LncRNA PRNCR1 regulates osteogenic differentiation in osteolysis after hip replacement by targeting miR-211-5p

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Running title: Role of PRNCR1 in osteolysis
Abstract

Background Osteogenic differentiation and osteolysis after hip replacement are both associated with bone metabolism. Interaction between the long non-coding RNA (lncRNA) prostate cancer non-coding RNA 1 (PRNCR1) and miR-211-5p was analyzed to illuminate their roles in osteogenic differentiation and osteolysis.

Methods The expression of PRNCR1, miR-211-5p and C-X-C chemokine receptor-4 (CXCR4) protein in tissues and mesenchymal stem cells (MSCs) were determined by qRT-PCR and western blot, separately. The osteogenic differentiation was assessed with Alkaline phosphatase (ALP) activity detection and ARS staining. The endogenous expressions of genes were modulated by recombinant plasmid and cell transfection. Combination condition and interaction between RNA and protein were determined with RIP and RNA pull-down assay, respectively. Interaction between miR-211-5p and CXCR4 was examined with Dual luciferase reporter assay.

Results PRNCR1 and CXCR4 were up-regulated in wear particles around prosthesis and in MSCs incubated with Polymethylmethacrylate (PMMA), while miR-211-5p was down-regulated. Repression of PRNCR1 weakened the inhibitory effect of wear particles on osteogenic differentiation. PRNCR1 positively regulated CXCR4 through inhibiting miR-211-5p. Wear particles regulated CXCR4 level through miR-211-5p to affect osteogenic differentiation of MSCs. Wear particles regulated the miR-211-5p level through PRNCR1 to affect osteogenic differentiation of MSCs.

Conclusion LncRNA PRNCR1 up-regulates CXCR4 through inhibiting miR-211-5p, which inhibits osteogenic differentiation and thereby leading to osteolysis after hip replacement.

Keywords osteolysis; osteogenic differentiation; PRNCR1; miR-211-5p; CXCR4
Introduction

Hip replacement is the reparation surgery for replacement of all or part hip joint with artificial hip joint[1], which has been used clinically for many years with better joint motion, early move around and decreased complications for staying long in bed[2]. However, complications of hip replacement such as postoperative infection, dearticulation, deep venous thrombosis, osteolysis, and prosthesis loosening are gradually increased, leading to prosthesis failure and bringing about additional suffering and burden for patients[3]. Osteolysis is caused by wear particles produced by friction between components of prosthesis and bone interface, and it’s the leading cause of prosthesis aseptic loosening[4]. With increasing wear particles produced, the formation of osteoclast was activated, and over-weight osteoclasts caused much bone resorption, which eventually led to osteolysis[5, 6].

As a result of excess of bone resorption and deficiency of bone formation, osteolysis is closely connected with osteogenic differentiation, which refers to the whole process of bone formation from mesenchymal stem cells (MSCs)[7]. Osteolysis after hip replacement is regulated by many factors related to osteogenic differentiation. CXCR4 is the primary transmembrane receptor of CXCL12 (stromal cell-derived factor-1, SDF-1)[8], and the CXCL12/CXCR4 signal axis plays an important role in mediating bone morphogenetic protein 9-induced osteogenic differentiation of MSCs[9]. A recent report revealed that metallic wear debris up-regulated CXCR4 expression in vitro and in vivo, in a dose-dependent manner, and the up-regulation was found in periprosthetic tissue from revision arthroplasty of failed metal-on-metal hip replacements, with radiographic evidence of osteolysis[10]. These studies demonstrated that CXCR4 was involved in osteolysis after hip replacement through mediating osteogenic differentiation of MSCs, which the specific regulatory mechanism remains unclear.

Previous study demonstrated that miR-211 acted as vital negative regulator of Runx2 to promote adipogenesis and suppress osteogenesis in bone mesenchymal stem cells (BMSCs)[11], and that miR-211 and autophagy-related gene 14 regulated osteoblast-like cell differentiation of human induced pluripotent stem cells[12]. The miR-21 expression also has been reported to be significantly up-regulated in the particle-induced osteolysis animal model[13]. MiR-211-5p is one of the members of miR-211 family that related to cell proliferation, apoptosis and drug sensitivity in hepatocellular carcinoma[14], and we predicted that it may also play a role in osteogenic
differentiation. Furthermore, bioinformatics analysis showed the complementary base pairs between miR-211-5p and the 3'UTR of CXCR4, indicating the potential binding site and interplay between them. In addition, up-regulation of long non-coding RNA (lncRNA) prostate cancer non-coding RNA 1 (PRNCR1) has been noted in colorectal cancer, which promoted cell cycle and cell proliferation[15], while its role in bone metabolism has not been noted. But it’s a breakthrough that the complementary base pairs was discovered between PRNCR1 and miR-211-5p by the bioinformatics analysis, which strongly hinting the close interaction between them.

We initiated this study to explore expression levels of lncRNA PRNCR1, miR-211-5p and CXCR4 in osteolysis after hip replacement, and to evaluate whether they interact with each other and exert an influence on osteogenic differentiation in periprosthetic osteolysis, aiming to offer a theoretical reference for prevention and treatment of osteolysis after hip replacement.

Materials and methods

Patient samples

From January 2016 to June 2016, 20 patients who received a revision surgery because of aseptic loosening of prosthesis after hip replacement were enrolled in this study. All the patients agreed and signed the documented informed consent for tissue donation for study before samples collection. Wear particles and normal tissues around the prosthesis of the hip joints in these patients were taken and stored at -80°C before analysis. This research was approved by the ethics committee of the Changzhou first people's hospital, the Third Affiliated Hospital of Soochow University and performed in accordance with the Helsinki Declaration.

