Prostate cancer cell-derived spondin 2 boosts osteogenic factor levels in osteoblasts via the PI3K/AKT/mTOR pathway

HONGBO WANG, MING ZHANG, WENCHENG LU and CHAO YUAN

Department of Spinal Surgery, Zaozhuang Municipal Hospital, Zaozhuang, Shandong 277101, P.R. China

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Abstract. Prostate cancer is the leading cause of cancer death among men worldwide. Bone metastasis is one of the main problems arising from prostate cancer. Spondin 2 is a diagnostic marker specific for prostate cancer; however, the role of spondin 2 in prostate cancer-driven osteogenesis remains unclear. The present study was carried out to explore the role of spondin 2 on prostate cancer cell-induced osteogenesis. In the present study, the expression of spondin 2 was analyzed in prostate cancer samples obtained from Gene Expression Omnibus. The supernatant of prostate cancer cells was used to treat the osteoblast precursor MC3T3-E1 cell line to determine the effect of spondin 2 on osteoblasts. The effect of spondin 2 on osteogenic factor production was also examined after neutralization with a spondin 2 antibody *in vitro* via reverse transcription-quantitative PCR. Furthermore, the effect of spondin 2 on the PI3K/AKT/mTOR pathway was assessed using a patient dataset from The Cancer Genome Atlas and *in vitro* via western blot analysis. In addition, an inhibitor of spondin 2 receptor (ATN-161) was used to explore the inhibition effect of spondin 2 receptor in MC3T3-E1 cells. The results showed that spondin 2 promoted *Osterix* and *Runx2* expression in osteoblasts, and this process was tightly associated with the activation of the PI3K/AKT/mTOR pathway. Moreover, it was demonstrated that the function of spondin 2 on prostate cancer-driven osteogenesis at least partly relied on the integrin receptor α5β1. These results demonstrated that spondin 2 boosts osteogenesis via the PI3K/AKT/mTOR pathway under conditions of prostate tumor progression.

Introduction

Prostate cancer (PCa) is the most frequently diagnosed malignant tumor and a major cause of cancer mortality (6.8%) in men worldwide (1). A main problem arising from PCa is bone tumor metastasis. A total of ~80% of patients with advanced PCa develop bone metastases and are treated with androgen deprivation therapy (2). Androgen receptor (AR) is a leading factor for the development of bone metastasis, and recent advances in therapeutic options for PCa highlight the necessity to block AR signaling (3). However, the role of AR in osteogenesis in PCa remains controversial and unclear. Therefore, for future therapeutic developments, it is essential to determine the underlying mechanism of PCa-driven osteogenesis.

Tumor-induced osteogenesis is a complex process that involves cell disengagement from the microenvironment *in situ*, degradation of the surrounding extracellular matrix, tumor cell dissemination and final proliferation of distant secondary bone tumors (4). Emerging evidence suggests that cytokines involved in the process mentioned above can also act as chemoattractants on pre-osteoblastic MC3T3-E1 cells and promote the secretion of osteogenic factors (5). Almost all osteogenic factors are activated via two important osteogenic transcription factors, which are runt-related transcription factor 2 (*Runx2*) and osteoblast-specific transcription factor *Osterix* (6-8). However, the upstream factors and signaling pathways regulating these two osteogenic factors are still poorly understood.

As a member of the F-spondin family of secreted extracellular matrix proteins, spondin 2 is encoded by the *SPON2* gene (9). Initially, spondin 2 was reported as a diagnostic marker specific for PCa (10,11). However, previous studies have shown that spondin 2 is overexpressed in the serum or tissue samples of malignant tumors, such as colorectal cancer and hepatocellular carcinoma (12,13). High levels of spondin 2 in colorectal cancer cells have been indicated to increase cell motility, thereby resulting in colorectal cancer metastasis in mice (14). Integrins are transmembrane heterodimers with α and β subunits that are considered to be major candidates as receptors for spondin 2 (15). Yang *et al* (16) analyzed the expression of integrins in MC3T3-E1 cells by flow cytometry and found high expression of integrin α5β1. Therefore, it was hypothesized that spondin 2 may play an important role in osteogenesis caused by PCa through integrin α5β1.

