Downregulation of miR-19a-3p promotes invasion, migration and bone metastasis via activating TGF-β signaling in prostate cancer

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Abstract. Constitutive activation of TGF-β signaling pathway is a well-documented mechanism responsible for the bone metastasis of prostate cancer (PCa). MicroRNAs (miRNAs) have been reported to be crucial for the activation of TGF-β signaling via targeting downstream components of TGF-β signaling pathway. Here, we report that miR-19a-3p is down-regulated in bone metastatic PCa tissues and cells. Upregulation of miR-19a-3p suppresses invasion, migration *in vitro* and inhibits bone metastasis *in vivo* in PCa cells. Conversely, silencing miR-19a-3p yields the opposite effect. Our results further demonstrate that miR-19a-3p inhibits invasion and migration abilities of PCa cells via targeting downstream effectors of TGF-β signaling, SMAD2 and SMAD4, resulting in the inactivation of TGF-β signaling. Therefore, our results uncover a novel mechanistic understanding of miR-19a-3p-induced suppressive role in bone metastasis of PCa, which will facilitate the development of effective cancer therapy methods against PCa.

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Introduction

Prostate cancer (PCa) is one of the most common cancers with indolent features in men (1). Distant bone metastasis is among the most preferential sites of metastasis in several human tumors, including breast cancer, prostate cancers, lung and kidney cancers (2,3). Therefore, clarifying in depth the molecular mechanisms underlying bone metastasis of PCa will facilitate the development of novel therapeutic avenues in the treatment of PCa.

TGF-β signaling is implicated in several physiological processes, including inhibiting cell proliferation, embryogenesis and bone remodeling (4). In cancers, TGF-β signaling has been identified to function as oncogene or tumor-suppressor dependent on the developmental stage and types of tumor (5,6). Accumulating studies have shown that TGF-β signaling is essential for the invasion and metastasis of tumor cells to bone in various cancers, such as breast cancer and melanoma (7,8). Importantly, TGF-β signaling has been demonstrated to play important roles in the development of bone metastasis of prostate cancer (9,10). Reportedly, therapy targeting TGF-β significantly reduced the metastasis of tumor cells to bone (9-11). Fournier and colleagues reported that SD208, a small-molecule inhibitor of the kinase activity of TGFBR1, significantly decreased the progression of PC-3 osteolytic metastases (12), indicating TGF-β signaling pathway is crucial for the bone metastasis of PCa cells. However, the molecular mechanism contributing to constitutive activation of TGF-β in PCa remains poorly known.

MicroRNAs (miRNAs) are a diverse group of small non-coding RNAs composed of 19-25 nucleotides, which mechanistically function by binding to the 3'-untranslated region (3'-UTR) of downstream mRNAs, leading to mRNA degradation or repression of translation (13,14). A growing body of literature has demonstrated that miRNAs not only play crucial roles in many biological processes including

proliferation, differentiation, cell cycle and apoptosis (13), but also regulate the progression and metastasis in various types of tumors (15-19). Furthermore, several miRNAs have been identified as critical mediators in the bone metastasis of human cancer (14,20-22). Our previous studies demonstrated that loss of wild-type P53 in PC-3 cells resulted in downregulation of miR-145, which further promoted bone metastasis of PCa via regulating several positive regulators of EMT, including ZEB2, and HEF1 (23-25). Therefore, these studies indicate that dysregulation of miRNAs plays a pivotal role in the bone metastasis of PCa.

In this study, we found that miR-19a-3p expression is dramatically decreased in bone metastatic PCa tissues and cells. Moreover, upregulation of miR-19a-3p suppresses, while silencing miR-19a-3p promotes invasion and migration *in vitro*. Importantly, upregulating miR-19a-3p repressed the osteolytic bone lesions *in vivo*. Our results further reveal that upregulating miR-19a-3p inhibits TGF-β signaling via targeting downstream effectors of TGF-β signaling SMAD2 and SMAD4, suppressing invasion and migration of PCa cells. Therefore, our results demonstrate that miR-19a-3p inhibits invasion and migration of PCa cells via directly targeting SMDA2 and SMAD4, indicating that miR-19a-3p play a tumor-suppressive role by suppressing invasion and migration ability in bone metastasis of PCa.

Materials and methods

Cell lines and cell culture. Human RWPE-1, DU145, LNCaP, 22Rv1, PC-3 and VCaP cells were obtained from the American Type Culture Collection (ATCC) and cultured according to the manufacturer's instructions. The C4-2B cell line was purchased from the MD Anderson Cancer Center. RWPE-1 cells were grown in defined keratinocyte-SFM (1X) (Invitrogen). LNCaP, 22Rv1, C4-2B and PC-3 cells were grown in RPMI-1640 medium (Invitrogen) supplemented with 10% FBS (Invitrogen), while DU145 and VCaP cells were grown in Dulbecco's modified Eagle's medium (Invitrogen) supplemented with 10% FBS. All cells were incubated at 37°C in a humidified atmosphere with 5% CO_2 and were routinely sub-cultured using 0.25% (w/v) trypsin-ethylenediamine-tetraacetic acid solution.

Patients and tumor tissues. In total 121 archived PCa tissues, including 76 non-bone metastatic PCa tissues and 45 bone metastatic PCa tissues were obtained during surgery or needle biopsy at The First People's Hospital of Guangzhou City (Guangzhou, China). Patients were diagnosed based on clinical and pathological evidence, and the specimens were immediately snap-frozen and stored in liquid nitrogen. For the use of these clinical materials for research purposes, prior patients' consents and approval from the Institutional Research Ethics Committee were obtained. The clinicopathological features of the patients are summarized in Table I. The median age (74-years), serum PSA level (76.5 μg/ml) and Gleason grade (7) in all 121 PCa tissues was used as the cut-off value for age, serum PSA level and Gleason grade, respectively. The median of miR-19a-3p expression in all 121 PCa tissues was used to stratify high and low expression of miR-19a-3p.