Reverse transcription and quantitative real-time PCR (qRT-PCR)

The qRT-PCR was performed for gene expression analysis. Total RNA was extracted from the wear particles, normal tissues around prosthesis or MSCs using the Trizol reagent (Invitrogen). Briefly, tissues or cells were kept in the centrifuge tube and mixed completely with 1ml Trizol; then 200μl chloroform was added and centrifuged for supernatant; with 500μl isopropanol added into the supernatant, the mixed solution was centrifuged and the precipitate was gently washed with 75% ethyl alcohol. Finally, the total RNA was dried in the air and dissolved with DEPC water. Quantity and quality of RNA were evaluated using the Nanodrop technology (Thermo Scientific) with A_{260}/A_{280} ratios between 1.9 and 2.1. 1μg of total RNA was taken and reverse-transcribed in a
20μl volume using M-MLV Reverse Transcriptase Kit (Promega), which is a recombinant DNA polymerase that synthesizes a complementary DNA (cDNA) strand from single-stranded RNA. This Reverse Transcriptase Kit is composed of First-Strand Buffer (including 250mM Tris-HCl with pH 8.3 at room temperature, 375mM KCl, 15mM MgCl2, and 50mM DTT), and the reaction was incubated at 37°C for 15min, then 85°C for 5sec. The obtained cDNA was mixed with SYBR Select Master Mix (Thermo Fisher) and amplified on an ABI 7900-fast thermocycler (Applied Biosystems). Primers were designed and synthesized by Sangon Biotech (Shanghai, China). The relative expression was calculated with the \(2^{-ΔΔCt}\) method.

**Protein extraction and western blotting**

Radio Immunoprecipitation Assay (RIPA) lysis buffer (Beyotime Biotechnology) containing protease inhibitors is commonly used in lysis of cells and tissues, for extraction of soluble proteins[16]. The wear particles, normal tissues around prosthesis or MSCs were treated with RIPA lysis buffer for 30 min on ice, and the acquired lysates were centrifuged at 12000g for 10 min at 4°C. The supernatants were collected and concentration of proteins was measured with a BCA protein assay kit (Thermo Fisher). Expression of proteins was analyzed by western blot. Proteins were separated by SDS-PAGE with electrophoresis system and transferred into the polyvinylidene difluoride (PVDF) membrane (Bio-Rad). The membrane was then blocked with 5% skimmed milk for 1 hour at RT, and then incubated with primary antibodies including anti-CXCR4 antibody (Abcam, 1:500) and anti-β-actin antibody (Abcam, 1:1000) at 4°C for overnight. The membrane was incubated with HRP-bounded antibodies for 1 hour and the target proteins were visualized by ECL Plus Western Blotting Substrate (Thermo Fisher). The β-actin protein was used as control to quantify protein level.

**Particle preparation**

Purified Polymethylmethacrylate (PMMA) particles used in this study were purchased from Polysciences. By using scanning electron microscope, the particles have been identified with a mean diameter of 0.33μm (0.33±0.019μm) and 90% of the particles have diameter less than 1μm. The particles were disinfected by torrefaction at 180°C for 6h and then washed in 70% ethanol solution twice. For decontamination from endotoxins, a Limulus Amoebocyte Lysate assay (Endosafe) was performed on PMMA particles. PMMA particles were finally suspended in sterile phosphate-buffered saline (PBS) solutions and stored at 4°C.
Cell culture

The human bone marrow-derived MSCs and HEK-293 cells were purchased from American Type Culture Collection (ATCC). The MSCs were divided into two groups: control, and 0.3% v/v PMMA, and they were maintained in the complete Dulbecco’s modified Eagle’s medium (DMEM, Gibco) supplemented with 10% fetal bovine serum (FBS, Gibco), 100U/ml penicillin, 100μg/ml streptomycin (Invitrogen), and 200ng/mL BMP2, and cultured at 37°C in a humidified atmosphere with 5% CO2, for 14 days. The 200ng/mL BMP2 was used for osteogenic induction. The medium was refreshed thrice a week.

The HEK-293 cells were widely used in the luciferase reporter assay for its high-density transfection[17], also in research concerning osteogenic differentiation[18]. The HEK-293 cells were cultured in the complete DMEM medium (Gibco) supplemented with 10% fetal bovine serum (FBS, Gibco), 100U/ml penicillin and 100μg/ml streptomycin (Invitrogen), and maintained at 37°C in a humidified atmosphere with 5% CO2.

Alkaline phosphatase (ALP) activity

ALP activity is a significant symbol for mature differentiation of osteoblast. The ALP activity was detected at 0, 1, 3, 7, and 14d after cell culture, respectively, using the Alkaline Phosphatase Assay Kit (Abcam). After collected and rinsed with PBS, MSCs were incubated and lysed with RIPA cell lysis buffer (Beyotime Biotechnology). The acquired cell lysate was centrifuged with 10,000 rpm/min for 5 min to get the supernatant, and incubated in ALP buffer (diethanolamine buffer solution, DEA, pH 9.8, Sigma-Aldrich) and p-nitrophenyl phosphate solution at 37°C for 30min with gentle shaking in the dark. Subsequently, the 0.2M NaOH final solution was added to each well to terminate the reaction. The absorbance at 405 nm was examined in an microplate reader (Promega) in triplicate to determine the ALP activity.

