The TGF-β signalling negative regulator PICK1 represses prostate cancer metastasis to bone

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Background: Constitutive activation of TGF-β signalling is a well-recognised mechanism in bone metastasis of prostate cancer (PCA). Protein Interacting with PRKCA 1 (PICK1) is a critical negative regulator of the TGF-β pathway. However, the clinical significance and biological role of PICK1 in PCA bone metastasis remain obscure.

Methods: PICK1 expression is evaluated by immunohistochemistry (IHC) in 198 PCA patients. Statistical analysis is performed to explore correlation between PICK1 expression and clinicopathological features in PCA patients. The biological role of PICK1 is examined in PC-3 and C4-2B cells in vitro and a mouse intracardial model in vivo.

Results: PICK1 expression is decreased in PCA tissues with bone metastasis and bone-derived cells and downregulation of PICK1 positively correlates with serum PSA level, Gleason grade and bone metastasis status in PCA patients. Overexpression of PICK1 suppresses PCA cell invasion and migration in vitro and bone metastasis in vivo. Our results further indicate downregulation of PICK1 is caused by miR-210-3p overexpression in PCA tissues with bone metastasis. Clinical negative correlation of PICK1 with miR-210-3p is confirmed in PCA tissues.

Conclusions: Our findings uncover a novel functionally and clinically relevant epigenetic regulatory mechanism for constitutive activation of TGF-β signalling in bone metastasis of PCA.

Prostate cancer (PCA) is one of the most commonly diagnosed cancers with indolent malignant features in men (Nelson et al, 2003). Despite great progress in the systemic treatment of PCA in recent years, distant bone metastasis remains the primary issue affecting the quality of life and survival time of PCA patients (Bubendorf et al, 2000). Therefore, a better understanding of the underlying mechanisms contributing to bone metastasis of PCA will facilitate the development of novel therapeutic strategies against PCA.

The TGF-β signalling pathway has important functions in several biological processes, including inhibiting cell proliferation, inducing cell differentiation, embryogenesis and bone remodelling.
caveolin-mediated endocytosis, ubiquitination and degradation of leads to enhanced lipid raft/caveolae localisation, resulting in the bone microenvironment, considerable attention has been given to the pro-bone metastasis roles of TGF-β signalling, where activation of TGF-β signalling drives tumour cell invasion and migration (Wang et al., 2015; Yu et al., 2016). For example, Fournier and colleagues reported that activation of TGF-β signalling upregulated PMEPA1, an important negative regulator of the TGF-β pathway, and that interrupting this negative feedback loop by PMEPA1 knockdown caused PCa cells to disseminate to bone marrow, ultimately increasing bone metastases in a mouse PCa model (Fournier et al., 2015). Furthermore, it is reported that therapy targeting TGF-β significantly attenuates metastasis of tumour cells to bone (Juaez and Guise, 2011; Hu et al., 2012; Wan et al., 2012). Although these studies demonstrate that the TGF-β pathway plays crucial roles in the bone metastasis of PCa, the molecular mechanism responsible for constitutive activation of TGF-β in PCa bone metastasis needs to be further elucidated.

Protein Interacting with PRKCA 1 (PICK1), which was initially found by a yeast two-hybrid system analysis for identifying proteins that interact with activated protein kinase C, alpha (PRKCA) (Staudinger et al., 1995), functions as an adaptor that binds to and guides the subcellular localisation and distribution of a set of membrane proteins (Staudinger et al., 1995). Previous studies have shown that PICK1 has important roles in regulating the cytoskeleton and neuron morphology, and PICK1 has been implicated in several diseases (Steinberg et al., 2006; Nakamura et al., 2011; Rocca et al., 2013; Rocca and Hanley, 2015; Kunicka et al., 2016). Recent studies implicate PICK1 dysregulation in the progression and metastasis of cancers (Cockbill et al., 2015). Notably, a study from Zhao et al. reported that PICK1 acts as an important negative regulator of TGF-β signalling in breast cancer cells by serving as a scaffold protein to enhance interaction between TGF-β receptor 1 (TβR1) and cavelin-1. Such interaction leads to enhanced lipid raft/caveolae localisation, resulting in cavelin-mediated endocytosis, ubiquitination and degradation of TβR1 (Zhao et al., 2012). However, the clinical significance and biological roles of PICK1 in bone metastasis of PCa are largely unknown.