RNA extraction, reverse transcription, and real-time PCR. Total RNA from tissues or cells were extracted using TRIzol (Life Technologies) according to the manufacturer's instructions. Messenger RNA (mRNA) and miRNA were reverse transcribed of total mRNA using the Revert Aid First Strand cDNA Synthesis kit (Thermo, USA) according to the manufacturer's protocol. Complementary DNA (cDNA) was amplified and quantified on ABI 7500HT system (Applied Biosystems, Foster City, CA, USA) using SYBR Green I (Applied Biosystems). The primers used in the reactions are listed in Table II. Real-time PCR was performed according to a standard method, as described previously (26). Primers for U6 and miR-19a-3p (miRQ0000073-1-2) were synthesized and purified by RiboBio (website for miR-19a-3p primer: http://www.ribobio.com/sitecn/product_info.aspx?id=200681) (Guangzhou, China). U6 or glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as endogenous controls for miRNA or mRNA respective. Relative fold expressions were calculated with the comparative threshold cycle ($2^{-\Delta\Delta Ct}$) method.

Plasmid, small interfering RNA and transfection. The human miR-19a-3p expression plasmid was generated by cloning the genomic pre-miR-19a-3p gene into retroviral transfer plasmid pMSCV-puro (Clontech Laboratories Inc., Tokyo, Japan) to generate plasmid pMSCV-miR-19a-3p. pMSCV-miR-19a-3p was cotransfected with the pIK packaging plasmid into 293FT cells using the standard calcium phosphate transfection method, as previously described (18). Thirty-six hours after the co-transfection, supernatants were collected and incubated with cells to be infected for 24 h in the presence of polybrene (2.5 μg/ml). After infection, puromycin (1.5 μg/ml) was used to select stably transduced cells over a 10-day period. The (CAGAC) 12/pGL3 TGF-β/Smad-responsive luciferase reporter plasmid and control plasmids (Clontech) were used to examine the transcriptional activity of TGF-β signaling quantitatively. The 3'-untranslated region (3'-UTR) of the human SMAD2 and SMAD4 were PCR-amplified from genomic DNA and cloned into pmirGLO luciferase reporter vector (Promega, Madison, WI, USA). The miArrest plasmids for anti-miR-19a-3p and negative control plasmids were constructed and cloned into pH1 plasmids by GeneChem (Shanghai, China). Anti-miR-19a-3p, small interfering RNA (siRNA) for SMAD2 (sc-37238) and SMAD4 (sc-29484) knockdown (50 nmol/l) were obtained from Santa Cruz (Dallas, TX, USA). The sequence of anti-miR-19a-3p is TCAG TTTTGCATAGATTTGCACA. Transfection of siRNAs and plasmids was performed using Lipofectamine 3000 (Life Technologies) according to the manufacturer's instructions.

Invasion and migration assays. Migration and invasion were assayed using Transwell chamber consisting of 8-μm membrane filter inserts (Corning) coated with or without Matrigel (BD Biosciences) as described previously (27). Briefly, the cells were trypsinized and suspended in serum-free medium. Then 1.5x10^5 cells were added to the upper chamber, and lower chamber was filled with the culture medium with 10% FBS. After incubated for 24-48 h, cells passed through the coated membrane to the lower surface, in which cells were fixed with

Table I. The relationship between miR-19a-3p expression and clinicopathological characteristics in 121 patients with prostate cancer.

Parameters	No. of cases	miR-19a-3p expression Low	miR-19a-3p expression High	P-value
Age (years)				
≤74	54	28	26	0.081
>74	67	33	34	
Differentiation				
Well/moderate	53	20	33	0.014[a]
Poor	68	41	27	
Serum PSA				
<76.5	56	22	34	
>76.5	65	39	26	0.023[a]
Gleason grade				
≤7	62	19	43	
>7	59	42	17	<0.001[a]
Operation				
TURP	42	22	20	
Needle biopsy	43	23	20	0.932
TURP+PP	7	3	4	
TURP+BO	19	11	8	
BO	10	6	4	
BM-status				
nBM	76	31	45	
BM	45	30	15	0.006[a]

PSA, prostate-specific antigen; TURP, transurethral resection prostate; PP, prior prostatectomy; BO, bilateral orchiectomies; SD, standard deviation; BM, bone metastasis.

Table II. The list of primers used in the reactions for real-time RT-PCR.

Real-time PCR primer	
SMAD2-up	CACGCTAGGAAAACAGCCTC
SMAD2-dn	TCGGAAGAGGAAGGAACAAA
SMAD4-up	TTGATCCTTTGGAAACAGTGAA
SMAD4-dn	GCCTTCCCACTCCCCTC
CTGF-up	GCTACCACATTTCCTACCTAGAAATCA
CTGF-dn	GACAGTCCGTCAAAACAGATTGTT
PTHRP-up	ACTCGCTCTGCCTGGTTAGA
PTHRP-dn	GGAGGTGTCAGACAGGTGGT
IL11-up	TGAAGACTCGGCTGTGACC
IL11-dn	CCTCACGGAAGGACTGTCTC
GAPDH-up	ATTCCACCCATGGCAAATTC
GAPDH-dn	TGGGATTTCCATTGATGACAAG

Luciferase assay. Cells ($4x10^4$) were seeded in triplicate in 24-well plates and cultured for 24 h. Cells were transfected with 250 ng (CAGAC) 12/pGL3 reporter luciferase plasmid, or 100 ng pmirGLO-PICK1-3'-UTR, luciferase plasmid, plus 5 ng pRL-TK *Renilla* plasmid (Promega) using Lipofectamine 3000 (Invitrogen) according to the manufacturer's instructions. Luciferase and *Renilla* signals were measured 36 h after transfection using a Dual Luciferase Reporter assay kit (Promega) according to the manufacturer's protocol.

RNA immunoprecipitation. Cells ($5x10^5$) were plated in 60-mm cell culture dishes, proliferating to 60-80% confluence after 24 h of culture, and the pIRESneo-FLAG/HA-Ago2 plasmid (10822; Addgene, Cambridge, MA, USA) were cotransfected into cells using Lipofectamine 3000, as previously described (30). After 48-h transfection, cells were washed and lysed in radioimmunoprecipitation buffer (Sigma-Aldrich) containing 10% proteinase inhibitor cocktail (Sigma-Aldrich) and 1 mM phenylmethylsulfonyl fluoride (Sigma-Aldrich). A fraction of the whole cell lysate was used for RNA isolation, and the remaining lysate was subjected to immunoprecipitation (IP) using an antibody against Ago2 (Abcam) or immunoglobulin G (IgG) (Abcam). RNA from whole cell lysates and RNA IP (RIP) fractions was extracted with TRIzol (Life Technologies) according to the manufacturer's instructions. The relative levels of mRNA were determined using real-time RT-PCR as described above. The relative mRNA enrichment in the RIP fractions was computed based on the ratio of relative mRNA levels in the RIP fractions and the relative mRNA levels in the whole cell lysates.