Plasmid construction

The pcDNA3.1 (Invitrogen) was used to construct the pcDNA-PRNCR1 or pcDNA-CXCR4 expression plasmids, with pcDNA3.1 as control. Briefly, the target gene PRNCR1 or CXCR4 was amplified with PCR and then purified with gel extraction. The purified product and pcDNA3.1 were linked with linkage with T4 DNA ligase (Takara) and integrated into pcDNA-PRNCR1 or pcDNA-CXCR4 recombinant plasmids after double enzymes restriction. The recombinant plasmids were cloned into E.coli to select and amplify the positive clones. After identified with
restriction enzyme analysis and sequence analysis (Sangon Biotech), the recombinant plasmids were extracted from positive clones for cell transfection.

**Cell transfection**

To modulate expression level of related genes in MSCs or HEK-293 cells, the negative control (NC), si-RNA, si-RNA-PRNCR1, miR-211-5p inhibitor, pre-NC, miR-211-5p mimic, pcDNA, pcDNA-PRNCR1, and pcDNA-CXCR4 were designed and synthesized by Biomics Biotechnologies for cell transfection. MSCs were seeded in 6-well plates at a density of \(4 \times 10^5\) cells/well. Then the transfection was performed in Opti-MEM (invitrogen) with 100nM of siRNA or 50nM miRNA (inhibitor or mimic) plasmid and an optimal volume of Lipofectamine2000 (Sigma) transfected into cells. Transfected cells were then cultured in regular medium 8h after transfection. At 48h after transfection, the genes expression was analyzed with qRT-PCR and western blot.

**Alizarin Red S (ARS) staining**

ARS staining was used to determine the calcification deposition in MSCs after cultured for 0, 1, 3, 7, and 14d in DMEM supplemented with 10% fetal bovine serum (FBS, Gibco), 100 U/ml penicillin, and 100 μg/ml streptomycin (Invitrogen), and 200ng/mL BMP2 for osteogenic induction. Cells were first fixed in neutral formalin buffer and washed with PBS for three times. Subsequent to further dehydration with ethanol (95%), the fixed cells were stained by Alizarin Red S solution for 1 minute. Cells were then soaked in acetone for 30 seconds followed by acetone-xylene 1:1 mixture solution for 15 seconds. The staining of calcification deposition was recorded under a microscope, and images were taken at the same time.

**RNA immunoprecipitation (RIP)**

RIP was performed to examine the binding between PRNCR1 and miR-211-5p with the RNA-Binding Protein Immunoprecipitation Kit (Millipore), which includes Magnetic Beads Protein A/G, RIP Wash Buffer, RIP Lysis Buffer, 0.5M EDTA, 10% SDS, Salt Solution I and II, Precipitate Enhancer, Normal Mouse IgG, Rabbit IgG Purified, Protease Inhibitor, RNase inhibitor, Proteinase K, Positive Control Antibody, and RIP primers. The Argonaute2 (AGO2) was used in RIP assay. HEK-293 cells were lysed with RIP lysis buffer and the acquired cell lysates were incubated with AGO2 antibody or normal mouse IgG. RNA-protein complexes were immunoprecipitated with protein A/G magnetic beads, and the beads bound complexes were
immobilized with magnetic. After the unbound materials washed off, the RNA was extracted by using Trizol (Invitrogen). The IP-western was used to detect the level of protein and qRT-PCR was performed to quantify the PRNCR1 and miR-211-5p.

**RNA pull-down assay**

RNA pull-down assay was conducted to determine the interaction between PRNCR1 and miR-211-5p. Briefly, the DNA probe complementary to PRNCR1 was synthesized and biotinylated by GenePharma Co., Ltd (Shanghai, China). RNA pull-down assay was carried out by the Magnetic RNA-Protein Pull-Down Kit (Thermo) according to its specification. 3μg PRNCR1 that labeled with streptavidin was diluted into 100μl with structure buffer, and then kept at 90°C for 5min. Then the streptavidin beads that washed with 60μl cell lysate were incubated at temperature for 1h with agitation. After the pre-treated RNA was incubated at temperature for 1h with agitation, the above washed 60μl streptavidin beads were added for incubation at temperature for 1h with agitation. The beads were washed with 1ml high-concentration salt solution twice, and subsequently washed with 1ml high-concentration salt solution twice. Finally, the RNA-binding protein complexes were washed and eluted for western blot or qRT-PCR analysis.

**Dual luciferase reporter assay**

Dual luciferase reporter assay was used to investigate the interaction relationship between miR-211-5p and CXCR4. The WT CXCR4 3’-UTR or MUT CXCR4 3’-UTR DNA segments containing the predicted miR-211-5p binding site was amplified from human genomic DNA and was then cloned into the pmirGLO plasmids (Promega) to form the reporter vector pmirGLO-CXCR4-WT or pmirGLO-CXCR4-MUT. Luciferase reporter plasmids plus miR-211-5p inhibitor or miR-211-5p mimic or their corresponding negative controls were co-transfected into HEK-293 cells using Lipofectamine2000 (Invitrogen). 48h after transfection, the luciferase activity was detected by the dual luciferase reporter assay system (Promega) and normalized to Renilla luciferase expression level. Each experiment was conducted in triplicate.

**Statistical analysis**

Quantified data were expressed as mean ± standard deviation, and all statistical analyses in this study were carried out by SPSS 21.0 software (SPSS Inc.). Differences between groups were performed by Student’s t test and a $P$ value of <0.05 was taken as statistically significant.

