed with 10 μl of SYBR Green PCR Master Mix (Thermo Fisher Scientific) and 1 μl upstream and downstream primers. The system was reacted at 95°C for 60 s, then at the conditions of 95°C for 30 s; Annealing Temperature for 30 s; 72°C for 30 s for 40 cycles. Finally, the DNA was detected at 95°C for 30 s and 5°C for 35 s. The 2−ΔΔCT method was used to determine the level of relative gene expression. All primer sequences are presented in Table S2.

**ELISA**

The protein content of Runx2, ALP, OPG, RANK and TRACP were determined by using enzyme-linked immunosorbsorbent assay kits (Abcam, Cambridge, MA, USA).

**Western blot analysis**

After treating the cells with miRNA mimics and inhibitors for 48 h, the cells were collected. 1×(10⁶) cells were cracked by 1 ml of RIPA and 10 μl of PMSF, then centrifuged at 12,000 r/min at 4°C for 4 min. The intermediate protein layer solution was removed, and the BCA protein quantification kit was used for protein quantification. Samples of each group were diluted to 50 μg/ml, and the diluted protein was mixed with Sample Buffer at the ratio of 4:1 and heated at 100°C for 5 min. Then Mixing Acrylamide, Resolving Buffer, Stacking Buffer, distilled water, 10% APS, and TEMED were mixed in proportion to make SDS-PAGE separation gel and stacking gel, and poured into the gel plate. The Prestained Protein Ladder and the sample were separately added into the sample wells of the gel plate, and the protein-loaded SDS-PAGE gel was subjected to vertical gel electrophoresis for 50 min. The polyvinylidene difluoride (PVDF) membrane was activated by methanol for 1 min and then transmembrane was performed. After that, the PVDF membrane was blocked by 5% fat-free milk containing TBST solution for 1 h. After blocking, the PVDF membrane was washed by TBST. The CCDC170 antibody (Thermo Fisher Scientific, rabbit polyclonal antibody-Catalog Number PA5-55537) was incubated at 25°C for 2 h at a dilution of 1:100, and the second antibody was incubated at 25°C for 1 h. Finally, Supersignal West Pico PLUS was used to fill the PVDF membrane and was placed in the iBright FL1000 (Thermo Fisher Scientific) for observation.

**Animals**

Twenty mice from Kunming (six weeks old, female) were purchased from the Experimental Animal Center of Chongqing Medical University (Chongqing, China). The mice were housed in a controlled facility at a constant temperature (25±2°C) and relative humidity (50±3%) with a 12/12 h light/dark cycle and free access to a standard mice chow diet and water. After a 1-week environmental adaptation test, the mice were divided into two groups (negative control group and CCDC170 siRNA group), ten in each group. Negative control group mice were tail vein injected with 100 μl PBS per week for 4 weeks, CCDC170 siRNA group mice were tail vein injected the mixture of 100 μl PBS and 50 μl CCDC170 2′-OMe siRNA per week for 4 weeks. Three days after the last injection, the mice were fasted for 18 h and then euthanized. Serum was collected from the mice and stored at −80°C for follow-up ELISA experiment. Spinal cord was collected and stored at −80°C for follow-up qPCR experiment. Tibia and femur were collected and fixed in 10% (v/v) buffered formaldehyde, paraffin embedded, and sectioned, before tartrate resistant acid phosphatase (TRAP) staining and observation with a BX43 microscope (Olympus, Tokyo, Japan), count the number of osteoclasts with three or more nuclei.

**µCT bone imaging**

For micro-computed tomography (µCT) analysis, Bruker MicroCT Skyscan 1272 system (Kontich, Belgium) with an isotropic voxel size of 10.0 μm was used to image the whole tibia and femur. Scans were conducted in 4%
Fig. 8. CCDC170 knockdown repressed bone formation in mice vivo. (A,B) The number of osteoclasts were increased. (C,D) Representative micro-computed tomography isosurface images. (E) The percent bone volume fraction (BV/TV) was decreased. (F) The trabecular number (Tb.N) was decreased. (G) The trabecular thickness (Tb.Th) was decreased. (H) The trabecular separation (Tb.Sp) was increased. (I) The BMD was decreased. The error bars show the standard of A-B deviation for ten biological replicates of a group and E-I deviation for four biological replicates of a group. P-values were calculated using a two-tailed Student's t-test. *P<0.05, **P<0.01, ***P<0.001.
paraformaldehyde and used an X-ray tube potential of 60 kV, an X-ray intensity of 166 μA, and an exposure time of 1700 ms. For trabecular bone analysis of the distalibia and femur, an upper 3 mm region beginning 0.8 mm proximal to the most proximal central epithysis of the femur was contoured.

