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Regulation of E3 ubiquitin ligase-1 (*WWP1*) by *microRNA-452* inhibits cancer cell migration and invasion in prostate cancer

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Background: *MicroRNA-224* (*miR-224*) and *microRNA-452* (*miR-452*) are closely located on the human chromosome Xq28 region. *miR-224* functions as a tumour suppressor by targeting tumour protein D52 (*TPD52*) in prostate cancer (PCa). Here, we aimed to investigate the functional significance of *miR-452* in PCa cells.

Methods: Functional studies of PCa cells were performed using transfection with mature miRNAs or siRNAs. Genome-wide gene expression analysis, *in silico* analysis, and dual-luciferase reporter assays were applied to identify miRNA targets. The association between *miR-452* levels and overall patient survival was estimated by the Kaplan–Meier method.

Results: Expression of *miR-452* was significantly downregulated in PCa tissues. Transfection with mature *miR-452* inhibited the migration and invasion of PCa cells. Kaplan–Meier survival curves showed that low expression of *miR-452* predicted a short duration of progression to castration-resistant PCa. WW domain-containing E3 ubiquitin protein ligase-1 (*WWP1*) was a direct target of *miR-452*, and knockdown of *WWP1* inhibited the migration and invasion of PCa cells. WWP1 was upregulated in PCa clinical specimens.

Conclusions: Regulation of the *miR-452–WWP1* axis contributed to PCa cell migration and invasion, and elucidation of downstream signalling of this axis will provide new insights into the mechanisms of PCa oncogenesis and metastasis.

Prostate cancer (PCa) is the most frequently diagnosed cancer and the second leading cause of cancer death among men in developed countries (Siegel *et al*, 2015). Multiple therapeutic options are available for patients with early stage PCa, and its prognosis is relatively favourable (Heidenreich *et al*, 2014a). In contrast, patients with advanced-stage PCa are initially treated with androgen deprivation therapy (ADT); however, their cancers eventually become resistant to ADT and progress to castration-resistant PCa (CRPC; Attard *et al*, 2016). Although several clinical trials for CRPC have been carried out, resulting in the availability of novel chemotherapeutic agents, these treatments provide limited benefits and are not considered curative (Heidenreich *et al*, 2014b; Crawford *et al*, 2015). Therefore, identification of effective biomarkers for detection of CRPC and understanding the molecular mechanisms of androgen-independent signalling and metastatic signalling pathways underlying PCa using current genomic approaches would help to improve therapies for and prevention of the disease.

MicroRNAs (miRNAs) are endogenous small RNA molecules (19–22 bases in length) that regulate protein-coding/non-protein-coding gene expression by translational repression or mRNA cleavage (Bartel, 2004). MicroRNAs are bioinformatically predicted

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to regulate more than one-third of the protein-coding genes in the human genome (Lewis *et al*, 2005; Friedman *et al*, 2009). Therefore, miRNAs act as fine-tuning regulators in almost all biological processes (Bartel, 2009). As for human cancers, a growing body of evidence has indicated that normal RNA regulatory networks can be disrupted by the aberrant expression of tumour-suppressive or oncogenic miRNAs in cancer cells (Garzon *et al*, 2009). Identification of aberrantly expressed miRNAs and novel network searches beginning from tumour-suppressive or oncogenic miRNAs has facilitated elucidation of the molecular mechanisms of cancer initiation, development, and metastasis.

Identification of aberrantly expressed miRNAs is the first step towards elucidating miRNA-based regulatory networks in PCa cells. Based on this, we constructed the miRNA expression signatures of PCa and CRPC using clinical specimens and identified tumour-suppressive miRNAs regulating novel oncogenic pathways (Fuse *et al*, 2012; Goto *et al*, 2015a). Interestingly, several miRNAs were found to be located within close proximity in the human genome, constituting a cluster of miRNAs. Our miRNA expression signatures of PCa showed that several clustered miRNAs were downregulated in cancer cells (Goto *et al*, 2015b). Focusing on the clustered miRNAs in our signatures, we have shown that the clustered miRNAs *miR-1/133a*, *miR-143/145*, *miR-23b/27b/24-1*, and *miR-221/222* function as tumour suppressors by targeting several oncogenic genes or pathways in PCa cells (Kojima *et al*, 2012; Goto *et al*, 2014a; Kojima *et al*, 2014; Goto *et al*, 2015a).