Western blotting. The proteins extracted from the cell lysates were loaded with 50 μg in each lane, which was further separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to polyvinylidene fluoride membranes (Millipore, Billerica, MA, USA). Western blotting was performed according to a standard method, as described previously (31). The membranes were probed with

4% paraformaldehyde and stained with hematoxylin. The cell count was done under a microscope (x100).

In vivo model of PCa bone metastasis. We used an intratibial injection model to detect whether upregulation of miR-19a-3p could reduce the bone metastatic capacity of PCa cells. Six male severe combined immunodeficient (SCID) mice at 3-4 weeks old were purchased from HFK Bio-Technology (Beijing, China). Each mouse was injected with stably selected PC-3/miR-19a-3p cells into its right tibia and with PC-3/vector cells into its left tibia as a matching control. The inoculation procedure was performed as previously described (28). Bone lesions were analyzed by using the following scoring standard based on X-ray examination (29): 0 (no lesion); 1 (minor lesions); 2 (small lesions); 3 (significant lesions with minor breaks in their margins); 4 (significant lesions with major breaks in peripheral lesions). The ethics approval statements for animal work were provided by The Institutional Animal Care and Use Committee of Sun Yat-Sen University Cancer Center. The ethics approval number for animal work was L102012016110D.
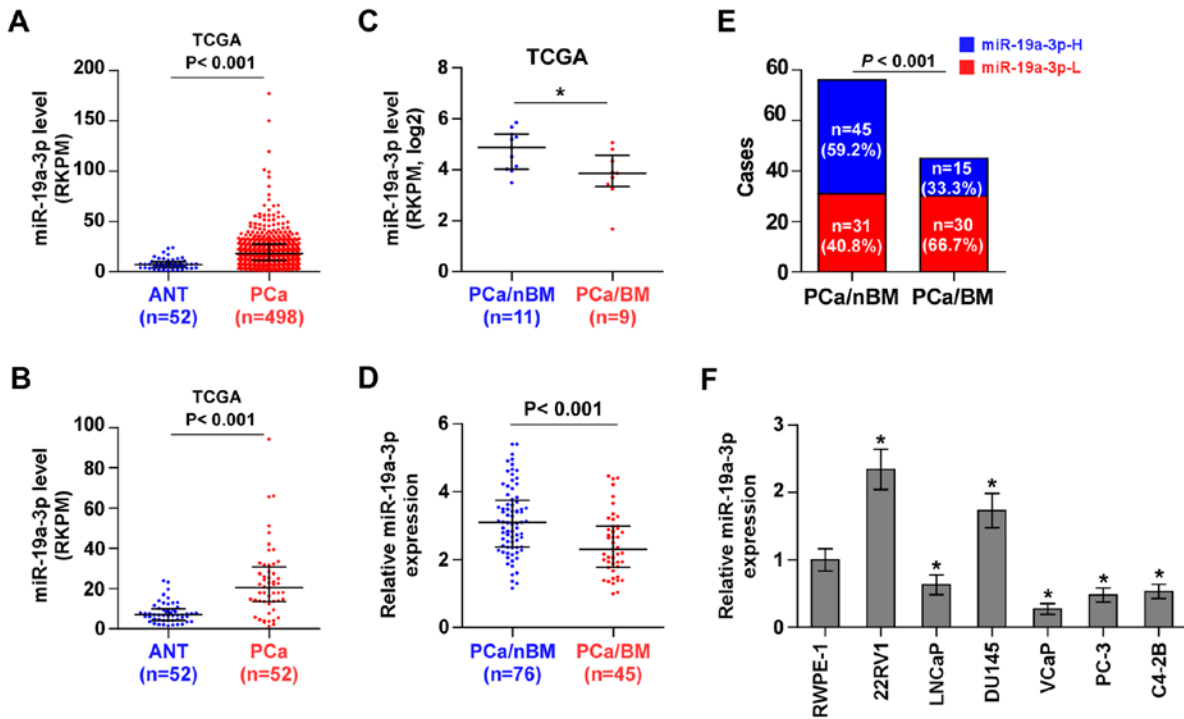
Figure 1. miR-19a-3p is downregulated in bone metastatic PCa tissues and cell lines. (A) miR-19a-3p expression level in 498 PCa tissues and 52 adjacent normal tissues (ANT) in the PCa datasets from TCGA. (B) miR-19a-3p expression level in 52 paired PCa tissues and the matched adjacent normal tissues (ANT) in the PCa datasets from TCGA. (C) miR-19a-3p expression level in bone metastatic PCa tissues (PCa/BM) was downregulated compared with non-bone metastatic PCa tissues (PCa/nBM) in the PCa datasets from TCGA. *P<0.05. (D) Expression of miR-19a-3p was downregulated in 7 bone metastatic PCa tissues compared with 18 non-bone metastatic PCa tissues. (E) Percentages and number of samples showing high or low miR-19a-3p expression in our PCa patients with different bone metastasis status. (F) Real-time PCR analysis of miR-19a-3p expression in normal prostate epithelial cell RWPE-1 and 6 PCa cell lines. U6 was used as endogenous controls. Each bar represents the mean values ± SD of three independent experiments. *P<0.05.

antibodies against SMAD2 (no. 5339; dilution, 1:1,000), SMAD4 (no. 38454; dilution, 1:1,000), pSMAD2/3 (no. 9510; diilution, 1:1,000), SMAD2/3 (no. 8685; dilution, 1:1,000) (Cell Signaling Technology, Beverly, MA, USA) and p84 (no. PA5-27816; dilution: 1:1,000) from Invitrogen overnight at 4˚C, and then incubated with horseradish peroxidase-conjugated secondary antibodies (Cell Signaling Technology) for 1 h at room temperature. Immune complexes were detected by enhanced chemiluminescence (Cell Signaling Technology). α-tubulin (Cell Signaling Technology) was used to correct for differences in protein loading from the control and experimental groups.