Data analysis and statistics

The data are presented as the mean±s.d. Statistical differences between two groups were determined by two-tailed Student’s t-test. Statistical differences among groups were analyzed by one-way ANOVA followed by the Student–Newman–Keuls’ multiple comparisons test. All experiments were performed independently at least three times with similar results, and representative experiments are shown. *P<0.05 was considered statistically significant. **P<0.01, ***P<0.001.

Acknowledgements

We thank reviewers and the editors for their constructive comments and suggestions to improve the manuscript.

Competing interests

The authors declare no competing or financial interests.

Author contributions

Data curation: J.L., R.Y.; Writing - original draft: X.L., Y.-G.L.; Writing - review & editing: F.T., X.Z.

Funding

This work was supported by Chongqing University Innovation Research Group Project (CXTBP20033) and the Natural Science Foundation of Chongqing.

Supplementary information

Supplementary information available online at https://bioc.biolistics.org/lookup doi:10.1242/bio.050930.supplemental

References

Albagha, O. M. and Ralston, S. H. (2003). Genetic determinants of susceptibility to osteoporosis. Endocrinol. Metab. Clin. 32, 65-81. doi:10.1001/s0038-8552-0200095-2

Ambros, V. (2004). The functions of animal microRNAs. Nature 431, 350-355. doi:10.1038/nature02871

Amores, S. L. and Zamore, P. D. (2013). Diversifying microRNA sequence and function. Nat. Rev. Mol. Cell. Biol. 14, 475-488. doi:10.1038/nrm3611

Cauley, J. A. (2013). Public health impact of osteoporosis. J. Gerontol. A. Biol. Sci. Med. Sci. 68, 1243-1251. doi:10.1093/gerona/glt093

Chen, W. J., Zhang, E. N., Zhong, Z. K., Jiang, M. Z., Yang, X. F., Zhou, D. M. and De, L. (2012). MicroRNA signatures associate with pathogenesis and progression of osteosarcoma. Cancer Res. 72, 1865-1877. doi:10.1158/0008-5472.CAN-11-2663

Juzenas, S., Saltenien, V., Kupcinskas, J., Link, A., Kiudelis, G., Jonaitis, L., Jarmalaitė, S., Kupcinskas, L., Malfertheiner, P. and Skiecevičienė, J. (2015). Analysis of deregulated microRNAs and their target genes in Gastric Cancer. PLoS ONE 10, e0132377. doi:10.1371/journal.pone.0132377

Kim, K. M. and Lim, S. K. (2014). Role of miRNAs in bone and their potential as therapeutic targets. Curr. Opin. Pharmacol. 16, 133-141. doi:10.1016/j.coph.2014.05.001

Lee, J. S., Song, D. W., Park, J. H., Kim, J. O., Cho, C. and Kim, D. H. (2017). miR-374 promotes myocardial hypertrophy by negatively regulating vascular endothelial growth factor receptor-1 signaling. BMB Rep. 50, 208-213. doi:10.5430/mmbr.2017.50.4.165

Lian, F., Cui, Y., Zhou, C., Gao, K. and Wu, L. (2015). Identification of a plasma founder microRNA panel as potential noninvasive biomarker for osteosarcoma. PLoS ONE 10, e0121499. doi:10.1371/journal.pone.0121499

Liu, B., Li, J. and Cairns, M. J. (2015). Identifying miRNAs, targets and functions. Brief. Bioinform. 15, 1-19. doi:10.1093/bib/bbs075

Lou, S., Sun, T., Li, H. and Hu, Z. (2018). Mechanisms of micro-RNA-mediated gene regulation in unicellular model alga Chlamydomonas reinhardtii. Biotechnol. Biofuels. 11, 244. doi:10.1186/s13068-018-1249-y

Mei, B., Wang, Y., Ye, Y. W., Huang, H., Zhou, Q., Chen, Y., Niu, Z., Yang, Z. and Huang, Q. Y. (2019). LncRNA ZBTB40-T1 mediated by osteoporosis GWAS risk SNPs suppresses osteogenesis. Hum. Genet. 138, 151-166. doi:10.1007/s00439-019-01969-y