The aim of the present study was to elucidate the function as well as the underlying mechanism of PCa cell-derived spondin 2 during PCa-driven osteogenesis. The detailed mechanisms of spondin 2 function in PCa-induced bone metastasis need to be further clarified in future studies.

Correspondence to: Dr Chao Yuan, Department of Spinal Surgery, Zaozhuang Municipal Hospital, 41 Longtou Road, Shizhong, Zaozhuang, Shandong 277101, P.R. China
E-mail: yuandf89@163.com

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Materials and methods

Cell culture and treatment. The human RWPE-1 cell line, PCa cell lines (LNCaP and C4-2 cells) and the osteoblastic cell line MC3T3-E1 were all purchased from American Type Culture Collection. RWPE-1 cells were cultured in keratinocyte serum-free medium supplemented with 25 μg/ml bovine pituitary extract, 5 ng/ml human recombinant epidermal growth factor, 100 U/ml penicillin and 100 μg/ml streptomycin (all from Invitrogen; Thermo Fisher Scientific, Inc.). LNCaP and C4-2 cells were cultured in DMEM (Invitrogen; Thermo Fisher Scientific, Inc.), while MC3T3-E1 cells were cultured in α-MEM (Invitrogen; Thermo Fisher Scientific, Inc.). The culture media were supplemented with 10% fetal bovine serum (FBS; Invitrogen; Thermo Fisher Scientific, Inc.), 100 U/ml penicillin and 100 μg/ml streptomycin with (or without) 0.5 μg/ml spondin 2 neutralizing antibody (cat. no. SP2021041B; Wuhan Dian Biotechnology Co., Ltd.), and cells were maintained in a humidified incubator at 37˚C with 5% CO_2. MC3T3-E1 cells were treated with 0.1 or 1.0 μg/ml spondin 2 recombinant protein (rSpondin 2; cat. no. RPF396Mu01; Cloud-Clone Corp.) for 24 h in a humidified incubator at 37˚C with 5% CO_2. Control group cells were treated with 1X PBS for 24 h in a humidified incubator at 37˚C with 5% CO_2. In the integrin α5β1 inhibitor assay, MC3T3-E1 cells were treated with 100 μM ATN-161 (MedChemExpress) for 24 h in a humidified incubator at 37˚C with 5% CO_2.

Collection of conditioned media (CM). LNCaP and C4-2 cells ($2x10^6$) were grown overnight in 100 mm culture dishes. After two washes with PBS, the cells were cultured in DMEM with 1% FBS for 48 h prior to collection of CM.

ELISA. The supernatants of normal prostate epithelial cells (RWPE-1) and PCa cells (LNCaP and C4-2) cultures were centrifuged at 1,000 x g for 15 min at 4˚C before the assay. Proteins were assessed using a spondin-2 ELISA kit (cat. no. JCSJ2862; Shanghai Jichun Industrial Co., Ltd.) according to the manufacturer's instructions.

Reverse transcription-quantitative PCR (RT-qPCR). Total RNA of MC3T3-E1 cells was extracted using TRIzol® reagent (Invitrogen; Thermo Fisher Scientific, Inc.). according to the protocol provided by the manufacturer. cDNA was synthesized using Prime Script RT Master Mix Kit (Takara Bio, Inc.) according to the manufacturer's instructions. RT-qPCR was performed in duplicate with a SYBR Premix Ex Taq™ kit (Takara Bio, Inc.) according to the manufacturer's instructions. PCR amplification conditions were as follows: Pre-denaturation at 95˚C for 15 sec; 45 cycles of denaturation at 95˚C for 5 sec and annealing/extension at 62˚C for 30 sec. The following primers were used in the present study: *Osterix* forward, 5'-GATGGCGTCCTCTCTGCTTG-3' and reverse, 5'-TCTTTGTGCCTCCTTTCCCC-3'; *Runx2* forward, 5'-GACGAGGCAAGAGTTTCACC-3' and reverse, 5'-GGACCGTCCACTGTCACTTT-3'; *Gapdh* forward, 5'-TCCACCACCCTGTTGCTGTA-3' and reverse, 5'-ACCACAGTCCATGCCATCAC-3'. Relative expression of the targeted genes was calculated using the $2^{\Delta\Delta Cq}$ method (17).