Statistical analysis. All values are presented as means ± standard deviation (SD). Significant differences were determined using SPSS 19.0 software (SPSS, Chicago, IL, USA). A paired Student's t-test was used to analyze the paired control group (vector or NC) and treatment group (miR-19a-3p or anti-miR-19a-3p) *in vitro* experiment. One-way ANOVA was used to determine statistical differences between multiple testing. An independent Student's t-test was used to analyze the paired control group (vector) and treatment group (miR-19a-3p) *in vivo* experiment. P<0.05 was considered statistically significant. All the experiments were repeated three times.

Results

miR-19a-3p is downregulated in bone metastatic PCa tissues and cell lines. To screen the aberrant miRNA expression

between bone metastatic PCa tissues and non-bone metastatic PCa tissues, we first analyzed the miRNA sequencing dataset of PCa from The Cancer Genome Atlas (TCGA) and found that miR-19a-3p expression was dramatically overexpressed in 498 PCa tissues compared with 52 ANT (Fig. 1A). Consistently, miR-19a-3p expression was overexpressed in 52 paired PCa tissues compared with the matched ANT (Fig. 1B). Interestingly, we found that miR-19a-3p expression was downregulated in bone metastatic PCa tissues compared with non-bone metastatic PCa tissues (Fig. 1C). To validate the miR-19a-3p expression in PCa tissues, real-time PCR was performed on 76 non-bone metastatic PCa tissues and 45 bone metastatic PCa tissues. As shown in Fig. 1D, miR-19a-3p expression was downregulated in bone metastatic PCa tissues compared with non-bone metastatic PCa tissues. The percentage of low miR-19a-3p expression was higher in PCa tissues with bone metastasis compared to PCa tissues without bone metastasis (Fig. 1E). We further examined miR-19a-3p expression in normal prostate cells (RWPE-1) and 6 PCa cell lines. Consistent with miR-19a-3p expression in PCa tissues, miR-19a-3p expression was elevated in primary PCa cells 22RV1 and brain metastatic PCa DU145 cells, but was differentially decreased in bone metastatic PCa cell lines (PC-3, C4-2B and VCaP) and lymph node metastatic cell line LNCaP (Fig. 1F). Statistical analysis of PCa tissue samples revealed that miR-19a-3p expression inversely correlated with differentiation, serum PSA levels, Gleason grade and bone metastasis status in PCa (Table I). Therefore, the published miRNA datasets and our results indicated that downregulation of miR-19a-3p may be implicated in bone metastasis of PCa.
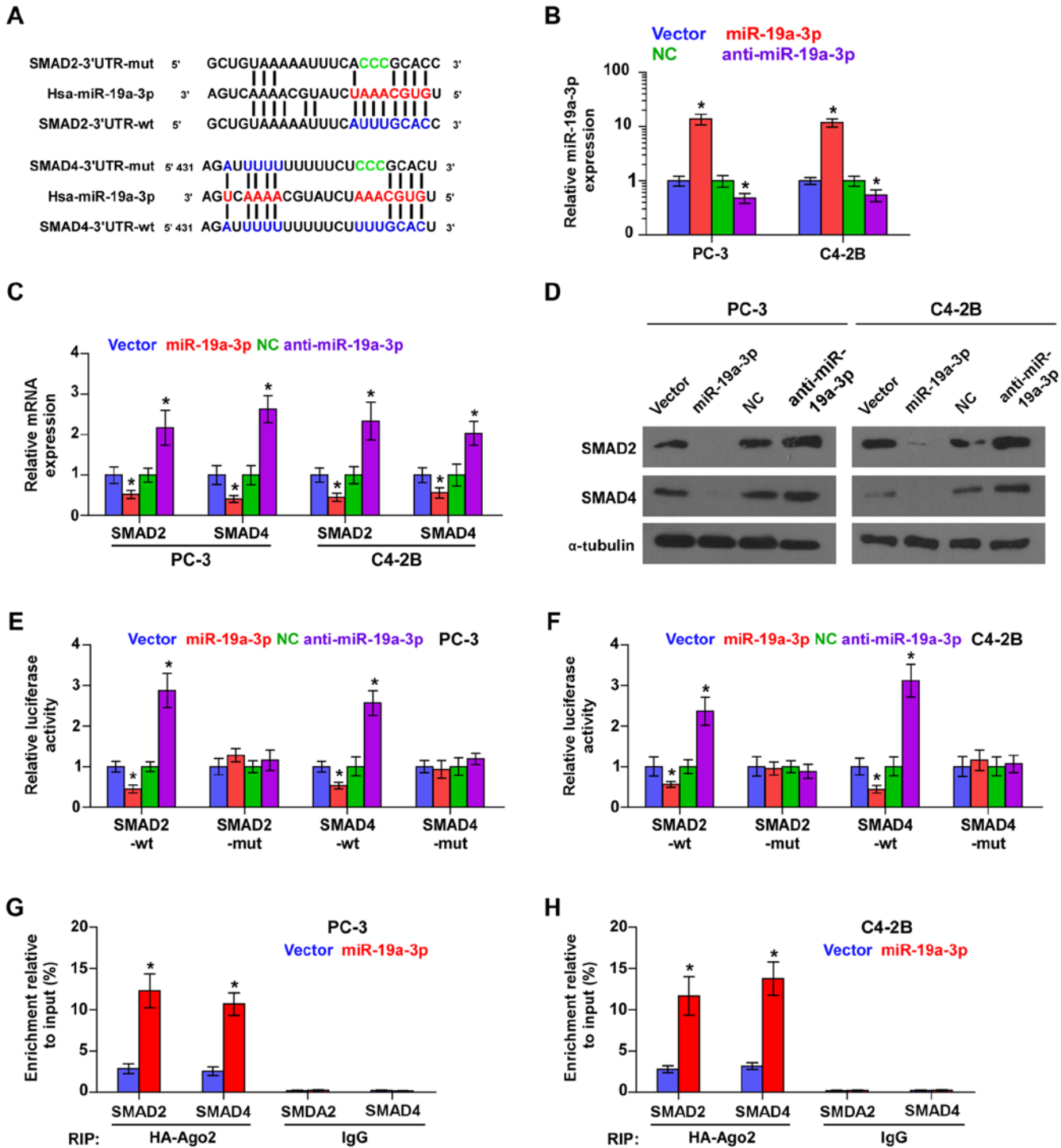
Figure 2. SAMD2 and SMAD4 are direct targets of miR-19a-3p in PCa cells. (A) Predicted miR-19a-3p targeting sequence and mutant sequences in 3'-UTRs of SMAD2 and SMAD4. (B) Real-time PCR analysis of miR-19a-3p expression in the indicated cells. Transcript levels were normalized by *U6* expression. *P<0.05. (C) Real-time PCR analysis of SMAD2 and SMAD4 expression in the indicated cells. Error bars represent the mean ± SD of three independent experiments. *P<0.05. (D) Western blot analysis of SMAD2 and SAMD4 expression in the indicated cells. α-tubulin served as the loading control. (E and F) Luciferase assay of the cells transfected with pmirGLO-3'-UTR reporter of SMAD2 and SMAD4 in the indicated cells. Error bars represent the mean ± SD of three independent experiments. *P<0.05. (G and H) miRNP IP assay showing the association between miR-19a-3p and SMAD2 and SAMD4 transcripts in PCa cells. Pulldown of IgG antibody served as the negative control. Error bars represent the mean ± SD of three independent experiments. *P<0.05.