In this study, we focused on *miR-452*, which forms a cluster with *miR-224* on the human chromosome Xq28 region. Our previous study revealed that *miR-224* inhibits cancer cell migration and invasion by directly regulating oncogenic tumour protein D52 (*TPD52*) in PCa cells (Goto *et al*, 2014b). However, the functional roles of *miR-452* in PCa cells are still unknown. The aim of this study was to investigate the functional significance of *miR-452* and the novel oncogenic pathways regulated by this miRNA in PCa cells.

We found that restoration of *miR-452* significantly inhibited cancer cell migration and invasion. WW domain-containing E3 ubiquitin protein ligase-1 (*WWP1*) was one of multiple targets of *miR-452* regulation in PCa cells and it was directly regulated by *miR-452* in PCa cells. Moreover, silencing of *WWP1* inhibited the migration and invasion of PCa cells. Discovery of the molecular targets and pathways regulated by tumour-suppressive *miR-452* will provide insights into the potential molecular mechanisms of PCa oncogenesis and metastasis, and will facilitate the development of novel diagnostic and therapeutic strategies for the treatment of the disease.

MATERIALS AND METHODS

Patients and clinical prostate specimens. Prostate specimens were obtained from patients admitted to Teikyo University Chiba Medical Centre Hospital from 2008 to 2013. Ninety patients with elevated prostate-specific antigen (PSA) levels underwent transrectal prostate needle biopsies. From the collected samples, 54 PCa tissues and 36 normal prostate tissues (non-PCa) were used. The patients' characteristics are summarised in Supplementary Table 1. For pathological verification, we obtained two needle biopsy specimens from the same region as used in this study, and one was pathologically proven to contain no cancerous tissue (designated the non-PCa specimens). Before prostate biopsies, written consent for tissue donation was obtained from each patient. The protocol was approved by the Institutional Review Board of Chiba University and Teikyo University. The definition of CRPC described by the European Association of Urology was used in this study (Heidenreich *et al*, 2014b).

Cell culture. Human PCa cells (PC3 and DU145 cells) were obtained from the American Type Culture Collection (Manassas, VA, USA) and maintained in RPMI-1640 medium supplemented with 10% foetal bovine serum in a humidified atmosphere of 5% CO_2 and 95% air at 37 °C.

RNA isolation. Total RNA was isolated using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. The quality of RNA was confirmed using an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA) as described previously (Goto *et al*, 2014a; Goto *et al*, 2015a; Kurozumi *et al*, 2016).

Quantitative real-time reverse transcription polymerase chain reaction (RT-qPCR). PCR was performed as previously described (Goto *et al*, 2014a; Goto *et al*, 2015a; Kurozumi *et al*, 2016). The expression levels of *miR-224* (Assay ID: 002099) and *miR-452* (Assay ID: 002329) were analysed by TaqMan RT-qPCR (TaqMan MicroRNA Assay; Applied Biosystems) and normalised to *RNU48* (Assay ID: 001006). TaqMan probes and primers for *WWP1* (P/N: Hs00366931_g1) and *GUSB* (P/N: Hs00939627_m1) as an internal control were obtained from Applied Biosystems (Assay-On-Demand Gene Expression Products).