Western blotting. MC3T3-E1 cells were collected and lysed using RIPA buffer (Boster Biological Technology) containing protease inhibitor cocktail and PMSF (Boster Biological Technology). For determining the protein concentration, a BCA method was used, and equal amounts (30 μg) of proteins were separated under 90 V via 10% SDS-PAGE and subsequently transferred onto PVDF membranes (MilliporeSigma). After blocking the membranes with 1X TBS-Tween (TBST; 0.05% Tween-20) containing 5% skimmed milk for 2 h at room temperature, the membranes were incubated with primary anti-phosphorylated (p)-PI3K (cat. no. 4228), anti-PI3K (cat. no. 4257), anti-p-AKT (cat. no. 4060), anti-AKT (cat. no. 9272), anti-p-mTOR (cat. no. 5536), anti-mTOR (cat. no. 2972) (all 1:1,000 dilution; Cell Signaling Technology, Inc.) and anti-GAPDH (1:1,000 dilution; cat. no. 60004-1-Ig; ProteinTech Group, Inc.) antibodies overnight at 4˚C. GAPDH was used as a normalization control. Membranes were rinsed in TBST, incubated with secondary anti-mouse IgG, AP-linked antibody (cat. no. 7056; 1:4,000 dilution; Cell Signaling Technology, Inc.) and anti-rabbit IgG, HRP-linked antibody (cat. no. 7074; 1:3,000 dilution; Cell Signaling Technology, Inc.) for 1 h at room temperature and then washed in 1X TBST. After incubation with the ECL Plus system (Amersham; Cytiva), signals were detected using the ImageQuant LAS 4000 mini system (GE Healthcare Bio-Sciences). The signals were detected using Adobe Photoshop CS3 software (Adobe Systems, Inc.).

Public database analysis. Gene expression data (GSE101607 dataset) were downloaded as raw signals from Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo) (18), and analyzed using the Geo2R tool from NCBI (https://www.ncbi.nlm.nih.gov/geo/geo2r). The differentially expressed genes were filtered by |log2FoldChangel>1 and FDR<0.05. Accordingly, a heatmap was generated using the 'pheatmap' package in R 3.6.1 (https://mirrors.tuna.tsinghua.edu.cn/CRAN/). Published gene expression profiles and clinical data of PCa patients were obtained from The Cancer Genome Atlas (TCGA) database (https://portal.gdc.cancer.gov). The data type was selected as 'count' and transformed into the transcript per million format. All patients with PCa were divided into two subgroups according to the median *SPON2* expression, namely *SPON2* low group (n=246) and *SPON2* high group (n=246). This part of data was analyzed via Gene Set Enrichment Analysis (GSEA v3.0; https://www.gsea-msigdb.org/gsea/index.jsp). GSEA is a computational method that determines whether an a priori defined set of genes shows statistically significant, concordant differences between two biological states (19).

Statistical analysis. Data are presented as the mean ± SD and were analyzed using GraphPad Prism software v6.01 (GraphPad Software, Inc.). Differences between two groups were assessed using a two-tailed unpaired Student's t-test. One-way analysis of variance tests with Bonferroni's post hoc test was used for multiple comparisons. The association between *SPON2* and the survival rate of patients with PCa was obtained using Kaplan-Meier analysis (the log-rank test was used to obtain the P-value) in GraphPad Prism software. $P<0.05$ was considered to indicate a statistically significant difference. All experiments were repeated at least three times.