miR-19a-3p targets effectors of TGF-β signaling SMAD2 and SMAD4. Using the publicly available algorithms TargetScan and miRanda, we found that SMAD2 and SMAD4 are potential targets of miR-19a-3p (Fig. 2A), both of which are critical downstream effectors of TGF-β signaling (32). Then, we further exogenously overexpressed miR-19a-3p

and endogenously silenced miR-19a-3p via virus transduction in PCa cells (Fig. 2B). Real time-PCR and western blot analysis showed that miR-19a-3p overexpression reduced, while silencing miR-19a-3p enhanced the mRNA and protein expression levels of SMAD2 and SMAD4 (Fig. 2C and D). To examine whether miR-19a-3p-mediated SMAD2 and SMAD4
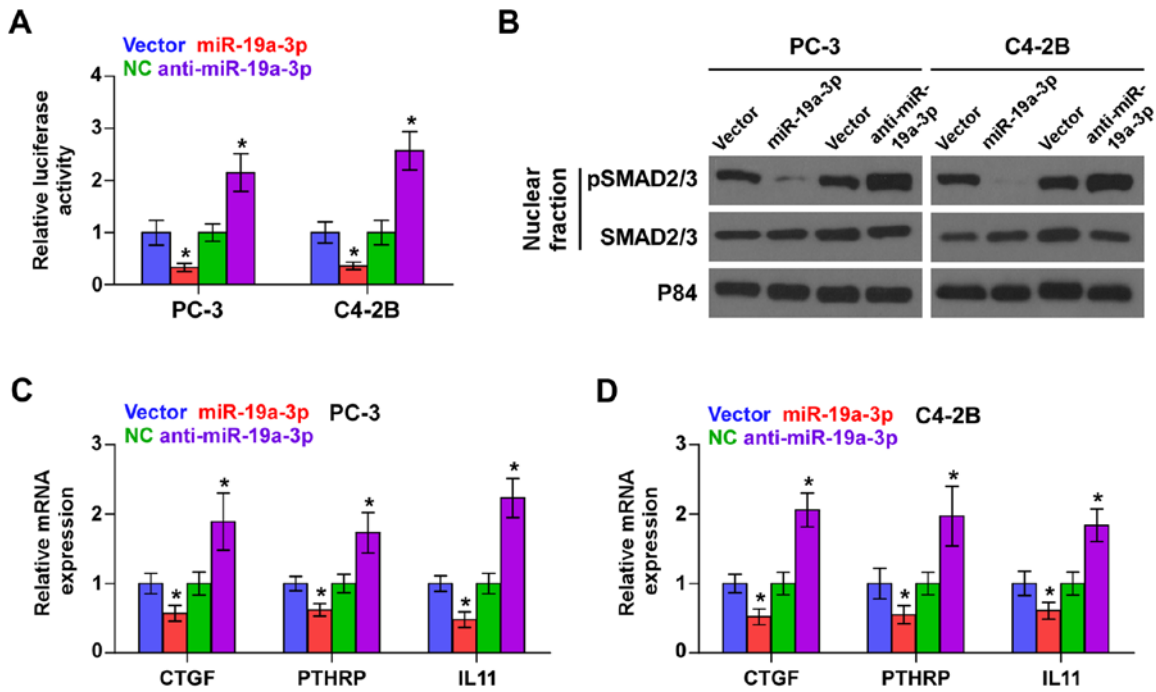
Figure 3. Downregulation of miR-19a-3p activates TGF-β signaling in PCa cells. (A) Transcriptional activity based on a TGF-β/Smad-responsive luciferase reporter as assessed in the indicated cells. Error bars represent the mean ± SD of three independent experiments. *P<0.05. (B) Western blot analysis showing that upregulation of miR-19a-3p decreased, while downregulation of miR-19a-3p increased nuclear translocation of pSMAD2/3 in PCa cells. The nuclear protein p84 was used as a nuclear protein marker. (C and D) Real-time PCR analysis of downstream bone metastasis-related genes of the TGF-β pathway, including CTGF, PTHRP and IL-11 in the indicated cells. Transcript levels were normalized to GAPDH expression. Error bars represent the mean ± SD of three independent experiments. *P<0.05.

downregulation occurs through miR-19a-3p-binding in the 3'-UTR of SMAD2 and SMAD4, the 3'-UTR of SMAD2 and SMAD4 were cloned into pmirGLO luciferase reporter vectors. As shown in Fig. 2E and F, miR-19a-3p overexpression decreased, whereas anti-miR-19a-3p increased, the luciferase reporter activity of SMAD2 and SMAD4, but not by the mutant 3'-UTR of SMAD2 and SMAD4 within miR-19a-3p-binding seed regions in PCa cells. Moreover, microribonucleoprotein (miRNP) immunoprecipitation (IP) assay showed a direct association of miR-19a-3p with SMAD2 and SMAD4 transcripts (Fig. 2G and G), further elucidating the direct repressive effects of miR-19a-3p on SMAD2 and SMAD4. Taken together, these results indicated that SMAD2 and SMAD4 are authentic targets of miR-19a-3p in PCa cells.