Transfection with mature miRNA and small-interfering RNA (siRNA). The following mature miRNA species were used in this study: Ambion Pre-miR miRNA precursor for *hsa-miR-452* (product ID: PM12509). The following siRNAs were used: Stealth Select RNAi siRNA; *si-WWP1* (cat no. HSS117118 and HSS117119; Invitrogen); and negative control miRNA/siRNA (P/N: AM17111, Applied Biosystems). RNAs were incubated with OPTI-MEM (Invitrogen) and Lipofectamine RNAiMAX reagent (Invitrogen). The transfection procedures and transfection efficiencies of miRNA in PC3 and DU145 cells were reported previously (Goto *et al*, 2014a; Goto *et al*, 2015a; Kurozumi *et al*, 2016).

Cell proliferation, migration, and invasion assays. Cell proliferation was determined by XTT assay using a Cell Proliferation Kit II (Roche Applied Sciences, Tokyo, Japan). Cell migration activity was analysed using uncoated Transwell polycarbonate membrane filters. Cell invasion was evaluated using modified Boyden chambers containing Transwell-precoated Matrigel membrane filter inserts. These assays were carried out as previously described (Goto *et al*, 2014a; Goto *et al*, 2015a; Kurozumi *et al*, 2016).

Genome-wide gene expression and *in silico* analyses for the identification of genes regulated by *miR-452*. We performed a combination of *in silico* and genome-wide gene expression analyses. First, genes regulated by *miR-452* were listed using the TargetScan database. Next, to identify upregulated genes in PCa, we analysed a publicly available gene expression data set in GEO (accession number: GSE29079). Finally, we carried out genome-wide gene expression analysis using *miR-452* transfectants of PC3 and DU145 cells. A SurePrint G3 Human GE 60K Microarray (Agilent Technologies) was used for expression profiling of miRNA transfectants in comparison with negative control miRNA transfectants. Finally, downregulated mRNAs containing *miR-452* target sites were listed as putative target genes.

Western blotting. Immunoblotting was performed with rabbit anti-WWP1 antibodies (1 : 700, ab43791; Abcam), and anti-GAPDH antibodies (1 : 1000, ab8245; Abcam) were used as an internal loading control. Membranes were washed and incubated with anti-rabbit IgG horseradish peroxidase-linked antibodies (7074; Cell Signaling Technology, Danvers, MA, USA). Complexes were visualised with Clarity Western ECL Substrate (Bio-Rad, Hercules, CA, USA), as described in our previous studies (Goto *et al*, 2014a; Goto *et al*, 2015a; Kurozumi *et al*, 2016).

Plasmid construction and dual-luciferase reporter assay. Partial wild-type sequences of the *WWP1* 3′ untranslated region (UTR) or those with deleted *miR-452* target sites (position 150–156 of the *WWP1* 3′ UTR) were inserted between the *Xho*I–*Pme*I restriction sites in the 3′ UTR of the hRluc gene in the psiCHECK-2 vector (C8021; Promega, Madison, WI, USA). The protocol for vector construction was described previously (Goto *et al*, 2014a; Goto *et al*, 2015a; Kurozumi *et al*, 2016).

Immunohistochemistry. A tissue microarray containing a total of 77 prostate specimens was used (Supplementary Table 2). The tissue microarray was obtained from Provitro (Berlin, Germany; Cat #401 2209, Lot #146.1 P020212, 26–46). Detailed information on all cancer specimens can be found at http://www.provitro.com/fileadmin/provitro-data/TMA/4012209.pdf. Immunostaining was evaluated using a previously described scoring method (Kojima *et al*, 2012). Tissue microarray was immunostained with an Ultra-Vision Detection System (Thermo Scientific, Fremont, CA, USA) following the manufacturer's protocol. Primary rabbit polyclonal antibodies against WWP1 (1:200, ab43791; Abcam) were used for immunochemistry. The slides were treated with biotinylated goat antibodies (Histofine SAB-PO kit; Nichirei, Tokyo, Japan).