MicroRNAs, which exhibit potent repressive activity on target genes by binding to the 3’ untranslated region (3’UTR) of messenger RNA (mRNA), are reported to be important mediators in bone metastasis of PCa (Wang et al., 2008; Pang et al., 2010; Guo et al., 2013; Ren et al., 2013, 2014). In this study, we found that PICK1 expression is decreased in PCa tissues with bone metastasis and bone metastatic cells and that reduced PICK1 expression positively correlates with the clinicopathological characteristics and bone metastasis status of PCa patients. Upregulation of PICK1 suppresses invasion and migration in vitro, as well as bone metastasis in PCa cells in vivo through repression of TGF-β signalling. Furthermore, our results demonstrate that high miR-210-3p expression is the main underlying epigenetic regulatory mechanism responsible for downregulation of PICK1 in PCa tissues with bone metastasis. Thus, our findings reveal a novel mechanism of constitutive activation of TGF-β signalling, resulting in the development of bone metastasis of PCa.
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**RESULTS**

**PICK1 is downregulated in PCa tissues with bone metastasis and bone metastatic cell lines.** To determine the clinical significance of PICK1 in PCa, we first analysed a publicly available PCa mRNA sequencing dataset and found that PICK1 expression was dramatically decreased in PCa tissues with bone metastasis compared with PCa tissues without bone metastasis (Figure 1A and B). We further examined expression of PICK1 in our 51 fresh PCa tissues and found it to be significantly lower in PCa tissues with bone metastasis compared with PCa tissues without bone metastasis (Figure 1C). In addition, the percentage of low-PICK1 expression was higher in PCa tissues with bone metastasis compared to PCa tissues without bone metastasis (Supplementary Figure 1A and B). Western blot analysis revealed downregulation of PICK1 protein expression in PCa tissues with bone metastasis (Figure 1D). Consistently, the mRNA and protein levels of PICK1 in PCa cell lines derived from bone metastases (PC-3, C4-2B and VCaP) were significantly lower than those in 22Rv1 cells derived from primary PCa, DU145 cells derived from brain metastasis and LNCaP cells derived from lymph node metastasis (Figure 1E and Supplementary Figure 1C). Immunohistochemical (IHC) staining of PICK1 in 198 PCa tissues was performed. The results showed that PICK1 was strongly expressed in PCa tissues without bone metastasis, but notably downregulated in PCa tissues with bone metastasis and further decreased in PCa bone tissues (Figure 1F). Through further analysis of the relationship between PICK1 expression and clinicopathological characteristics, we found that the level of PICK1 negatively correlated with serum prostate-specific antigen (PSA) levels (P<0.001), the Gleason grade (P=0.015) and the bone metastasis status (P<0.001) in PCa (Supplementary Tables S3 and S4). Collectively, these results implicate reduced PICK1 expression in the bone metastasis of PCa.

**PICK1 inhibits bone metastasis of PC-3 cells.** To determine the role of PICK1 in PCa bone metastasis *in vivo*, we first generated PCa cell lines stably overexpressing PICK1 by ectopically overexpressing PICK1 via retrovirus infection (Supplementary Figure 2). A mouse model of bone metastasis was employed, whereby a luciferase control or PICK1-overexpressing PC-3 cells were inoculated into the left cardiac ventricle of male nude mice to monitor the development of distant bone metastasis loci by BLI. As shown in Figure 2A and B, PICK1-overexpressing PC-3 cells caused fewer bone metastases compared with the control group by both X-ray and BLI. Hematoxylin and eosin (H&E) staining of sectioned tumours from the tibias of injected mice demonstrated that increased PICK1 expression significantly decreased the tumour burden in bone (Figure 2C). Furthermore, PICK1-overexpressing cells resulted in fewer metastatic foci and smaller...
Supplementary Figure 3A and B). These results indicate that capacities of PCa cells both in the absence and presence of TGF-
that upregulating PICK1 inhibited the invasion and migration downstream bone metastasis-related genes of the TGF-
analysis showed that PICK1 overexpression repressed multiple translocation in PCa cells (Figure 3B). Moreover, real-time PCR revealed that upregulation of PICK1 decreased pSMAD2/3 nuclear motif CAGAC. Cellular fractionation and western blotting analysis which consists of 12 tandem copies of the Smad/DNA binding TGF-
upregulating PICK1 suppressed the transcriptional activity of the of PICK1 on TGF-