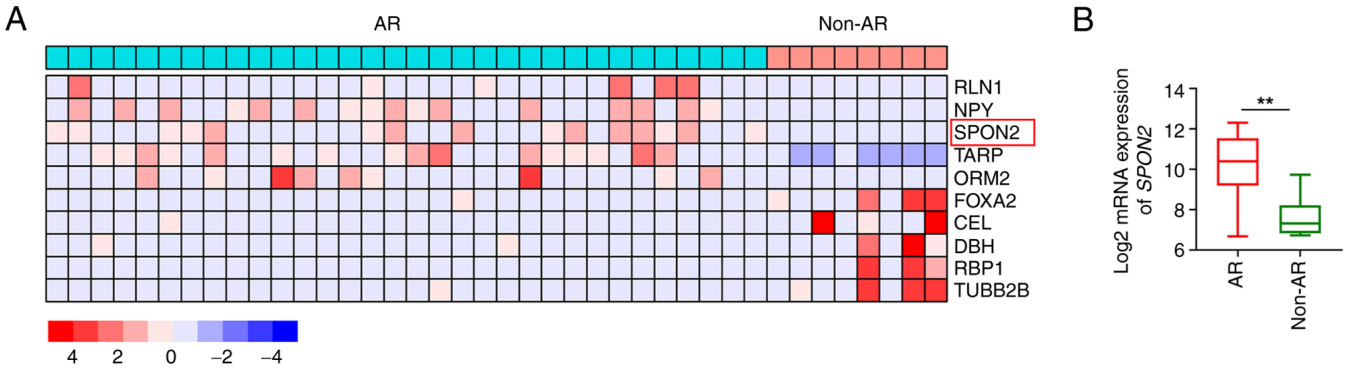

Figure 1. *SPON2* is highly expressed in AR-positive prostate cancer. (A) Heatmap of top five up- and downregulated differentially expressed genes between the AR-positive and non-AR-positive groups in the GSE101607 dataset. (B) Boxplots showing the expression levels of *SPON2* in the GSE101607 dataset. **P<0.01. AR, androgen receptor; *SPON2*, spondin 2.