Statistical analysis. The relationships between two groups and the numerical values obtained by RT-qPCR were analysed using Mann–Whitney *U* tests. Spearman's rank test was used to evaluate the correlations between the expression of *miR-224* and *miR-452*. The relationships among >3 variables and numerical values were analysed using the Bonferroni-adjusted Mann–Whitney *U* test. Survival analysis was analysed by the Kaplan–Meier method and log-rank test, using Stat Mate software (version 4.01, ATMS Co., Tokyo, Japan). All other analyses were performed using Expert StatView (version 5, SAS Institute Inc., Cary, NC, USA).

RESULTS

Expression levels of *miR-224* and *miR-452* in PCa specimens and cell lines. Database analysis demonstrated that these miRNAs were closely located on human chromosome Xq28 within 1000 base pairs, forming a cluster (Figure 1A).

We analysed the expression levels of *miR-224* and *miR-452* in non-PCa ($n=36$) and PCa ($n=54$) clinical specimens. The median PSA level of patients with non-PCa specimens was 7.315 ng/ml (range, 4.3–35.5 ng ml^{-1}). On the other hand, PSA levels in patients with PCa were high, with a median of

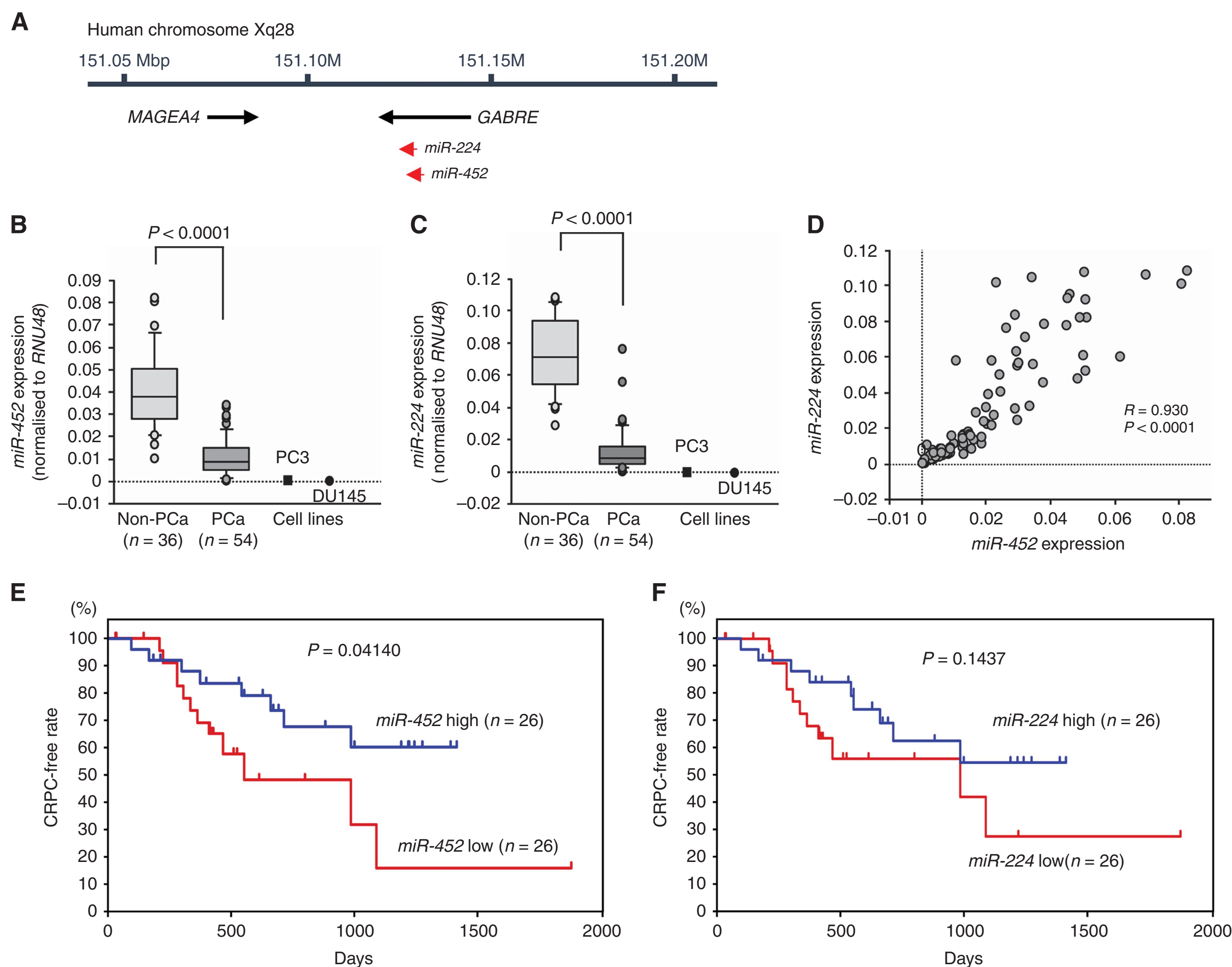

Figure 1. Location and expression levels of clustered *miR-224/452* and its associations with CRPC progression-free interval. (**A**) Location of the *miR-224/452* cluster in the human genome. (**B**) Expression levels of *miR-452* in PCa clinical specimens and cell lines. *RNU48* was used for normalisation. (**C**) Expression levels of *miR-224* in PCa clinical specimens. *RNU48* was used for normalisation. (**D**) Correlations among the relative expression levels of *miR-224* and *miR-452*. (**E**) Kaplan–Meier analysis for expression level of *miR-452* and CRPC-free rate. (**F**) Kaplan–Meier analysis for expression level of *miR-224* and CRPC-free rate.

212 ng ml^{-1} (range, 3.45–3750 ng ml^{-1}). Forty-four patients with PCa had advanced disease with metastasis to lymph nodes, bone, or other sites (Supplementary Table 1).

