Exosomal miR-1275 Secreted by Prostate Cancer Cells Modulates Osteoblast Proliferation and Activity by Targeting the SIRT2/RUNX2 Cascade

Zihao Zou1, Ranran Dai1, Nan Deng1, Wei Su2, and Ping Liu1

Abstract
Prostate cancer (PCa) is one of the most frequently diagnosed malignancies and the second leading cause of cancer mortality among men worldwide. Modulation of osteoblast activity is involved in PCa metastasis, and miR-1275 is also reported to regulate PCa metastasis; however, the association between cancer-derived exosomal miR-1275 and osteoblast activity is unclear. Here, we isolated exosomes from PC3-derived conditioned medium by ultracentrifugation. We found that miR-1275 could be transferred from PCa cells to osteoblasts via exosomes. Exosomal miR-1275 significantly accelerated the proliferation of osteoblasts and the expression levels of osteoblast-specific genes, such as osteocalcin (OCN), type I collagen (COL-1), and osteopontin (OPN). Moreover, exosomal miR-1275 increased the expression of RUNX2, a master modulator of osteoblast activity, by down-regulation of SIRT2, which in turn influenced the growth and activity of osteoblasts. Our findings indicate that PCa-derived exosomal miR-1275 promotes the proliferation and activity of osteoblasts via modulation of SIRT2/Runx2 signaling.

Keywords
prostate cancer, exosomes, miR-1275, SIRT2, osteoblast proliferation

Introduction
Prostate cancer (PCa) is one of the most frequently diagnosed malignancies and the second leading cause of cancer mortality among men worldwide1. The incidence of PCa is increasing worldwide in recent years. Each year 1.6 million men are diagnosed with PCa and 366,000 men die of this disease2. The communication between PCa cells and the metastatic microenvironment is crucial for PCa metastasis3,4. Understanding the molecular mechanism underlying the communication between PCa cells and osteoblasts will be crucial for developing novel therapeutic strategies against metastatic PCa.

Exosomes are small extracellular vesicles, which are involved in multiple cellular processes, such as tumor initiation, growth, progression, metastasis, drug resistance, and immune escape5. Exosomes contains many genetic material, proteins, and lipids and act as potential signaling molecules between cancer cells and the surrounding cells6. Exosomes also carry different nucleic acids, such as microRNAs (miRNAs) miRNAs are a group of small endogenous non-coding nucleotides that modulate cell growth, activity, and metabolism through posttranscriptional regulation of gene expression7. It has been reported that cancer cells deliver their exosomes comprising miRNAs to the metastatic site and modify the microenvironment, thus facilitating cancer metastasis8. The intercommunication between different cells via exosomal miRNAs is suggested to be an important way of interaction between tumor cells and the tumor microenvironment9. Multiple studies have been demonstrated that miR-1275 plays an important role in cancer progression10–12. Using the whole miRNome analysis, Nam et al. have

1 Department of urology, Third Affiliated Hospital of Guangzhou Medical University, Guangzhou, Guangdong, P.R. China
2 Department of Urology, Zhujiang Hospital, Southern Medical University, Guangzhou, Guangdong, P.R. China

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Corresponding Author:
Zihao Zou, Department of urology, Third Affiliated Hospital of Guangzhou Medical University, Guangzhou, Guangdong, P.R. China.
Email: dannis1984@gzhmu.edu.cn
shown that miR-1275 is a potential candidate associated with PCa metastasis\textsuperscript{13}. Controlling osteoblast activity is suggested to be important for the progress of PCa metastasis\textsuperscript{14,15}. However, the function of exosomal miR-1275 from PCa cells in osteoblasts remains unclear.

In this study, we found that miR-1275 could be released from PC3 cells and transferred to hFOB1.19 osteoblasts. Treatment with exosomes produced from miR-1275-overexpressing PC3 cells (miR-1275-exo) significantly increased the proliferation and activity of osteoblasts, whereas treatment with miR-1275 inhibitor abolished this effect. Moreover, our data demonstrated that miR-1275-exo exerted its function by regulating (Sirtuin 2) SIRT2/Runt-related transcription factor 2 (RUNX2) cascade. Our findings revealed an enhancing effect of exosomal miR-1275 on osteoblast activity.