**Results**
Altered expression of lncRNA PRNCR1, miR-211-5p and CXCR4 in wear particles around prosthesis

To investigate the role of PRNCR1 in osteolysis after hip replacement, its expression level in prosthetic wear particles was assessed. Expression level of lncRNA PRNCR1, miR-211-5p and CXCR4 was analyzed in wear particles around prosthesis (n=12) and normal tissues around prosthesis (n=12) in patients underwent a revision surgery because of aseptic loosening of prosthesis after hip replacement. It showed that expression of PRNCR1 was markedly increased in wear particles around prosthesis compared with normal tissues around prosthesis (Figure 1A), while expression of miR-211-5p was decreased (Figure 1B), and expression of CXCR4 protein was increased (Figure 1C). The clear up-regulation of PRNCR1 in prosthetic wear particles may imply some potential role of it in aseptic loosening of prosthesis after hip replacement.

Altered expression of PRNCR1, miR-211-5p and CXCR4 in MSCs incubated with PMMA

To explore the influence of PRNCR1 on osteogenic differentiation, its expression in MSCs incubated with PMMA was measured. MSCs were divided into control group and PMMA group (with 0.3% v/v PMMA), and cultured in complete medium with 200mg/mL BMP2 for 14 days for osteogenic induction. The ALP activity was detected at 0, 1, 3, 7, and 14d after cell culture, respectively. The ALP activity in PMMA group at 1 to 14d was far lower than that in control group (Figure 2A). The expression levels of Runx2, Osterix, and Osteocalcin (OCN) in PMMA group at 1 to 14d were also far lower than that in control group (Figure 2B). The level of ARS in PMMA group from 1 to 14d was much less than that in control group (Figure 2C). The expression of PRNCR1 was significantly higher in PMMA group from 3 to 14d than that in control group, while expression of miR-211-5p from 1 to 14d was much lower than that in control group, and expression of CXCR4 mRNA and protein from 1 to 14d was evidently higher than that in control group (Figure 2D). These findings demonstrated that PMMA treatment clearly suppressed the osteogenic differentiation of MSCs. In addition, the expression of PRNCR1 was significantly elevated by PMMA during osteogenic differentiation of MSCs, suggesting that PRNCR1 may play a role in osteogenic differentiation affected by PMMA.

Repression of PRNCR1 weakened the inhibitory effect of wear particles on osteogenic differentiation

Due to the up-regulation of PRNCR1 during osteogenic differentiation of MSCs incubated with
PMMA, we clarified its influence on osteogenic differentiation by knocking down it. MSCs that induced with BMP2 for 14d for osteogenic induction were divided into four groups: control group, PMMA group, PMMA + siRNA group and PMMA + siRNA-PRNCR1 group. The PMMA was added into after the siRNA or siRNA-PRNCR1 was transfected into MSCs for 48h. The results suggested that PMMA inhibited the ALP activity, while siRNA-PRNCR1 reversed the inhibitory effect (Figure 3A); PMMA suppressed the expression of Runx2, Osterix and OCN, while siRNA-PRNCR1 reversed the expression of them (Figure 3B). PMMA decreased the levels of ARS, while siRNA-PRNCR1 reversed the decrease (Figure 3C). Our data suggested that inhibition of PRNCR1 weakened the inhibitory effect of wear particles on osteogenic differentiation.

**MiR-211-5p was regulated by PRNCR1 in HEK-293 cells**

The binding site of PRNCR1 and miR-211-5p was predicted by TargetScan and microrna.org (Figure 4A). Subsequently, combination condition and interaction between RNA and protein were determined. AGO2 antibody was used in the RIP assay, and the level of PRNCR1 and miR-211-5p was examined. Compared with IgG, plenty of PRNCR1 and miR-211-5p were detected in the AGO2 antibody (Figure 4B). In the RNA pull-down assay, AGO2 in the pull-down complex of PRNCR1 was analyzed by western blot, with NC as control (Figure 4C). The miR-30d-5p gathered extensively in the pull-down complex of DGCR5, while it increased slightly in NC (Figure 4D). It revealed that the miR-211-5p is a target of PRNCR1.

**CXCR4 was regulated by PRNCR1 through miR-211-5p in MSCs**

To identify the relationship among PRNCR1, miR-211-5p, and CXCR4, MSCs that induced with BMP2 for 14d for osteogenic induction were divided into several groups: NC, PMMA, PMMA+siRNA, PMMA+siRNA-PRNCR1, PMMA+siRNA-PRNCR1+NC, PMMA+siRNA-PRNCR1+miR-211-5p inhibitor. The results showed that siRNA-PRNCR1 inhibited the expression of CXCR4 mRNA and protein, while miR-211-5p inhibitor promoted the expression of CXCR4 mRNA and protein (Figure 5A). Comparison among groups of control, pcDNA-PRNCR1, pcDNA-PRNCR1 + pre-NC, pcDNA-PRNCR1 + miR-211-5p mimic revealed that pcDNA-PRNCR1 promoted the expression of CXCR4 mRNA and protein, while miR-211-5p mimic repressed the expression of CXCR4 mRNA and protein (Figure 5B). We showed that PRNCR1 regulated CXCR4 through miR-211-5p in MSCs.
CXCR4 was negatively regulated by miR-211-5p in HEK-293 cells

Binding site of miR-211-5p and WT 3’ UTR of CXCR4 was predicted by TargetScan and microrna.org software (Figure 6A), and we explored the interaction between them. Compared with Pre-NC, with miR-211-5p over-expressed by miR-211-5p mimic transfection, the activity of WT 3’ UTR of CXCR4 was inhibited, and the expression of CXCR4 mRNA and protein was also suppressed. No significant difference was noted in the activity of MUT 3’ UTR of CXCR4 between the two groups (Figure 6B). Compared with NC, with miR-211-5p down-regulated by miR-211-5p inhibitor, the activity of WT 3’ UTR of CXCR4 was up-regulated, and the expression of CXCR4 mRNA and protein was also up-regulated. No significant difference was found in the activity of MUT 3’ UTR of CXCR4 between the two groups (Figure 6C). These results demonstrated that CXCR4 was negatively regulated by miR-211-5p via binding with its 3’ UTR.