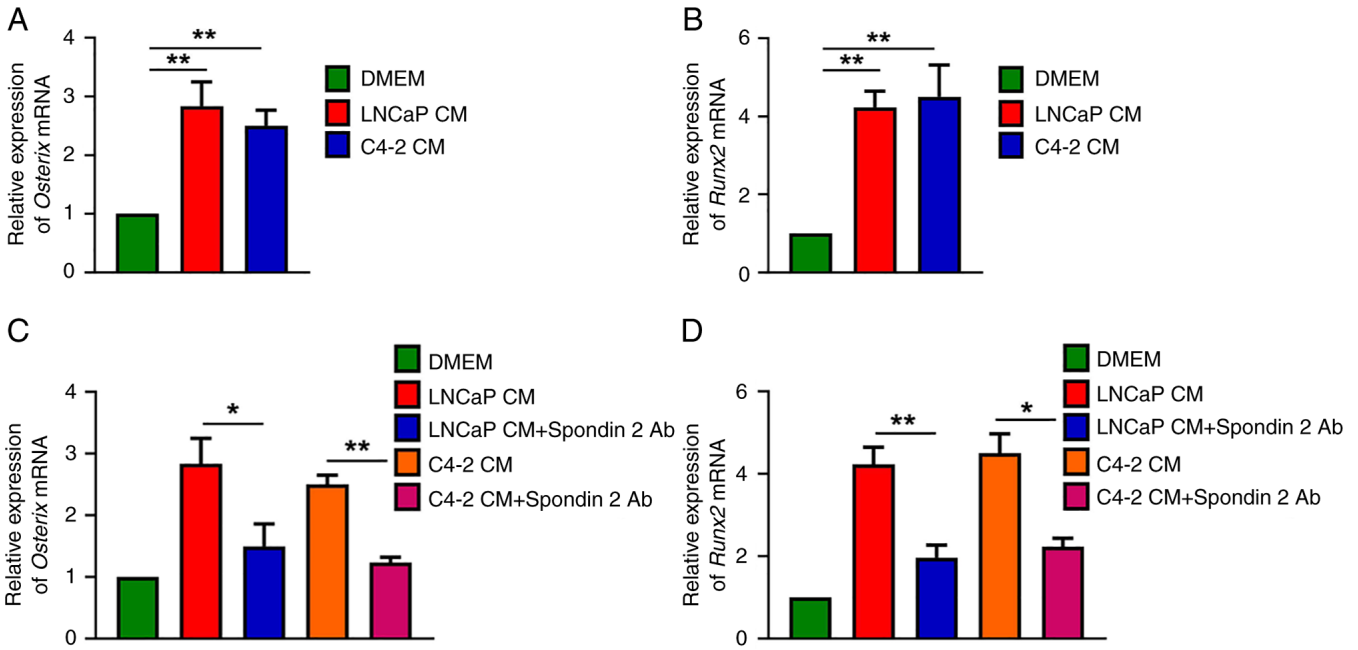

Figure 2. Prostate cancer cell-derived spondin 2 enhances the production of osteogenic factors in osteoblasts. MC3T3-E1 cells were treated with CM from LNCaP or C4-2 cell lines. The mRNA levels of (A) *Osterix* and (B) *Runx2* in MC3T3-E1 cells were measured using RT-qPCR analysis. The results were normalized to *Gapdh*. MC3T3-E1 cells were treated with CM from LNCaP or C4-2 cell lines and spondin 2 antibody. The mRNA levels of (C) *Osterix* and (D) *Runx2* in MC3T3-E1 cells were measured using RT-qPCR analysis. The results were normalized to *Gapdh*. *P<0.05 and **P<0.01. CM, conditioned medium; RT-qPCR, reverse transcription-quantitative PCR; *Runx2*; runt-related transcription factor 2; Ab, antibody.

Results

SPON2 expression is increased in patients with AR-positive PCa. Patients with castration-resistant PCa (GSE101607 dataset) were divided into two subgroups according to AR positivity, namely the AR-positive group (n=32) and the non-AR-positive group (n=8). By setting $\log_2$ (fold change) at ±1 and P<0.05, 687 differentially expressed genes (DEGs) between the AR-positive and the non-AR-positive group were identified with 188 upregulated DEGs and 499 downregulated DEGs. According to the heat map of the top five most up- and downregulated DEGs (Fig. 1A and Table SI), *SPON2* was indicated to be highly expressed in AR-positive group (Fig. 1B). Consistent with this, ELISA results also showed that spondin 2 protein was secreted from PCa cells. Spondin 2 protein in the supernatants of PCa cells was significantly increased compared with normal prostate epithelial cells (235.13±61.82 pg/ml in LNCaP and 280.02±90.50 pg/ml in C4-2 cells vs. 34.97±12.60 pg/ml in RWPE-1 cells) (Fig. S1). In the present study, the association of *SPON2* with the survival of patients with PCa based on TCGA data was also analyzed. The data showed that high level of *SPON2* was associated with poor prognosis, but there was no significant difference between the two groups (Fig. S2).

PCa cell-derived spondin 2 promotes osteogenic factor production in osteoblasts. Next, the osteoblastic classification of PCa bone metastasis was sought to be determined, based on the evidence of tumor cell-derived osteogenic factors from two AR-positive PCa cell lines (LNCaP and C4-2), leading

to increased bone formation. Compared with untreated MC3T3-E1 cells, it was found that CM from the LNCaP and C4-2 cells enhanced *Osterix* and *Runx2* mRNA expression in osteoblasts (Fig. 2A and B). To examine whether spondin 2 was a critical factor in PCa cells, CM was treated with spondin 2 antibody. The results showed that spondin 2 antibody effectively reduced *Osterix* and *Runx2* mRNA synthesis after treatment with PCa cell CM (Fig. 2C and D), indicating that PCa cell-derived spondin 2 promotes the production of osteogenic factors in osteoblasts.

SPON2 is positively associated with the PI3K/AKT/mTOR pathway in patients. The expression levels of *SPON2* were further analyzed using TCGA PCa dataset, and it was found that the expression levels of *SPON2* were elevated in tumor compared with normal tissues (Fig. 3A). Furthermore, patients with PCa were divided into two subgroups according to the median *SPON2* expression, namely *SPON2* low group (n=246) and *SPON2* high group (n=246). As a previous study indicated that the PI3K/AKT/mTOR pathway could regulate the migration and invasion of PCa (20,21), it was then explored whether *SPON2* expression was associated with the PI3K/AKT/mTOR pathway. According to GSEA using TCGA PCa dataset, high expression of *SPON2* was positively associated with the enrichment of the PI3K/AKT/mTOR signaling pathway (Fig. 3B).