Methods

Cell Culture

Human PC3 prostate cancer cells and hFOB1.19 osteoblasts were obtained from the American Type Culture Collection (ATCC, Manassas, Virginia, USA). PC3, LNCaP, C4-2B, and RWPE-1 cells were cultured in RPMI1640 medium and hFOB1.19 cells were culture in Dulbecco’s modified eagle medium (DMEM)/F12 medium (HyClone, Logan, Utah, USA). All media were supplemented with 100 U/ml penicillin, 100 µg/ml streptomycin (Seyotin, guangzhou, China), and 10% fetal bovine serum (Gibco, Waltham, Massachusetts, USA). The cells were maintained at 37°C in a humidified atmosphere with 5% CO\textsubscript{2}.

Cell Transfection and co-Culture Assay

PC3 (3 × 10\textsuperscript{5} cells/well) cells were seeded in 6-well plates. miR-1275 mimics, miR-1275 inhibitors or control miRNAs were transfected into PC3 cells by using lipopectamine 2000 reagent (Thermofisher, Waltham, Massachusetts, USA) following the manufacturer’s protocol. The co-culture assay was performed using Transwell inserts (Corning, NY, USA). 1 × 10\textsuperscript{5} PC3 transfected with miR-1275 mimics were added to the upper chamber, while 2 × 10\textsuperscript{5} hFOB1.19 osteoblasts were seeded into the lower chamber. After incubation for 24 h, osteoblasts were collected and used for RNA isolation.

Exosome Isolation

Exosomes were isolated from the PC3-derived conditioned medium by ultracentrifugation. Conditioned medium was collected from 48 h cell cultures, followed by centrifugation at 500 g for 20 min and 40 minutes at 10,000 g. After filtration with a 0.2 µm sterilized filter, the supernatants were ultracentrifuged at 100,000 g for 70 min at 4°C. The exosomes were washed once with phosphate buffer saline (PBS), followed by a second ultra-centrifuged at 100,000 g for 70 min at 4°C. The exosomes were resuspended in PBS finally. The size distribution of the exosomes was observed on a TEM-1400plus transmission electron microscope (TEM). The concentration of exosomes was determined using a Bicinchoninic AcidProtein Assay Kit (Seyotin, Guangzhou, China)

Exosome Uptake Assay

Exosomes were labeled using the PKH67 Green Fluorescent Cell Linker Kit according to the manufacturer’s instructions. In brief, 100 µl exosomes isolated from PC3 cells with different treatments were diluted in 1 ml diluent C from the kit and 6 µl PKH26 dye was diluted in 1 ml diluent C. The dilutions were mixed continuously for 30 s and kept at room temperature for 5 min. Then 2 ml 10% BSA(Seyotin, Guangzhou, China) in PBS was added to quench the staining. Labeled exosomes were washed in 10 ml PBS and collected by centrifugation at 100,000 g for 70 min. hFOB1.19 cells were then incubated with exosomes for 24 h. After washing with PBS, cells were fixed with 4% paraformaldehyde for 10 minutes. Nuclei were stained with DAPI solution. Images were captured by a confocal laser scanning microscope LSM410 (Carl Zeiss, Germany)

Real-Time PCR

Total RNA from cells was isolated using TRIzol reagent (Biowater, Guangzhou, China) and total RNA from exosomes was extracted using the mirVana miRNA Isolation Kit. The RNA was reverse transcribed into first-strand cDNA using the High Capacity cDNA Reverse Transcription Kit (Seyotin, Guangzhou, China), according to the manufacturer’s instructions. qRT-PCR was performed using the Power SYBR Green Master Mix (Seyotin, Guangzhou, China) and conducted on an ABI 7500 (Applied Biosystems, NY, USA). The amplification conditions were as follows: 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 45 s. GAPDH, U6 (cellular), or Cel-mir-39 (exosomal) were used as the internal controls for mRNA and miRNA, respectively. The relative expression was calculated using 2\textsuperscript{ΔΔCt} method. The primers used for qRT-PCR were as follows: miR-1275 forward, 5’-CTCTGTGAGAGGTGGTG-3’ and miR-1275 reverse, 5’-TCTGGTGGAAAATAG-3’; U6 forward, 5’-GCTCGGAGACATATACAAA-3’ and U6 reverse, 5’-GCTTCACAGGAATTGCAGTCT-3’; Cel-miR-39-3p forward, 5’-GGTTACCGGATTGGTAACT-3’; Cel-miR-39-3p reverse, 5’-GAGGGAGAGGAAAGGGA-3’; OCN forward: 5’-GGTTACCGGAGAGGAAAGGGA-3’; OCN reverse: 5’-GGTTACCGGAGAGGAAAGGGA-3’; COL-I forward: 5’-ACAGATCGGGAGACACTCCGT-3’; COL-I reverse: 5’-ACAGATCGGGAGACACTCCGT-3’
5′-TTTGTATTCACTACGTCTTGCC-3′ Runx2 forward: 5′-CCCCTCTCTAAGGAAGCTGACG-3′ and Runx2 reverse: 5′-AACGGCCCAGTTCTGAGACGACCTGA-3′; GAPDH forward: 5′-ATGGAATCCCATCACCACCATT-3′ and GAPDH reverse: 5′-GGTTGAGCAGGCGTTTTATT-3′.