Emerging evidence demonstrates that PICK1 is a critical negative regulator of the TGF-β pathway (Zhao et al, 2012); thus, we examined the effect of PICK1 on TGF-β signalling. As shown in Figure 3A, upregulating PICK1 suppressed the transcriptional activity of the TGF-β/Smad-responsive luciferase reporter plasmid CAGA12, which consists of 12 tandem copies of the Smad/DNA binding motif CAGAC. Cellular fractionation and western blotting analysis revealed that upregulation of PICK1 decreased pSMAD2/3 nuclear translocation in PCa cells (Figure 3B). Moreover, real-time PCR analysis showed that PICK1 overexpression repressed multiple downstream bone metastasis-related genes of the TGF-β pathway in both the absence and presence of ectopic TGF-β (Figure 3C–K). Overall, our results demonstrate that PICK1 inhibits TGF-β signalling activity in PCa cells.

Furthermore, in vitro invasion and migration assays showed that upregulating PICK1 inhibited the invasion and migration capacities of PCa cells both in the absence and presence of TGF-β (Supplementary Figure 3A and B). These results indicate that PICK1 downregulation promotes invasion and migration in PCa cells by activating TGF-β signalling.

Overexpression of miR-210-3 causes PICK1 downregulation in PCa tissues with bone metastasis. To clarify the underlying mechanism of reduced PICK1 expression in PCa tissues with bone metastasis, we first analyzed the deletion status from a genetic perspective in a PCa dataset from The Cancer Genome Atlas (TCGA) and found a deletion rate of only 0.2% among all 496 TCGA PCa samples (Supplementary Figure 4A). This result indicates that epigenetic regulation may be involved in PICK1 downregulation in PCa tissues with bone metastasis. To clarify the underlying mechanism of reduced PICK1 expression in PCa tissues with bone metastasis.

osteolytic areas of metastatic tumours, as well as longer survival and bone metastasis-free survival rates compared to the control group (Figure 2D–G). Therefore, these findings indicate that upregulation of PICK1 inhibits the bone metastasis of PCa.

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As miRNAs, small endogenous non-coding RNAs that post transcriptionally regulate target genes by binding to their 3’UTR, leading to degradation, are involved in cancer (Bartel, 2009), we investigated whether miRNAs participate in PICK1