Downregulation of miR-19a-3p activates TGF-β signaling in PCa cells. As SMAD2 and SMAD4 are critical effectors of TGF-β signaling, we further investigated the effect of miR-19a-3p on TGF-β signaling activity and found that miR-19a-3p overexpression reduced, while silencing miR-19a-3p enhanced the transcriptional activity of a TGF-β/Smad-responsive luciferase reporter plasmid known as CAGA12 composed of 12 tandem copies of a Smad/DNA binding element CAGAC sequence in PCa cells (Fig. 3A). Cellular fractionation and western blot analysis revealed that upregulation of miR-19a-3p decreased, while miR-19a-3p silencing increased pSMAD2/3 nuclear translocation in PCa cells (Fig. 3B). Real-time PCR analysis showed that upregulating miR-19a-3p decreased expression levels of multiple TGF-β signaling downstream bone metastasis-related target genes including CTGF, PTHRP and IL11 in PCa cells.

Conversely, silencing miR-19a-3p increased expression of these downstream genes in PCa cells (Fig. 3C and D). Thus, these results demonstrate that downregulation of miR-19a-3p activates TGF-β signaling pathway in PCa cells.

Downregulation of miR-19a-3p promotes invasion and migration via activating TGF-β signaling pathway in PCa cells. We examined whether miR-19a-3p is involved in the invasion and migration PCa cells. Transwell-Matrigel invasion assay was used to assess the invasive ability of PCa cells and the result indicated that miR-19a-3p overexpression dramatically decreased, while silencing miR-19a-3p increased, the invasive ability of PC-3 and C4-2B cells (Fig. 4A). Furthermore, migration assay revealed that upregulating miR-19a-3p decreased, while silencing miR-19a-3p increased the migration capability of PCa cells (Fig. 4B). These results indicated that miR-19a-3p inhibits invasion and migration ability of PCa cells *in vitro*.

We further explored the functional significance of TGF-β signaling in the stimulatory role of miR-19a-3p downregulation in PCa cells using TGF-β signaling inhibitor SD208. As shown in Fig. 4C, SD208 abrogated the TGF-β signaling activity enhanced by miR-19a-3p downregulation in PCa cells. Furthermore, the stimulatory effects of miR-19a-3p on invasion and migration of PCa cells were impaired by SD208 respectively (Fig. 4D and E). Therefore, these results imply that downregulation of miR-19a-3p promotes invasion and migration via activating TGF-β signaling pathway in PCa cells.

Upregulation of miR-19a-3p inhibits bone lesions of PC-3 cells in vivo. To demonstrate further the effect of miR-19a-3p on the development of PCa bone metastasis *in vivo*, an intratibial
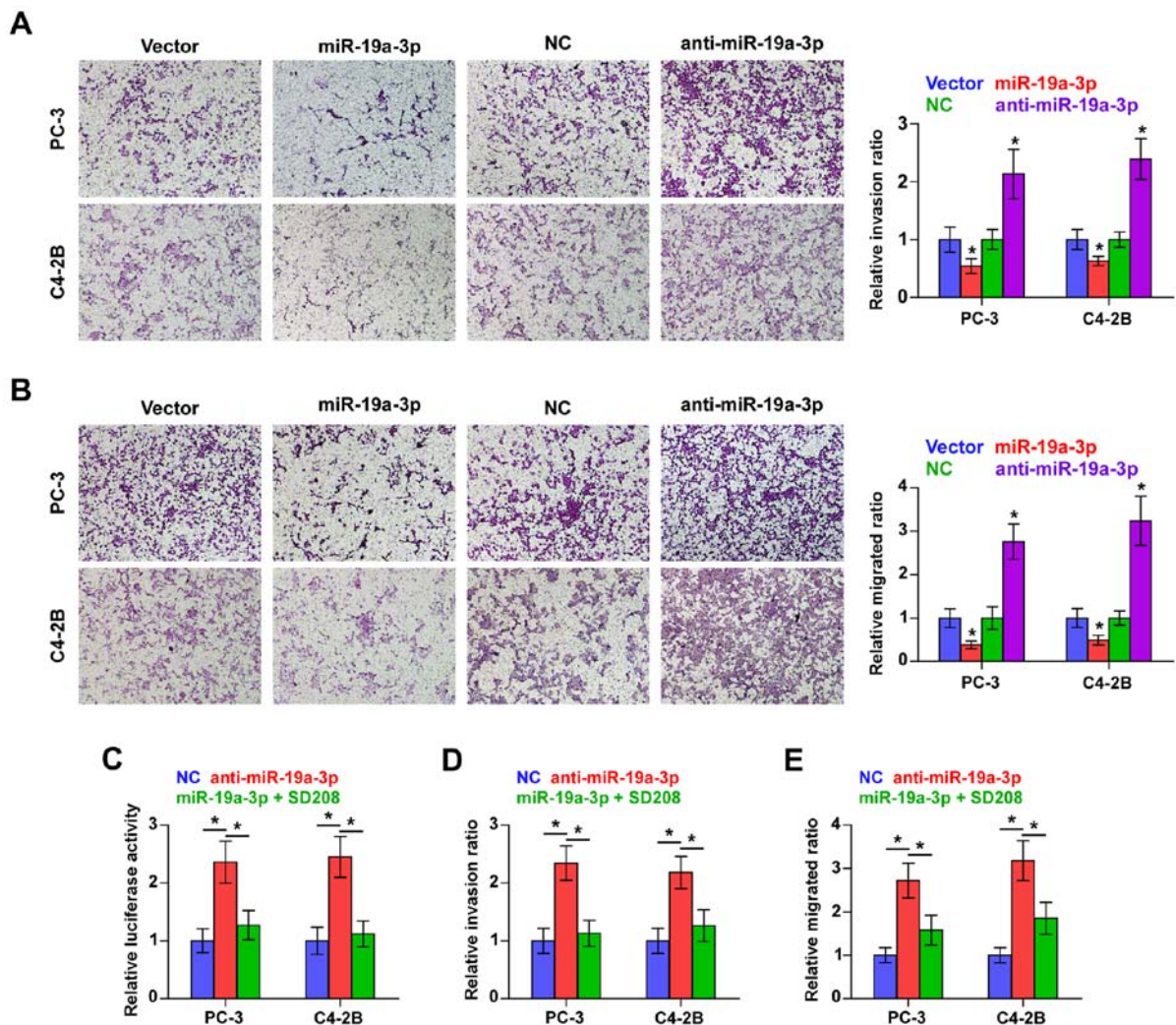
Figure 4. miR-19a-3p inhibits the invasion and migration of PCa cells *in vitro*. (A) Overexpression of miR-19a-3p suppressed, while miR-19a-3p silencing increased the invasive ability of PC-3 and C4-2B cells. Each bar represents the mean values ± SD of three independent experiments. *P<0.05. (B) Overexpression of miR-19a-3p suppressed, while miR-19a-3p silencing increased the migration ability of PC-3 and C4-2B cells. Each bar represents the mean values ± SD of three independent experiments. *P<0.05. (C) TGF-β signaling inhibitors SD208 attenuated the stimulatory effect of miR-19a-3p downregulation on TGF-β transcriptional activity in the indicated cells, respectively. Error bars represent the mean ± SD of three independent experiments. *P<0.05. (D and E) TGF-β signaling inhibitors SD208 attenuated the stimulatory effect of miR-19a-3p silencing on invasion and migration ability in the indicated cells, respectively. Error bars represent the mean ± SD of three independent experiments. *P<0.05.