Cell Counting Kit-8 (CCK-8) Assay

hFOB1.19 osteoblasts (3 × 103 cells/well) were placed in 96-well plates. The viability of hFOB1.19 was determined by the CCK-8 assay kit (Seyotin, Guangzhou, China). The absorbance was detected at 450 nm on a plate reader every day.

Luciferase Reporter Assay

The wild-type 3′-UTR and the 3′-UTR mutant that contains the mutated miR-1275 binding site of SIRT2 were amplified by PCR and inserted into the pGL3-control vector (Promega, Madison, Wisconsin, USA) to construct luciferase reporter vectors. 293 T cells were seeded in 24-well plates and were co-transfected with the firefly luciferase reporter vector and the Renilla luciferase control vector with miR-1275 mimics or control mimics by Lipofectamine 2000 (Thermofisher, Waltham, Massachusetts, USA). 24 h post-transfection, the firefly and Renilla luciferase activities were detected using a Dual-Luciferase Reporter Assay System (Promega, Madison, Wisconsin, USA). The firefly luciferase activity was normalized to Renilla luciferase activity.

Western Blotting

Cells were washed twice with PBS and lysed using RIPA lysis buffer (Seyotin, Guangzhou, China). The protein concentration was measured using the BCA method. The protein samples were separated by SDS-polyacrylamide gel electrophoresis and transferred to PVDF membrane. The membrane was blocked with 5% non-fat milk and incubated with antibodies overnight at 4°C. After washing three times with Tris buffered saline tween (TBST) (Seyotin, Guangzhou, China), the membrane was incubated with HRP-coupled second antibody. The protein bands were detected by Immobilon Western kit (Seyotin, Guangzhou, China).

Statistical Analysis

All data were presented as the mean ± SD. Statistical analyses were performed with SPSS 19.0 (SPSS Inc. USA). Analysis between two groups were assessed by two-tailed Student’s t-test while comparisons of more than two groups were performed with one-way multivariate analysis of variance (ANOVA) followed by Turkey’s test. A P-value < 0.05 was considered statistically significant.

Results

Characterization of Exosomes from PCa Cells

PC3 cells cultured in the exosome-free medium were transfected with miR-1275 or control miRNA and exosomes were isolated from the culture medium by centrifugation. The morphology of the isolated exosomes was examined by TEM. As shown in Fig. 1A, exosomes from PC3 cells over-expressing miR-1275 (miR-1275-exo) or control miRNA (NC-exo) exhibited spherical morphology with a diameter range of 50 ~ 100 nm. To further characterize the isolated exosomes, the expression levels of exosomal markers TSG-101 and CD63 were detected by Western blot. Both NC-exo and miR-1275-exo were positive for TSG-101, CD63, CD9, and Calnexin (Fig. 1B). We then determined the expression of miR-1275 in exosomes using qRT-PCR. The expression of miR-1275 in miR-1275-exo was significantly higher than that in No-exo (Fig. 1C). Moreover, we found that miR-1275 could elevate the proliferation of PC3 cells (Fig. S1) and higher expression of miR-1275 was detected from exosomes isolated from LNCaP and C4-2B cells compared with normal prostate epithelial cells RWPE-1 (Fig. S2).

miR-1275 is Transferred to Osteoblasts Via Exosomes

miRNAs can be transferred from cells to cells via exosomes to execute their function. To address whether miR-1275 could be transferred from PCa cells to osteoblasts via exosomes, we labeled miR-1275-exo and NC-exo with PKH67 (green), followed by incubation with osteoblasts. After 72 h of incubation, PKH67 lipid dye was observed in the osteoblasts (Fig. 2A), indicating a transfer of exosomes to osteoblasts. Treatment with miR-1275-exo resulted in a remarkable increase in miR-1275 expression in osteoblasts, while treatment with NC-exo had no significant effect on miR-1275 level (Fig. 2B). In addition, the expression of miR-1275 in osteoblasts co-cultured with miR-1275-transfected PC3 cells was examined and found that the expression of miR-1275 in the co-cultured osteoblasts was increased (Fig. 2C).