Wear particles regulated the expression of CXCR4 through miR-211-5p to influence the osteogenic differentiation of MSCs

To investigate the influence of miR-211-5p and CXCR4 on osteogenic differentiation, MSCs that induced with BMP2 for 14d for osteogenic induction were divided into several groups: NC, PMMA, PMMA + pre-NC, PMMA + miR-211-5p mimic, PMMA + miR-211-5p mimic + pcDNA, PMMA + miR-211-5p mimic + pcDNA-CXCR4. The PMMA was added into after other materials were transfected into MSCs for 48h. The results indicated that PMMA repressed the ALP activity, while miR-211-5p mimic reversed the inhibitory effect, which was further reversed by pcDNA-CXCR4 (Figure 7A). PMMA inhibited the expression of Runx2, Osterix and OCN, while miR-211-5p mimic reversed the inhibitory effect, which was further reversed by pcDNA-CXCR4 (Figure 7B). PMMA decreased the levels of ARS, while miR-211-5p mimic reversed the decrease, which was reversed by pcDNA-CXCR4 again (Figure 7C).

Wear particles regulated the expression of miR-211-5p through PRNCR1 to influence the osteogenic differentiation of MSCs

Finally, we attempted to confirm the impact of PRNCR1 on osteogenic differentiation and its regulatory effect on miR-211-5p. MSCs that induced with BMP2 for 14d for osteogenic induction were divided into several groups: NC, PMMA, PMMA + siRNA, PMMA + siRNA-PRNCR1, PMMA + siRNA-PRNCR1 + NC, PMMA + siRNA-PRNCR1 + miR-211-5p inhibitor. The PMMA was added into after other materials were transfected into MSCs for 48h. It indicated that
PMMA inhibited the ALP activity, while siRNA-PRNCR1 reversed the ALP activity, which was further reversed by miR-211-5p inhibitor (Figure 8A). PMMA inhibited the expression of Runx2, Osterix and OCN, while siRNA-PRNCR1 reversed the expression levels of them, which was further reversed by miR-211-5p inhibitor (Figure 8B). PMMA decreased the levels of ARS, while siRNA-PRNCR1 reversed the decrease, which was reversed by miR-211-5p inhibitor again (Figure 8C). Above all, we proved that wear particles regulated the expression of miR-211-5p through PRNCR1 to influence the osteogenic differentiation of MSCs.

**Discussion**

By exploring the interaction between lncRNA PRNCR1 and miR-211-5p, and between miR-211-5p and CXCR4, and their impacts on osteogenic differentiation, this study demonstrated that PRNCR1 exerted an inhibitory effect on osteogenic differentiation by up-regulating expression of CXCR4 through inhibiting miR-211-5p, which contributed to osteolysis after hip replacement. These findings may provide a novel strategy for prevention of osteolysis after hip replacement and other bone metabolism diseases related to osteogenic differentiation.

Loss of bone stock around the prosthesis after hip replacement is precisely because of simulation of bone resorption or inhibition of bone formation, known as periprosthetic osteolysis[19, 20], which is closely connected with osteogenic differentiation. Generally, wear particles around the prosthesis inhibited the ability of osteoblasts to secrete mineralized bone matrix and inhibited its ability to proliferate, which was adverse to the osteogenic differentiation process and subsequently contributed to the osteolysis process[21]. It has been reported that various types of wear debris particles (including PMMA) behaved differently in the proliferation, migration, differentiation, and functions of osteogenic cells, playing a significant role in the pathogenesis and process of debris-associated aseptic prosthetic loosening [22, 23]. However, it has been proved that PMMA particle-induced inhibition of osteoprogenitor differentiation and proliferation was not due to secreted inhibitory factors[24]. Here, we revealed the underlying mechanism of PMMA particles repressing osteogenesis, by negatively affecting osteogenic differentiation of MSCs via PRNCR1/miR-211-5p/CXCR4 signaling, and thus causing osteolysis after hip replacement, reconfirming the particle-associated dysregulation of osteogenic differentiation.

The genetic polymorphisms of PRNCR1 has been identified to be closely associated with the risk of prostate cancer, colorectal cancer, and gastric cancer[25-27]. PRNCR1 also has been reported to be
up-regulated in colorectal cancer, and it promoted cell cycle and cell proliferation[15], while its impact on bone metabolism has not been paid attention yet. In the present study, increased expression of PRNCR1 was observed in per-iprosthetic osteolysis tissues and MSCs incubated with PMMA, implying its potential role in osteogenic differentiation of MSCs and osteolysis. MiR-211-5p was identified as a novel target of PRNCR1 in our study, with miR-211-5p initially implicated in per-iprosthetic osteolysis. Previous study has proved that MSCs migration was enhanced by miR-211 overexpression, improving the therapeutic efficacy of MSCs transplantation[28]. Here we demonstrated that wear particles up-regulated CXCR4 through miR-211-5p to affect osteogenic differentiation of MSCs, which contributed to osteolysis. But it has been claimed that age-related CXCR4-deficiency impaired osteogenic differentiation potency of mouse BMSCs[29], which seemed to be contrary to our results. These two opposite findings may be explained that expression of CXCR4 in osteogenic differentiation was not only age-related but also dose-dependent[10], which deserved further exploration in the future.