injection mouse model was established. As shown in Fig. 5A, the skeletal lesions of the animals in the left tibia injected with PC-3/vector cells were significantly larger than those in the right tibia injected with PC-3/miR-19a-3p cells, indicating that upregulating miR-19a-3p inhibited the bone invasive abilities of PC-3 cells *in vivo*. Histological analysis of H&E staining showed that the extent and areas of skeletal lesions observed in the scoring of X-rays were significantly smaller tumors and less bone invasion in mice injected with PC-3/miR-19a-3p cells compared with PC-3/vector cells (Fig. 5B and C). These observations indicated that the upregulation of miR-19a-3p represses osteolytic bone lesions of PCa cells in bone.

SMAD2 and SMAD4 mediate the stimulatory effects of anti-miR-19a-3p on TGF-β signaling activity, invasion and migration in PCa cells. To further investigate whether SMAD2 and SMAD4 contribute to the TGF-β signaling activity, invasion and migration abilities induced by miR-19a-3p downregulation in PCa cells, we used RNA interference to knock down the

SMAD2 and SMAD4 in miR-19a-3p-downregulation in PCa cells. As shown in Fig. 6, individual silencing SMDA2 and SMAD4 significantly attenuated the stimulatory effects of anti-miR-19a-3p on TGF-β signaling activity, invasion and migration abilities in PCa cells. These results indicated that SMAD2 and SMAD4 mediate the stimulatory effects of anti-miR-19a-3p on TGF-β signaling activity, invasion and migration in PCa cells.

Discussion

In the present study, our results indicate that miR-19a-3p expression is markedly downregulated in bone metastatic PCa tissues and cells, which is consistent with the results of publicly available PCa datasets. Furthermore, upregulating miR-19a-3p suppresses, while downregulating miR-19a-3p enhances invasion and migration *in vitro*. Importantly, upregulating miR-19a-3p represses the osteolytic bone lesions *in vivo*. Our results further demonsrate that overexpression of miR-19a-3p inhibits TGF-β signaling via targeting downstream effectors
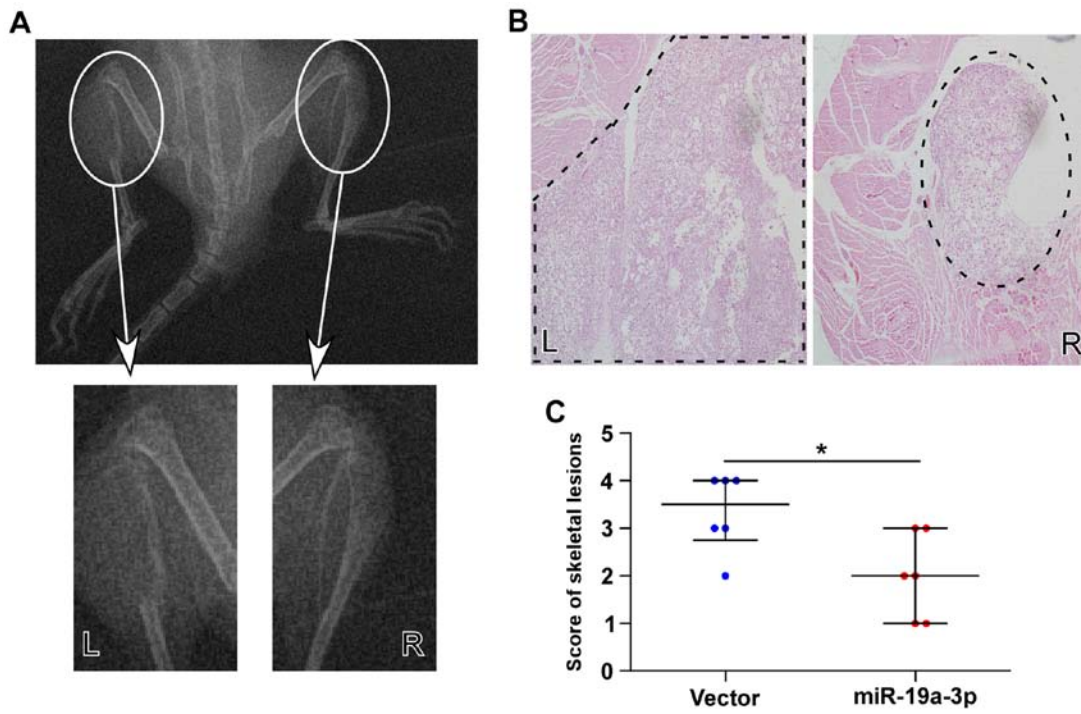
Figure 5. miR-19a-3p inhibits the osteolytic bone lesions of PC-3 cells *in vivo*. (A) The skeletal lesions of the animals in the left tibia injected with PC-3/vector cells were significantly larger than those in the right tibia injected with PC-3/miR-19a-3p cells (n=6). (B) Representative images of sections sliced from the indicated tumors and stained with H&E staining. (C) The extents and areas of skeletal lesions were assessed by X-ray scores. *P<0.05.
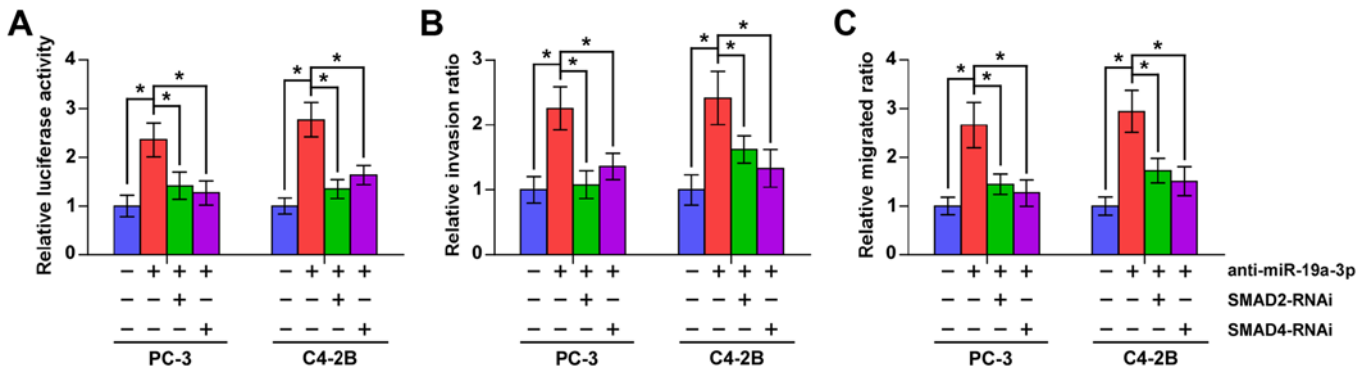