Exosomal miR-1275 Enhances Osteoblast Proliferation and Activity

Next, we determined whether exosomal miR-1275 could affect the proliferation of osteoblasts. CCK-8 assay showed treatment with miR-1275-exo significantly increased the viability of osteoblasts compared with NC-exo (Fig. 3A). Transfection with miR-1275 mimics further extended the enhancement in osteoblast viability induced by miR-1275-exo. On the contrary, treatment with miR-1275 inhibitor blocked the effect of miR-1275-exo (Fig. 3A). In addition, the mRNA and protein levels of osteogenesis-related genes, such as osteocalcin (OCN), type I collagen (COL-1), and osteopontin (OPN) were higher in miR-1275-exo group than in NC-exo group, as characterized by qRT-PCR, Western blot, and immunofluorescence staining (Fig 3B–D).
Treatment with miR-1275 mimics further increased the expression of OCN, COL-1, and OPN induced by miR-1275-exo, whereas transfection with miR-1275 inhibitor abolished the increase induced by miR-1275-exo (Fig 3B–D). Together, these results suggest that exosomal miR-1275 has a positive effect on osteoblast activity.
SIRT2 is a Target of miR-1275

To explore how miR-1275 modulates osteoblast activity, we search the potential downstream targets of miR-1275 by mRNA target-predicting algorithms (TargetScan and miRDB). Among the potential targets, SIRT2 was predicted to be the target of miR-1275 by these three bioinformatic softwares, which has been suggested to be important for the regulation of osteogenic differentiation16, and thus was chosen as the candidate target for further studies (Fig. 4A). To confirm that SIRT2 is a target of miR-1275, luciferase report assay was performed. The activity of wild-type 3′-UTR of SIRT2 was significantly decreased after overexpressing miR-1275, while there was no significant change in the activity of the mutated 3′-UTR of SIRT2 (Fig. 4B). Transfection of miR-1275 markedly repressed the expression of SIRT2 (Fig. 4C). Consistently, treatment with miR-1275-exo also resulted in a downregulation of SIRT2 (Fig. 4D). Overexpression of SIRT2 increased the viability of osteoblasts, whereas knockdown of SIRT2 resulted in the opposite result (Fig. 3). Moreover, overexpression of SIRT2 restrained the proliferation of osteoblasts increased by miR-1275-exo (Fig. 4E, F). Collectively, these results indicated that SIRT2 is a target of miR-1275.

Exosomal miR-1275 Enhances Osteoblast Activity Via SIRT2/RUNX2 Cascade

RUNX2 is a transcription factor that plays an important role in osteoblast activity via modulation of osteoblast-specific genes. We found that RUNX2 could positively regulate OCN expression (Fig. S4), which was consistent with previous studies17,18. We then investigated whether SIRT2 could affect RUNX2 expression. Western blot analysis showed that overexpression of SIRT2 suppressed the protein level of RUNX2, whereas siRNA-mediated silencing of SIRT2 elevated the expression of RUNX2 (Fig. 5A). Moreover, we found that the activity of RUNX2 promoter was decreased after overexpression of SIRT2 (Fig. 5B). In agreement with this result, the mRNA level of RUNX2 was suppressed upon SIRT2 overexpression and increased following SIRT2 knockdown (Fig. 5C). Treatment with miR-1275-exo decreased SIRT2 expression but increased RUNX2 expression and co-treatment with miR-1275 inhibitor abolished these effects (Fig. 5D). Overexpression of SIRT2...
abrogated the elevation in RUNX2 expression induced by miR-1275-exo (Fig. 5E). Furthermore, knockdown of RUNX2 counteracted the increased expression of OCN, as well as the viability of osteoblasts induced by miR-1275-exo (Fig. 5F, G). Taken together, these data indicate that exosomal miR-1275 accelerates osteoblast activity through the regulation of SIRT2/RUNX2 cascade (Fig. 5H).