Apart from the above three factors, expression of Runx2, Osterix and OCN were also investigated in this study, all of which were closely related to osteogenic differentiation. Runx2 is one of the special transcription factors of osteocyte that promotes osteoblast differentiation, playing key role in bone formation[30]. Osterix is one of the crucial transcript factors that positively modulate osteoblast differentiation, and the expression level of Osterix is considered as the symbol of differentiated degree of osteoblasts[31]. OCN is synthesized and secreted by osteocyte, and its level can reflect the activity status of osteocyte, especially for the latest formed osteocyte[32]. Our study showed that particles suppressed the expression of Runx2, Osterix and OCN, along with osteogenic differentiation inhibited, emphasizing their functions in bone metabolism.

In the present study, we elucidated the pivotal role of PRNCR1 in osteogenic differentiation and osteolysis after hip replacement, offering a potential and effective target for treatment of osteolysis after hip replacement. Regarding of the influence of PRNCR1 on osteoclast and bone resorption, much further studies are needed in the future.

**Conflict of interest**

The authors declare no conflict of interest.

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None
Ethical approval:
The study was approved by ethics committee of Changzhou first people's hospital.

Consent for publication
The study was undertaken with the patient's consent.

Availability of data and material
Not applicable

Authors' contributions
ZT put forward the concept of the study, designed the study, prepared the manuscript and contributed to the statistical analysis. XS contributed to the data acquisition. ZG edited the manuscript. All authors read and approved the final manuscript.

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References
1. Learmonth ID, Young C, Rorabeck C: The operation of the century: total hip replacement. Lancet 2007, 370(9597):1508-1519.
2. Shan L, Shan B, Graham D, Saxena A: Total hip replacement: a systematic review and meta-analysis on mid-term quality of life. Osteoarthritis Cartilage 2014, 22(3):389-406.
3. Attinger M, Siebenrock K: Total hip replacement: between normal rehabilitation and complication. Praxis (Bern 1994) 2014, 103(24):1439-1446.
4. Howie DW, Neale SD, Haynes DR, Holubowycz OT, McGee MA, Solomon LB, Callary SA, Atkins GJ, Findlay DM: Periprosthetic osteolysis after total hip replacement: molecular pathology and clinical management. Inflammopharmacology 2013, 21(6):389-396.
5. Sun SX, Guo HH, Zhang J, Yu B, Sun KN, Jin QH: BMP-2 and titanium particles synergistically activate osteoclast formation. Braz J Med Biol Res 2014, 47(6):461-469.
6. Gallo J, Vaculova J, Goodman SB, Konttinen YT, Thyssen JP: Contributions of human tissue analysis to understanding the mechanisms of loosening and osteolysis in total hip replacement. Acta Biomater 2014, 10(6):2354-2366.
7. Nombela-Arrieta C, Ritz J, Silberstein LE: The elusive nature and function of mesenchymal stem cells. Nat Rev Mol Cell Biol 2011, 12(2):126-131.
8. Kim DS, Kim YS, Bae WJ, Lee HJ, Chang SW, Kim WS, Kim EC: The role of SDF-1 and CXCR4 on odontoblastic differentiation in human dental pulp cells. Int Endod J 2014, 47(6):534-541.
9. Liu C, Weng Y, Yuan T, Zhang H, Bai H, Li B, Yang D, Zhang R, He F, Yan S et al: CXCL12/CXCR4 signal axis plays an important role in mediating bone morphogenetic protein 9-induced osteogenic differentiation of mesenchymal stem cells. Int J Med Sci 2013, 10(9):1181-1192.
10. Drynda A, Singh G, Buchhorn GH, Awiszus F, Ruetschi M, Feuerstein B, Kliche S, Lohmann CH: Metallic wear debris may regulate CXCR4 expression in vitro and in vivo. J Biomed Mater Res A 2015, 103(6):1940-1948.
11. Huang J, Zhao L, Xing L, Chen D: MicroRNA-204 regulates Runx2 protein expression and mesenchymal progenitor cell differentiation. Stem Cells 2010, 28(2):357-364.
12. Ozeki N, Hase N, Hiyama T, Yamaguchi H, Kawai-Asano R, Nakata K, Mogi M: MicroRNA-211
and autophagy-related gene 14 signaling regulate osteoblast-like cell differentiation of human induced pluripotent stem cells. Exp Cell Res 2017, 352(1):63-74.

13. Zhou Y, Liu Y, Cheng L: miR-21 expression is related to particle-induced osteolysis pathogenesis. J Orthop Res 2012, 30(11):1837-1842.

14. Jiang G, Wen L, Deng W, Jian Z, Zheng H: Regulatory role of miR-211-5p in hepatocellular carcinoma metastasis by targeting ZEB2. Biomed Pharmacother 2017, 90:806-812.