**Figure 1. PICK1 is downregulated in PCa tissues with bone metastasis.** (A) PICK1 expression was dramatically reduced in PCa tissues with bone metastasis (PCa/BM) compared with PCa tissues without bone metastasis (PCa/nBM) based on analysis of the TCGA PCa sequencing dataset (PCa/nBM, n = 12; PCa/BM, n = 10). P = 0.002. (B) PICK1 expression was dramatically decreased in PCa/BM compared with PCa/nBM based on analysis of the GSE46602 PCa sequencing dataset (PCa/nBM, n = 14; PCa/BM, n = 22). P = 0.003. (C) Real-time PCR analysis of PICK1 expression in 23 freshly collected non-bone metastatic and 28 bone metastatic PCa tissue samples. Transcript levels were normalised to GAPDH expression. Lines represent median and lower/upper quartiles. P < 0.001. (D) Western blotting analysis of PICK1 expression in 4 PCa/nBM and 4 PCa/BM. α-Tubulin served as the loading control. (E) Western blotting analysis of PICK1 expression in PCa cells. α-Tubulin served as the loading control. (F) Immunohistochemical staining of PICK1 protein expression in representative samples of PCa/nBM, PCa/BM and bone of PCa are shown.
downregulation in PCa tissues with bone metastasis. Using the publicly available algorithms TargetScan and miRanda, we identified 29 broadly conserved miRNAs with the potential to bind to the 3'UTR of PICK1. To validate the ability of these predicted miRNAs to directly bind to the PICK1 3'UTR, an RNA immunoprecipitation (RIP) analysis with the MS2 binding protein (MS2bp) was performed. A plasmid expressing the 3'UTR of PICK1 containing the MS2-binding sequence was constructed and cotransfected together with the MS2bp-YFP expression vector into PC-3, C4-2B and VCaP cells. The transcript-specific binding of YFP protein in combination with miRNA complexes was precipitated with a YFP antibody. Quantitative real-time PCR analysis revealed strong enrichment of nine miRNAs in binding complexes, indicating the potent binding ability of these miRNAs to the PICK1 3'UTR (Figure 4A). We further evaluated these nine miRNAs and found that only miR-338-3p, miR-210-3p and miR-34a-5p significantly correlated with bone metastasis of PCa in the TCGA PCa dataset (Figure 4B). As miRNAs suppress target genes and PICK1 negatively regulates PCa bone metastasis, only those miRNAs that correlated positively with PCa bone metastasis would inhibit PICK1 expression, resulting in bone metastasis of PCa. Accordingly, miR-210-3p was the only potential candidate in this scenario (Figure 4B), and miR-210-3p expression should be elevated in PCa tissues with bone metastasis compared with PCa tissues without bone metastasis. Indeed, publicly available PCa data sets and our PCa tissues revealed significantly elevated levels of miR-210-3p expression in PCa tissues with bone metastasis and bone metastasis cell lines (Figure 4C and D and Supplementary Figure 5). Furthermore, real-time PCR and western blot analysis revealed that enhanced miR-210-3p expression decreased while silenced miR-210-3p increased the mRNA and protein levels of PICK1 in PCa cells (Figure 4E and F). In addition, a luciferase assay using in PCa cells showed that miR-210-3p overexpression attenuated and miR-210-3p inhibition increased reporter activity driven by the PICK1 3'UTR but not a mutant miR-210-3p seed region (Figure 4G). Collectively, these results demonstrate that miR-210-3p overexpression leads to reduced expression of PICK1 in PCa tissues with bone metastasis.

miR-210-3p activates TGF-β signalling and promotes invasion and migration by targeting PICK1 in PCa cells. We first examined the effects of miR-210-3p on TGF-β signalling activity and migration and invasion abilities in PCa cells. As shown in Figure 5A–E, silencing miR-210-3p inhibited pSMAD2/3 nuclear translocation, TGF-β signalling activity and downstream target gene expression. Moreover, downregulation of miR-210-3p repressed migration and invasion capacities of PC-3 cells (Figure 5F and G). Further analysis showed that silencing PICK1...
Transcript levels were normalised to GAPDH expression. Error bars represent the mean ± s.d. of three independent experiments. *P<0.05, **P<0.01.

reversed the inhibitory effects of reduced miR-210-3p expression on pSMAD2/3 nuclear translocation, TGF-β signalling activity and downstream target gene expression in PCa cells. In addition, downregulating PICK1 restored the miR-210-3p silencing-induced inhibition of migration and invasion in PCa cells. These findings demonstrate that miR-210-3p promotes TGF-β signalling in PCa cells, as well as migration and invasion by repressing PICK1.

Clinical correlation of miR-210-3p with PICK1 in human PCa tissues. To investigate the clinical significance of miR-210-3p and PICK1, miR-210-3p expression and the mRNA levels of PICK1 were examined in 51 fresh PCa tissues. As shown in Figure 6A, the negative correlation between PICK1 mRNA and miR-210-3p expression was verified in our PCa tissue samples, consistent with the TCGA PCa tissue analysis (Figure 6B). Indeed, PICK1 expression was inversely correlated with miR-210-3p expression (r = −0.697, P<0.05) and downstream bone metastasis-related genes of TGF-β signalling, CTGF (r = −0.791, P<0.05), PTHRP (r = −0.763, P<0.05) and IL-11 (r = −0.686, P<0.05), in human PCa and bone tissues (Figure 6C–G). Thus, our results indicate that decreased PICK1 expression due to miR-210-3p promotes bone metastasis via TGF-β signalling in PCa (Figure 6H).
Within the context of cancer progression, emerging evidence implicates PICK1 in the formation of cell–cell boundaries, cell movement, and positioning during development, whereas loss of PICK1 leads to dissociation of epithelial cells via disruption of adherens junctions, resulting in invasion and metastasis of cancer cells (Son et al., 2014). Accordingly, aberrant expression of PICK1 has been linked to more aggressive and metastatic tumour phenotypes. For example, in astrocytic tumours, exogenous expression of PICK1 effectively represses the migration and invasion capacity of cells (Cockbill et al., 2015). However, the clinical significance and biological role of PICK1 in bone metastasis of PCA remain unknown. In this study, we found PICK1 expression to be dramatically decreased in PCA tissues with bone metastasis and in bone metastatic cells and even further decreased in bone tissues of PCA. Furthermore, upregulating PICK1 represses prostate cancer metastasis to bone in vivo, as well as bone metastasis of PCA cells in vitro. In clinical PCA cases, the level of PICK1 in PCA tissues negatively correlated with the serum PSA level, Gleason grade and bone metastasis status. Thus, our results demonstrate the tumour-suppressive role of PICK1 in the bone metastasis of PCA. Intriguingly, Zhang et al. reported overexpression of PICK1 in breast cancer cells, correlating with shortened overall survival. In addition, silencing PICK1 in MDA-MB-231 cells decreased proliferation and tumourigenicity both in vitro and in vivo, supporting an oncogenic role of PICK1 in human breast cancer (Zhang et al., 2010). However, the specific mechanism responsible has not yet been elucidated. Collectively, our results, in combination with other studies, indicate that the pro- and anti-tumour roles of PICK1 are environmental and cancer-type dependent.