Spondin 2 activates the PI3K/AKT/mTOR pathway in osteoblasts. To investigate the specific effect of spondin 2 on osteogenic factor production in osteoblasts, MC3T3-E1 cells were treated with rSpondin 2. Different concentrations of rSpondin 2 were used according to a previous study (22), and in the present study it was observed that two concentrations of rSpondin2 (0.1 and 1.0 μg/ml) showed the most highly promoting effect on the transcription of *Osterix* and *Runx2* as well as the phosphorylation of PI3K, AKT and mTOR. RT-qPCR analysis showed that rSpondin 2-induced MC3T3-E1 cells had higher transcriptional levels of *Osterix* and *Runx2* compared with control cells in a concentration-dependent manner (Fig. 4A and B). To further investigate the underlying mechanism, the activity of the PI3K/AKT/mTOR pathway was further explored. The protein levels of PI3K, p-PI3K, AKT, p-AKT, p-mTOR and mTOR were measured in osteoblasts cultured with various concentrations of rSpondin 2. According to the results, the phosphorylation of PI3K, AKT and mTOR were significantly increased after treatment with rSpondin 2 in a concentration-dependent manner, compared with control cells. On the other hand, the total PI3K, AKT and mTOR levels remain unchanged (Fig. 4C).

Inhibition of integrin α5β1 suppresses the PI3K/AKT/mTOR pathway in osteoblasts. Spondin 2 is known to bind to integrin receptors (9). Integrins α5 and β1 are known to be expressed in osteoblasts on the bone surface (23). In order to determine whether spondin 2 activated the PI3K/AKT/mTOR pathway via integrin α5β1, an integrin α5β1 inhibitor (ATN-161) was used to determine its effects on spondin 2-mediated osteogenic factor production. According to RT-qPCR analysis, ATN-161 significantly inhibited spondin 2-induced mRNA expression of *Osterix* and *Runx2* (Fig. 5A and B). Furthermore, western

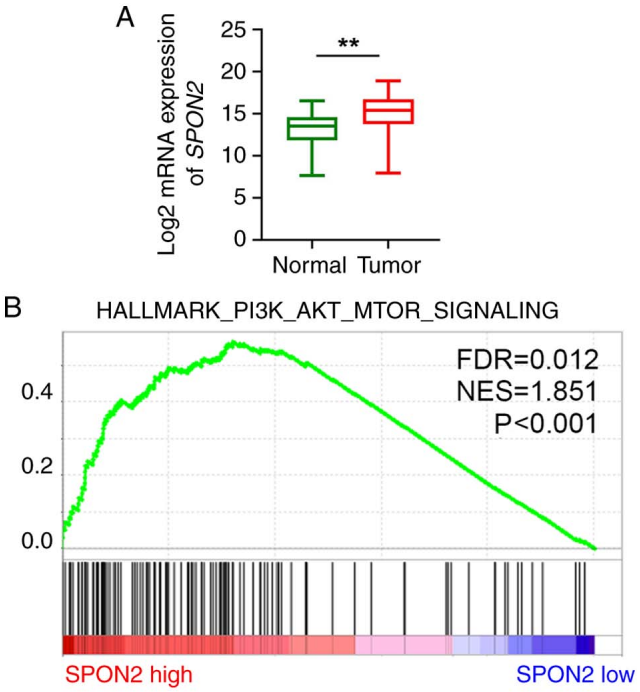

Figure 3. *SPON2* is positively associated with the PI3K/AKT/mTOR pathway. (A) Boxplots showing the expression levels of *SPON2* in the TCGA PCa dataset. (B) Gene Set Enrichment Analysis of the PI3K/AKT/mTOR signaling pathway in the *SPON2* high group compared with the *SPON2* low group in the TCGA PCa dataset. **P<0.01. FDR, false discovery rate; NES, normalized enrichment score; PCa, prostate cancer; *SPON2*, spondin 2.