Mo, X. B., Lu, Z., Yang, H. Z., Zhang, Z. L., Deng, F. Y. and Lei, S. F. (2015). Genotype-association analysis identified novel genes associated with bone mineral density. PLoS ONE 10, e0121811. doi:10.1371/journal.pone.0121811

Mehr, A. M. and Mott, J. L. (2015). Microarray analysis of murine microRNA biology. Semin. Liver. Biol. 25, 303-011. doi:10.1053/j.semliverb.2014.10.001

Mullin, B. H., Walsh, J. P., Zheng, H. F., Brown, S. J., Surdulicas, G. L., Curtis, C., Breen, G., Budidridge, F., Richards, J. B., Spector, T. D. et al. (2016). Genome-wide association study using family-based cohorts identifies the WLS and BCOSCC170/ESR1 loci as associated with bone mineral density. BMC Genomics 17, 136. doi:10.1186/s12864-016-2481-o

Mullin, B. H., Zhao, J. H., Brown, S. J., Perry, J. R. B., Luan, J., Zheng, H. F., Langenberg, C., Budidridge, F., Scott, R., Wareham, N. J. et al. (2017). Genome-wide association study meta-analysis for quantitative ultrasound parameters of bone identification of five novel loci for bone density and attenuation. Hum. Mol. Genet. 26, 2791-2802. doi:10.1093/hmg/ddx174

Niu, G., Li, B. and Sun, L. (2015). MicroRNA-153 inhibits osteosarcoma cells proliferation and invasion by targeting TGF-β. PLoS ONE 10, e0119225. doi:10.1371/journal.pone.0119225

Pan, X., Li, Z., Zhao, L., Quan, J., Zhou, L., Xu, J., Xu, W., Guan, X., Li, H., Yang, S. et al. (2018). microRNA-572 functions as an oncogene and a potential biomarker for renal cell carcinoma prognosis. Oncol. Rep. 40, 3092-3101. doi:10.26891/tor.2018.6649

Paul, P., Chakraborti, A., Sarkar, D., Langthasa, M., Rahman, M., Bari, M., Singh, R. S., Malaker, A. K. and Chakraborti, S. (2018). Interplay between miRNAs and human diseases. J. Cell. Physiol. 233, 2007-2018. doi:10.1002/jcp.25854

Poddar, S., Kesharwani, D. and Datta, M. (2017). Interplay between the miRNome and the epigenetic machinery: Implications in health and disease. J. Cell. Physiol. 232, 2938-2945. doi:10.1002/jcp.25819

Qin, L., Liu, Y., Wang, Y., Wu, G., Chen, J., Ye, W., Yang, J. and Huang, Q. Y. (2016). Computational characterization of osteoporosis associated SNPs and genes identified by genome-wide association studies. PLoS ONE 11, e0150070. doi:10.1371/journal.pone.0150070

Ralston, S. H. and Uitterlinden, A. G. (2010). Genetics of osteoporosis. Endoc. Rev. 31, 629-662. doi:10.1210/er.2009-0044

Sakib, O. L. and Farber, C. R. (2017). Using GWAS to identify novel therapeutic targets for osteoporosis. Transl. Res. 181, 15-26. doi:10.1016/j.trsl.2016.10.009