Several lines of evidence demonstrate that TGF-β signalling is constitutively activated in bone metastases of various types of cancer, including breast cancer, melanoma and PCA (Yin et al., 2013). Furthermore, upregulating the potential ability to bind to the 3′UTR of PICK1 with PCa bone metastasis. *P < 0.05. (C) Real-time PCR analysis of miR-210-3p expression in 108 PCa/nBM tissues and 90 PCa/BM tissues. Transcript levels were normalised to U6 expression. Lines represent median and lower/upper quartiles. P < 0.001. (D) miR-210-3p expression levels were markedly elevated in PCa/BM tissues compared with PCa/nBM tissues, as assessed by analysis of the TCGA PCA miRNA sequencing dataset (nBM, n = 11; BM, n = 9). P = 0.04. (E) Real-time PCR analysis of PICK1 expression in the indicated cells. Transcript levels were normalised to U6 expression. Error bars represent the mean ± s.d. of three independent experiments. *P < 0.05. (F) Western blotting of PICK1 expression in the indicated cells. α-Tubulin served as the loading control. (G) Luciferase assay of cells transfected with the pmirGLO-PICK13′UTR reporter in miR-210-3p-overexpressing and -silenced PC-3 and C4-2B cells. *P < 0.05.
Expression activates TGF-β, colleagues reported that upregulation of the negative regulator of migration and invasion, and lung metastasis of melanoma cells further inhibits the epithelial-mesenchymal transition (EMT), reduced expression of PICK1 in PCa tissues can activate TGF-β signalling, which results in tumour cell metastasis in different types of cancer. In gallbladder carcinoma, ectopic expression of miR-20a activated TGF-β signalling by targeting SMAD7, which induced EMT and enhanced metastasis (Chang et al., 2013). Bonci et al reported that concomitant loss of miR-15/miR-16 and gain of miR-21 in PCa cells aberrantly activated TGF-β signalling, which mediated local invasion, distant bone marrow colonisation and osteolysis (Bonci et al., 2016). In this study, we found that overexpression of miR-210-3p activated TGF-β signalling and promoted the bone metastatic capacities of PCa cells in vitro and that silencing of PICK1 activates TGF-β signalling and promotes bone metastatic abilities of PCa cells. Importantly, we found that overexpression of miR-210-3p is the primary mechanism contributing to PICK1 downregulation in bone metastatic PCa tissues. Taken together, our findings reveal a novel mechanism responsible for activating TGF-β signalling, which contributes to bone metastasis of PCa (Figure 6H).

**CONCLUSION**

Our results demonstrate that downregulation of PICK1 elicited by miR-210-3p overexpression activates the TGF-β pathway, which further promotes bone metastasis in PCa, indicating that the miR-210-3p/PICK1/TGF-β signalling axis plays an important role in PCa bone metastasis. These findings contribute to a comprehensive understanding of the mechanism responsible for activation of the TGF-β pathway, facilitating the development of an effective therapeutic strategy against bone metastasis of PCa.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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