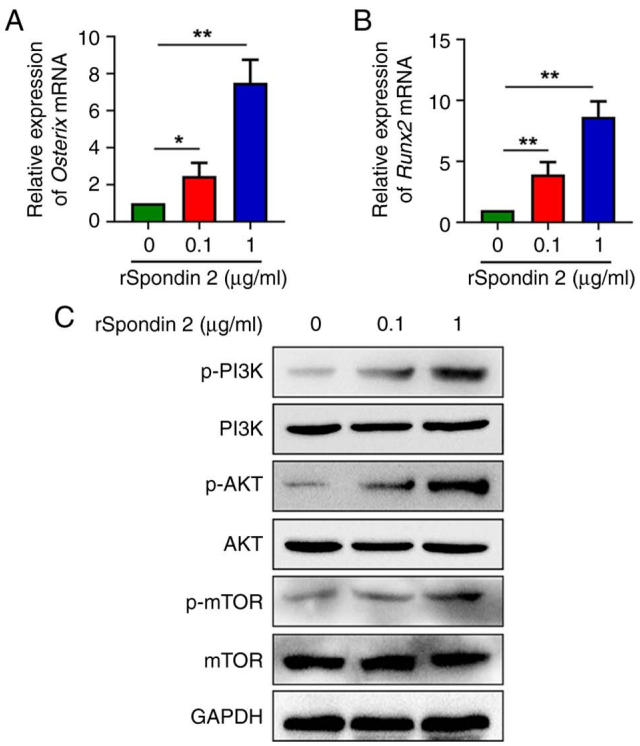

Figure 4. Spondin 2 activates the PI3K/AKT/mTOR pathway. MC3T3-E1 cells were treated with 0.0, 0.1 or 1.0 μg/ml rSpondin 2. The mRNA levels of (A) *Osterix* and (B) *Runx2* in MC3T3-E1 cells were measured using reverse transcription-quantitative PCR analysis. The results were normalized to *Gapdh*. (C) The expression levels of p-PI3K, PI3K, p-AKT, AKT, p-mTOR and mTOR in MC3T3-E1 cells were determined by western blot analysis. *P<0.05 and **P<0.01. rSpondin 2, recombinant spondin 2; *Runx2*; runt-related transcription factor 2; p, phosphorylated.

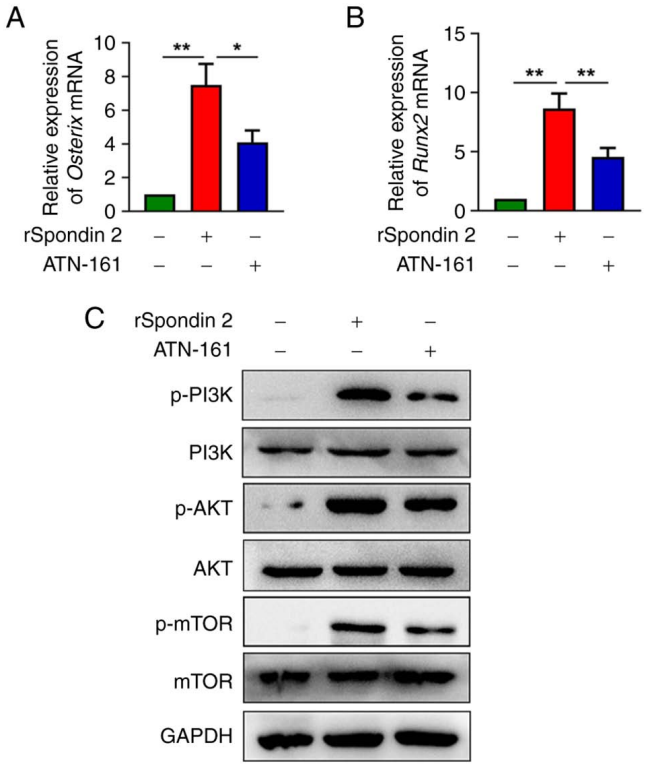

Figure 5. ATN-161 inhibits the PI3K/AKT/mTOR pathway. MC3T3-E1 cells were treated with 1.0 μg/ml rSpondin 2 and 10.0 μM ATN-161. The mRNA levels of (A) *Osterix* and (B) *Runx2* in MC3T3-E1 cells were measured using reverse transcription-quantitative PCR analysis. The results were normalized to *Gapdh*. (C) The expression levels of p-PI3K, PI3K, p-AKT, AKT, p-mTOR and mTOR in MC3T3-E1 cells were determined by western blot analysis. *P<0.05 and **P<0.01. rSpondin 2, recombinant spondin 2; *Runx2*; runt-related transcription factor 2; p, phosphorylated.