15. Yang L, Qiu M, Xu Y, Wang J, Zheng Y, Li M, Xu L, Yin R: Upregulation of long non-coding RNA PRNCR1 in colorectal cancer promotes cell proliferation and cell cycle progression. Oncol Rep 2016, 35(1):318-324.

16. Ngoka LC: Sample prep for proteomics of breast cancer: proteomics and gene ontology reveal dramatic differences in protein solubilization preferences of radioimmunoprecipitation assay and urea lysis buffers. Proteome Sci 2008, 6:30.

17. Backliwal G, Hildinger M, Hasija V, Wurm FM: High-density transfection with HEK-293 cells allows doubling of transient titers and removes need for an a priori DNA complex formation with PEI. Biotechnol Bioeng 2008, 99(3):721-727.

18. Gu Y, Ma L, Song L, Li X, Chen D, Bai X: miR-155 Inhibits Mouse Osteoblast Differentiation by Suppressing SMADS Expression. Biomed Res Int 2017, 2017:1893520.

19. O’Neill SC, Queally JM, Devitt BM, Doran PP, O’Byrne JM: The role of osteoblasts in peri-prosthetic osteolysis. Bone Joint J 2013, 95-B(8):1022-1026.

20. Wang Z, Liu N, Liu K, Zhou G, Gan J, Wang Z, Shi T, He W, Wang L, Guo T et al: Autophagy mediated CoCrMo particle-induced peri-implant osteolysis by promoting osteoblast apoptosis. Autophagy 2015, 11(12):2358-2369.

21. Jonitz-Heincke A, Lochner K, Schulze C, Pohle D, Pustlauk W, Hansmann D, Bader R: Contribution of human osteoblasts and macrophages to bone matrix degradation and proinflammatory cytokine release after exposure to abrasive endoprosthetic wear particles. Mol Med Rep 2016, 14(2):1491-1500.

22. Hou Y, Cai K, Li J, Chen X, Lai M, Hu Y, Luo Z, Ding X, Xu D: Effects of titanium nanoparticles on adhesion, migration, proliferation, and differentiation of mesenchymal stem cells. Int J Nanomedicine 2013, 8:3619-3630.

23. Jiang Y, Jia T, Gong W, Wooley PH, Yang SY: Effects of Ti, PMMA, UHMWPE, and Co-Cr wear particles on differentiation and functions of bone marrow stromal cells. J Biomed Mater Res A 2013, 101(10):2817-2825.

24. Chiu R, Ma T, Smith RL, Goodman SB: Kinetics of polymethylmethacrylate particle-induced inhibition of osteoprogenitor differentiation and proliferation. J Orthop Res 2007, 25(4):450-457.

25. Sattarifard H, Hashemi M, Hassanzarei S, Narouie B, Bahari G: Association between genetic polymorphisms of long non-coding RNA PRNCR1 and prostate cancer risk in a sample of the Iranian population. Mol Clin Oncol 2017, 7(6):1152-1158.

26. Li L, Sun R, Liang Y, Pan X, Li Z, Bai P, Zeng X, Zhang D, Zhang L, Gao L: Association between polymorphisms in long non-coding RNA PRNCR1 in 8q24 and risk of colorectal cancer. J Exp Clin Cancer Res 2013, 32:104.

27. Li L, Jia F, Bai P, Liang Y, Sun R, Yuan F, Zhang L, Gao L: Association between polymorphisms in long non-coding RNA PRNCR1 in 8q24 and risk of gastric cancer. Tumour Biol 2016, 37(1):299-303.
28. Hu X, Chen P, Wu Y, Wang K, Xu Y, Chen H, Zhang L, Wu R, Webster KA, Yu H et al: MiR-211/STAT5A Signaling Modulates Migration of Mesenchymal Stem Cells to Improve its Therapeutic Efficacy. Stem Cells 2016, 34(7):1846-1858.

29. Guang LG, Boskey AL, Zhu W: Age-related CXC chemokine receptor-4-deficiency impairs osteogenic differentiation potency of mouse bone marrow mesenchymal stromal stem cells. Int J Biochem Cell Biol 2013, 45(8):1813-1820.

30. Vimalraj S, Arumugam B, Miranda PJ, Selvamurugan N: Runx2: Structure, function, and phosphorylation in osteoblast differentiation. Int J Biol Macromol 2015, 78:202-208.

31. Shi K, Lu J, Zhao Y, Wang L, Li J, Qi B, Li H, Ma C: MicroRNA-214 suppresses osteogenic differentiation of C2C12 myoblast cells by targeting Osterix. Bone 2013, 55(2):487-494.

32. Kondo A, Tokuda H, Matsushima-Nishiwaki R, Kuroyanagi G, Yamamoto N, Mizutani J, Kozawa O, Otsuka T: Rho-kinase limits BMP-4-stimulated osteocalcin synthesis in osteoblasts: regulation of the p38 MAP kinase pathway. Life Sci 2014, 96(1-2):18-25.

Figure legends

Figure 1 Altered expression of lncRNA PRNCR1, miR-211-5p and CXCR4 in wear particles around prosthesis. (A) The expression of PRNCR1 in wear particles around prosthesis (n=12) and normal tissues around prosthesis (n=12) in patients underwent a revision surgery because of aseptic loosening of prosthesis after hip replacement was quantified by qRT-PCR. *P<0.05 vs. normal. (B) The expression of miR-211-5p in wear particles around prosthesis (n=12) and normal tissues around prosthesis (n=12) was quantified by qRT-PCR. *P<0.05 vs. normal. (C) The expression of CXCR4 protein in wear particles around prosthesis (n=12) and normal tissues around prosthesis (n=12) was analyzed by western blot, with β-actin served as control.