**Discussion**

Modulation of osteoblast activity is suggested to be crucial in PCa metastasis. In the present study, we found that PC3 cells could transferred miR-1275 to osteoblasts via exosomes. Moreover, we identified that PC3-derived exosomal miR-1275 promoted the proliferation and activity of osteoblasts.

Exosomes play important roles in the occurrence, development, treatment, and prognosis of various diseases. miRNAAs can be transferred from cancer cells to the recipient cells to facilitate tumorigenesis and metastasis. Disruption of the dynamic balance between osteoblasts and osteoclasts could lead to PCa bone metastasis. It has been reported that exosomal miRNA from PCa cells can increase the activity of osteogenesis cells and thus contribute to PCa metastasis, but whether it has an function in osteoblast activity is unknown. Herein, we showed that exosomal miR-1275 derived from PCa significantly enhanced the growth of osteoblasts and increased the expression of osteogenesis-related genes, indicating an increased activity of osteoblasts.

**SIRT2 is a target of miR-1275.** (A) The wild-type (WT) and mutated (Mu) binding sites of miR-1275 in the 3'UTR of SIRT2 are shown. (B) Luciferase report assay was performed in osteoblasts transfected with WT or mutated SIRT2 3'UTR in the presence (or absence) of miR-1275. (C) The effect of miR-1275 on the expression of SIRT2 was detected by Western blot. (D) The effect of exosomal miR-1275 on the expression of SIRT2 was detected by Western blot. (E) Overexpression of SIRT2 reduced osteoblast growth increased by exosomal miR-1275. *P < 0.05, **P < 0.01, n = 3. Data represent the mean ± SD of three separate experiments; comparison was performed with one-way ANOVA followed by Tukey’s post hoc test.
Moreover, it has been reported that SIRT2 is associated with osteogenic activity\textsuperscript{16,26}. Consistently, herein, we showed that SIRT2 is a target of miR-1275 and exosomal miR-1275 increased osteoblast activity by downregulation of SIRT2.

RUNX2 is a key transcription factor controlling the proliferation and activity of osteoblasts\textsuperscript{27}. It has been reported that the deletion of RUNX2 in osteoblasts disrupts postnatal bone formation\textsuperscript{28}. Deficiency of Runx2 resulted in an absence of osteoblasts and bone in mice\textsuperscript{29}. Ectopic expression of Runx2 is effective for transdifferentiation of preadipocytes into fully differentiated bone-forming osteoblasts and enhances osteoblastic activity via regulation of osteoblastic genes\textsuperscript{17,18}. In the present study, we found that RUNX2 was a downstream target of SIRT2 and exosomal miR-1275 upregulation RUNX2 by decreasing SIRT2 expression. Moreover, we confirmed that exosomal miR-1275 modulated osteoblast activity by the modulation SIRT2/RUNX2 cascade.

**Conclusion**

In summary, our data demonstrated that PCa-derived exosomal miR-1275 significantly increased the proliferation and activity of osteoblasts, which was mediated by the regulation of SIRT2/RUNX2 pathway. Therefore, exosomal miR-1275 is an important enhancer of osteoblast activity.

**Abbreviations**

ATCC: American Type Culture Collection  
BCA: Bicinchoninic acid  
CCK-8: Cell Counting Kit-8  
COL-I: Type I collagen  
DMEM: Dulbecco’s modified eagle medium  
FBS: Fetal bovine serum  
OCN: Osteocalcin  
OPN: Osteopontin  
PBS: Phosphate buffer saline  
TBST: Tris buffered saline tween  
TEM: Transmission electron microscope  
RUNX2: Runt-related transcription factor 2

**Authors’ Contributions**

ZHZ designed and performed the experiments, analysed the data and wrote the manuscript. ND and PZJ carried out the experiments. WS and PL performed some of the research. All authors read and approved the final manuscript. ZPZ helped to submit the manuscript.

**Availability of Data and Materials**

For data availability, please contact the corresponding author.

**Declaration of Conflicting Interests**

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

**Ethical Approval**

This study was approved by our institutional review board.

**Statement of Human and Animal Rights**

This article does not contain any studies with human or animal subjects.
Statement of Informed Consent

There are no subject in this article and informed consent is not applicable.

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ORCID iD

Zihao Zou https://orcid.org/0000-0001-6429-2397

Supplemental Material

Supplemental material for this article is available online.

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