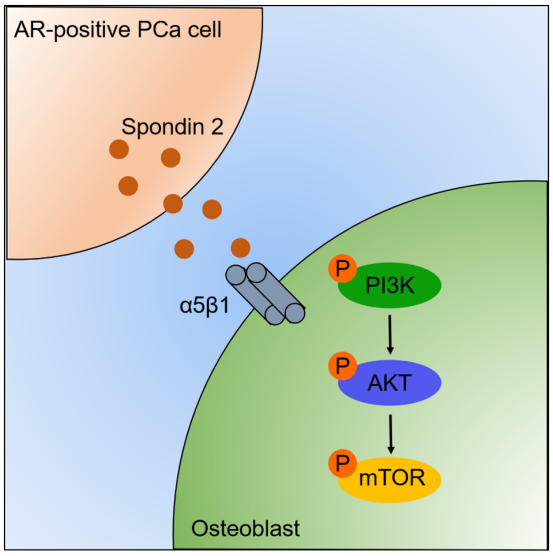

Figure 6. Schematic diagram summarizing the mechanism of spondin 2 function. Spondin 2 secreted from AR-positive PCa cells increases *Osterix* and *Runx2* synthesis in osteoblasts via the PI3K/AKT/mTOR signaling pathway. AR, androgen receptor; PCa, prostate cancer; p, phosphorylated.

blot analysis indicated that ATN-161 significantly inhibited spondin 2-mediated PI3K, AKT and mTOR phosphorylation (Figs. 5C and S3).

Discussion

A previous study has shown that *SPON2* is a new serum and histological diagnostic biomarker for PCa (11). Similarly, the present study found that spondin 2 facilitated osteogenic factor production induced by PCa cells, and *SPON2* was upregulated in AR-positive tumors of patients with PCa. Moreover, it was identified that PCa cell-derived spondin 2 promoted the osteogenic activity of osteoblasts by activating the PI3K/AKT/mTOR pathway.

The disruption of homeostasis between osteoblasts is involved in PCa-induced osteogenesis (2,24). These processes are regulated by tumor cell-derived cytokines, such as bone morphogenetic protein, platelet-derived growth factor, insulin-like growth factor and extracellular calcium (25-28). As an extracellular matrix protein, increased levels of spondin 2 in serum are correlated with high incidence of osteogenesis induced by prostate tumor cells (29). According to previous studies, spondin 2 is a critical regulator of cancer progression; however, its underlying mechanism in osteoblast activity remains to be elucidated (11-13). On the other hand, Runx2 can promote the differentiation of mesenchymal stem cells towards osteoblasts, while Osterix plays an important role in osteoblast differentiation (30,31). On this basis, it was identified that spondin 2 derived from PCa cells significantly enhanced the expression of the osteogenic genes Runx2 and Osterix, indicating an increased activity of osteoblasts.

The PI3K/AKT/mTOR signaling pathway has been indicated to participate in cell proliferation, inflammation, immunity and tumorigenesis (32-34). Inhibition of the PI3K/AKT/mTOR signaling pathway can suppress tumor growth and tumor-induced osteogenesis (35,36). Moreover, the PI3K/AKT/mTOR pathway has been reported to be a potential target in castration resistant PCa (37,38). However, whether spondin 2 upregulates the PI3K/AKT/mTOR signaling during osteogenesis driven by PCa progression requires further investigation. In the present study, it was indicated that spondin 2 secreted by AR-positive PCa cells could promote the differentiation of osteoblast precursors to mature osteoblasts (showed by the increased expression of Runx2 and Osterix) through the PI3K/AKT/mTOR signaling pathway. As a well-known receptor of spondin 2, integrin α5β1 plays a significant role in bone formation (23), and the present study showed that spondin 2 receptor inhibitor ATN-161 could inhibit spondin 2-mediated PI3K, AKT and mTOR phosphorylation in osteoblast precursor MC3T3-E1 cells.

The present study explored the role of spondin 2 on osteogenesis caused by PCa cells *in vitro*, while the association between spondin 2 and bone metastasis in animal models as well as patients with PCa requires further investigation.

In summary, the current study demonstrated that spondin 2 derived from AR-positive PCa cells could effectively enhance PCa-induced osteogenesis through activation of the PI3K/AKT/mTOR signaling cascade (Fig. 6). Furthermore, to the best of our knowledge, the present study was the first to demonstrate that integrin α5β1 is involved in spondin 2-regulated osteogenesis in PCa cells *in vitro*.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

Experiments were conceived and designed by CY and HW. HW and MZ performed the experiments and data analysis, prepared the figures and wrote the manuscript. WL helped with the experimental operation and data analysis. CY participated in manuscript writing. CY and HW confirm the authenticity of all the raw data. All authors have read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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