Figure 2 Altered expression of lncRNA PRNCR1, miR-211-5p and CXCR4 in MSCs with PMMA. (A) The ALP activity was determined in MSCs cultured with or without PMMA for different days to assess the osteogenic differentiation. (B) The expression levels of Runx2, Osterix, and OCN in MSCs were determined by qRT-PCR. (C) The ARS staining was used to evaluate the osteogenic differentiation of MSCs after osteogenic induction. (D) The expression of PRNCR1, miR-211-5p and CXCR4 mRNA in MSCs was determined by qRT-PCR. The expression of CXCR4 protein in MSCs at different days was analyzed by western blot, with β-actin served as control. *P<0.05 vs. control.

Figure 3 Repression of PRNCR1 weakened the inhibitory effect of wear particles on osteogenic differentiation. (A) The ALP activity was determined in MSCs at different groups to assess the osteogenic differentiation. (B) The expression levels of Runx2, Osterix and OCN in MSCs were examined by qRT-PCR. (C) The ARS staining was used to evaluate the osteogenic differentiation
of MSCs after osteogenic induction. *P<0.05 vs. control. &P<0.05 vs. PMMA + siRNA.

**Figure 4** MiR-211-5p was regulated by PRNCR1 in HEK-293 cells. (A) The binding site between PRNCR1 and miR-211-5p was predicted by TargetScan and microrna.org. (B) RIP was performed to examine the binding between PRNCR1 and miR-211-5p, and the expression of AGO2 protein was detected by western blot, and the expression of PRNCR1 and miR-211-5p were quantified by qRT-PCR. *P<0.05 vs. IgG. (C) RNA pull-down assay was conducted to determine the interaction between PRNCR1 and miR-211-5p, and the expression of AGO2 protein was detected by western blot. (D) The expression of miR-211-5p was determined by qRT-PCR. *P<0.05 vs. Beads.

**Figure 5** CXCR4 was regulated by PRNCR1 through miR-211-5p in MSCs. (A) The expression of CXCR4 in MSCs was determined by qRT-PCR. *P<0.05 vs. control. &P<0.05 vs. siRNA. #P<0.05 vs. NC. The expression of CXCR4 protein in MSCs was detected by western blot, with β-actin served as control. (B) The expression of CXCR4 in MSCs was determined by qRT-PCR. *P<0.05 vs. control. &P<0.05 vs. pre-NC. The expression of CXCR4 protein in MSCs was analyzed by western blot, with β-actin served as control.

**Figure 6** CXCR4 was negatively regulated by miR-211-5p in HEK-293 cells. (A) The binding site between miR-211-5p and 3'-UTR of WT CXCR4 was predicted by TargetScan and microrna.org software. (B) The luciferase activity was detected to show the interaction between miR-211-5p and CXCR4. The expression levels of CXCR4 mRNA and protein in HEK-293 cells were analyzed by qRT-PCR and western blot, respectively. *P<0.05 vs. pre-NC. (C) The luciferase activity was detected to show the interaction between miR-211-5p and CXCR4. The expression levels of CXCR4 mRNA and protein in HEK-293 cells were analyzed by qRT-PCR and western blot, respectively. *P<0.05 vs. NC.

**Figure 7** Wear particles regulated the expression of CXCR4 through miR-211-5p to influence the osteogenic differentiation of MSCs. (A) The ALP activity was determined in MSCs at different groups to assess the osteogenic differentiation. (B) The expression levels of Runx2, Osterix and OCN in MSCs were detected by qRT-PCR. (C) The ARS staining was used to detect the osteogenic differentiation of MSCs after osteogenic induction. *P<0.05 vs. control. &P<0.05 vs. pre-NC. #P<0.05 vs. pcDNA.

**Figure 8** Wear particles regulated the expression of miR-211-5p through PRNCR1 to influence the osteogenic differentiation of MSCs. (A) The ALP activity was determined in MSCs at different
groups to evaluate the osteogenic differentiation. (B) The expression levels of Runx2, Osterix and OCN in MSCs were detected by qRT-PCR. (C) The ARS staining was used to detect the osteogenic differentiation of MSCs after osteogenic induction. *P<0.05 vs. control. &P<0.05 vs. siRNA. †P<0.05 vs.NC.
A  

| 3' | ucCGCU - UCCU - AC -- UGUUUCCCu |
|----|---------------------------------|
| WT | 5' ucGUGGUAGGGACUGUAGAAAAGGGAa |
|    |                                  |
| MUT| 5' ucCGCU- UCCU- AC - - UGUUUCCCu |
|    |                                  |
|    | 3' CXCR4 3'-UTR                  |

B

**Relative Luciferase Activity**

|          | pre-NC | miR-211-5p mimic |
|----------|--------|-----------------|
| WT       |        | *               |
| MUT      | *      |                 |

**Relative CXCR4 mRNA Expression**

|          | pre-NC | miR-211-5p mimic |
|----------|--------|-----------------|
| WT       |        | *               |
| MUT      | *      |                 |

C

**Relative Luciferase Activity**

|          | NC     | miR-211-5p inhibitor |
|----------|--------|---------------------|
| WT       |        | *                   |
| MUT      | *      |                     |

**Relative CXCR4 mRNA Expression**

|          | NC     | miR-211-5p inhibitor |
|----------|--------|---------------------|
| WT       |        | *                   |
| MUT      | *      |                     |