Saetrom, P., Biesinger, J., Li, S. M., Smith, D., Thomas, L. F., Majzoub, K., Rivas, G. E., Aluini, J., Rossi, J. J., Krontris, T. G. et al. (2009). A risk variant in an miR-125b binding site in BMPR1B is associated with breast cancer
pathogenesis. Cancer Res. 69, 7459-7465. doi:10.1158/0008-5472.CAN-09-1201
Sapkota, Y., Steinthorsdottir, V., Morris, A. P., Fassbender, A., Rahmioglu, N., De, I., Buring, J. E., Zhang, F., Edward, T. L., Jones, S. et al. (2017). Meta-analysis identifies five novel loci associated with endometriosis highlighting key genes involved in hormone metabolism. Nat. Commun. 8, 15539. doi:10.1038/ncomms15539
Stacey, S. N., Sulem, P., Zanon, C., Gudjonsson, S. A., Thorleifsson, G., Helgason, A., Jonasdottir, A., Besenbacher, S., Kostic, J. P., Fackenthal, J. D. et al. (2010). Ancestry-shift refinement mapping of the C6orf97-ESR1 breast cancer susceptibility locus. PLoS Genet. 6, e1001029. doi:10.1371/journal.pgen.1001029
Suzuki, H. I., Young, R. A. and Sharp, P. A. (2017). Super-enhancer–mediated RNA processing revealed by integrative MicroRNA network analysis. Cell 68, 1000-1014.e15. doi:10.1016/j.cell.2017.02.015
Tang, P., Xiong, Q., Ge, W. and Zhang, L. (2014). The role of microRNAs in osteoclasts and osteoporosis. RNA Biol. 11, 1355-1363. doi:10.1080/15476286.2014.996462
Tu, M., Tang, J., He, H., Cheng, P. and Chen, C. (2017). MiR-142-5p promotes bone repair by maintaining osteoblast activity. J. Bone. Miner. Metab. 35, 255-264. doi:10.1007/s00774-016-0757-8
Veeraraghavan, J., Tan, Y., Cao, X. X., Kim, J., Wang, X., Charnness, G. C., Maiti, S. N., Cooper, L. J., Edwards, D. P., Contreras, A. et al. (2014). Recurrent ESR1-CCDC170 rearrangements in an aggressive subset of oestrogen receptor-positive breast cancers. Nat. Commun. 5, 4577. doi:10.1038/ncomms5577
Villalobos, M., Jiménez, R. F., Estrada, K., Parra, A. Y., González, A., Patiño, N., Castillejos, M., Quiterio, M., Fernandez, J. C., Ibarra, B. et al. (2017). A pilot genome-wide association study in postmenopausal Mexican-Mestizo women implicates the RMND1/CCDC170 locus associated with bone mineral density. Int. J. Genomics. 2017, 1-13. doi:10.1155/2017/5831020
Wu, X., Zhao, X. and Miao, X. (2018). MicroRNA-374b promotes the proliferation and differentiation of neural stem cells through targeting Hes1. Biochem. Biophys. Res. Commun. 503, 593-599. doi:10.1016/j.bbrc.2018.06.044
Xiao, M., Li, J., Li, W., Wang, Y., Wu, F., Xi, Y., Zhang, L., Ding, C., Luo, H., Li, Y. et al. (2017). MicroRNAs activate gene transcription epigenetically as an enhancer trigger. RNA Biol. 14, 1326-1334. doi:10.1080/15476286.2015.1112487
Xie, Y., Chen, Y., Zhang, L., Ge, W. and Tang, P. (2017). The roles of bone-derived exosomes and exosomal microRNAs in regulating bone remodelling. J. Cell. Mol. Med. 21, 1033-1041. doi:10.1111/jcmm.13039
Yamamoto, M., Yamamoto, Y., Fujiiwa, S., Sueta, A., Yamamoto, S., Hayashi, M., Tomiguchi, M., Takeshita, T. and lwase, H. (2015). C6ORF97-ESR1 breast cancer susceptibility locus: influence on progression and survival in breast cancer patients. Eur. J. Hum. Genet. 23, 949-956. doi:10.1038/ejhg.2014.219
Yan, L., Cai, K., Liang, J., Liu, H., Liu, Y. and Gui, J. (2017). Interaction between miR-572 and PPP2R2C, and their effects on the proliferation, migration, and invasion of nasopharyngeal carcinoma (NPC) cells. Biochem. Cell Biol. 95, 578-584. doi:10.1139/bcb-2016-0237
Zhang, X., Liu, J., Zang, D., Wu, S., Liu, A., Zhu, J., Wu, G., Li, J. and Jiang, L. (2015). Upregulation of miR-572 transcriptionally suppresses SOCS1 and p21 and contributes to human ovarian cancer progression. Oncotarget 6, 15180-15193. doi:10.18632/oncotarget.3737
Zhao, X., Xu, D., Li, Y., Zhang, J., Liu, T., Ji, Y., Wang, J., Zhou, G. and Xie, X. (2014). MicroRNAs regulate bone metabolism. J. Bone. Miner. Metab. 32, 221-231. doi:10.1007/s00774-013-0537-7
Zuo, Z., Ye, F., Liu, Z., Huang, J. and Gong, Y. (2019). MicroRNA-153 inhibits cell proliferation, migration, invasion and epithelial-mesenchymal transition in breast cancer via direct targeting of RUNX2. Exp. Ther. Med. 17, 4693-4702. doi:10.3892/etm.2019.7470