Figure 6. Downregulation of SMAD2 and SMAD4 reverse the miR-19a-3p-downregulation-induced invasion and migration of PCa cells. (A) The stimulatory effects of silencing miR-19a-3p on the transcriptional activity of a TGF-β/Smad-responsive luciferase reporter were abrogated by individual downregulation of SMAD2 and SMAD4. Error bars represent the mean ± SD of three independent experiments. *P<0.05. (B and C) The stimulatory effects of silencing miR-19a-3p on the invasion and migration abilities of PCa cells were abrogated by individual downregulation of SMAD2 and SMAD4. Error bars represent the mean ± SD of three independent experiments. *P<0.05.

of TGF-β signaling SMAD2 and SMAD4, which further suppresses invasion and migration of PCa cells. In addition, the stimulatory effects of downregulating miR-19a-3p on the TGF-β signaling activity, invasion and migration abilities in PCa cells are reversed by individual silencing of SMAD2 and SMAD4. Therefore, these results present our improved understanding of miR-19a-3p-induced tumor suppressive role in bone metastasis of PCa.

Accumulating studies indicate that constitutive activation of TGF-β signaling is reported in bone metastasis of several human cancers. Kang and colleagues reported that Smad4 was essential for the induction of IL-11, a gene implicated in bone metastasis in this mouse model system, which further contributed to the formation of osteolytic bone metastases

in breast cancer (33). Moreover, a study by Javelaud *et al* (7) revealed that TGF-β promoted osteolytic bone metastases in melanoma cells by stimulating the expression of prometastatic factors via the Smad pathway. In PCa, Fournier and colleagues reported that upregulation of the negative regulator of the TGF-β pathway PMEPA1 by TGF-β signaling inhibited bone metastasis of PCa. Importantly, breaking the negative feed-back loop by PMEPA1 silencing promoted bone metastases *in vivo* (12). These studies demonstrate that TGF-β signaling plays an important role in bone metastasis of cancer, including PCa. In the present study, we found that ectopic expression of miR-19a-3p expression suppressed activation of TGF-β signaling via directly repressing SMAD2 and SMAD4 expression, which further inhibited the invasion and migration *in*

vitro and bone metastasis *in vivo* in PCa cells. Therefore, our results present a novel mechanism responsible for the inhibitory effects of miR-19a-3p on the invasion, migration and bone metastasis in PCa cells.

miR-19a-3p has been reported to be upregulated in multiple human cancers, including cutaneous squamous cell carcinoma, colorectal cancer myeloma, follicular lymphoma, gastric cancer and astrocytoma, which contributed to cancer cell proliferation and metastasis via various mechanisms (34-39). However, the expression of miR-19a-3p has also been reported to be downregulated in breast cancer and non-melanoma skin cancer (40-42). These findings suggested that miR-19a-3p functions as an oncomir or tumor suppressive miRNA depending on the tumor type. However, the expression level and biological role of miR-19a-3p in bone metastasis of PCa remain largely unknown. In this study, we found that miR-19a-3p expression was downregulated in bone metastatic PCa tissues and cells compared with non-bone metastatic PCa tissues and cell lines. Upregulating miR-19a-3p suppressed the invasion, migration and osteolytic capability of PCa cells via targeting downstream effectors of TGF-β signaling, SMAD2 and SMDA4, leading to inactivation of TGF-β signaling. Moreover, the stimulatory effects of miR-19a-3p silencing on the invasion and migration in PCa cells were attenuated by individual silencing of SMAD2 and SMAD4. Therefore, our findings demonstrate that downregulation of miR-19a-3p promotes invasion, migration and bone metastasis of PCa cells via activating TGF-β signaling pathway. Furthermore, several studies have shown that miR-19a-3p was identified in the serum of cancer patients, including colorectal cancer and astrocytoma (35,39,43), suggesting that miR-19a-3p may serve as a serum marker for the diagnosis of cancer. However, whether miR-19a-3p may be used as a serum marker for the diagnosis of PCa bone metastasis needs to be further validated of in a larger series of studies.

In conclusion, our results demonstrate that tumor-suppressive miR-19a-3p inhibits the invasion, migration and osteolytic capability of PCa cells via targeting SMAD2 and SMDA4, leading to inactivation of TGF-β signaling. Thus, improved understanding of the specific role of downregulation of miR-19a-3p in the bone metastasis of PCa will increase our knowledge of the development of PCa bone metastasis, which will help to develop new therapeutic strategies against PCa.

Acknowledgements

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