The expression levels of *miR-224* and *miR-452* were significantly downregulated ($P<0.0001$) in PCa tissues compared with non-PCa tissues (Figure 1B and C). Furthermore, Spearman's rank test showed positive correlations between the expression of *miR-224* and *miR-452* ($R=0.930$ and $P<0.0001$; Figure 1D).

We have previously published data showing the functional significance of *miR-224* and its target *TPD52* (Goto *et al*, 2014b). Therefore, in this study, we performed additional analyses of *miR-452*.

Associations between the expression levels of *miR-452* and CRPC progression in PCa tissues. Among 54 patients with PCa, 52 underwent ADT with luteinising hormone-releasing hormone agonist and anti-androgens. A total of 20 patients progressed to CRPC despite combined androgen blockade (Supplementary Table 1). The risk of progression to CRPC was evaluated in patients with high *vs* low *miR-452* expression. Low expression of *miR-452* was associated with shorter progression-free interval ($P=0.0414$; Figure 1E). However, there was no association between expression level of *miR-224* and progression-free interval ($P=0.1437$; Figure 1F).

Effects of restoring *miR-452* expression on cell proliferation, migration, and invasion in PC3 and DU145 cells. To investigate the functional roles of *miR-452*, we performed gain-of-function studies using miRNA transfection in PC3 and DU145 cells. Cell proliferation was not inhibited in *miR-452* transfectants in comparison with mock- or miR-control-transfected PC3 cells (Figure 2A). However, *miR-452* transfection significantly inhibited cell migration as compared with mock- or miR-control-transfected PC3 and DU145 cells ($P<0.0001$; Figure 2B). Similarly, Matrigel invasion assays demonstrated that cell invasion activity was significantly inhibited in *miR-452* transfectants in comparison with mock- or miR-control-transfected PC3 and DU145 cells ($P<0.0001$; Figure 2C). The representative micrographs of migration and invasion assays are shown in Supplementary Figure 1.

To investigate the synergistic effects of *miR-224* and *miR-452*, we performed migration assay with cotransfection of *miR-224* and *miR-452* in PC3, but they did not show synergistic effects of these miRNAs transfection (Supplementary Figure 2).

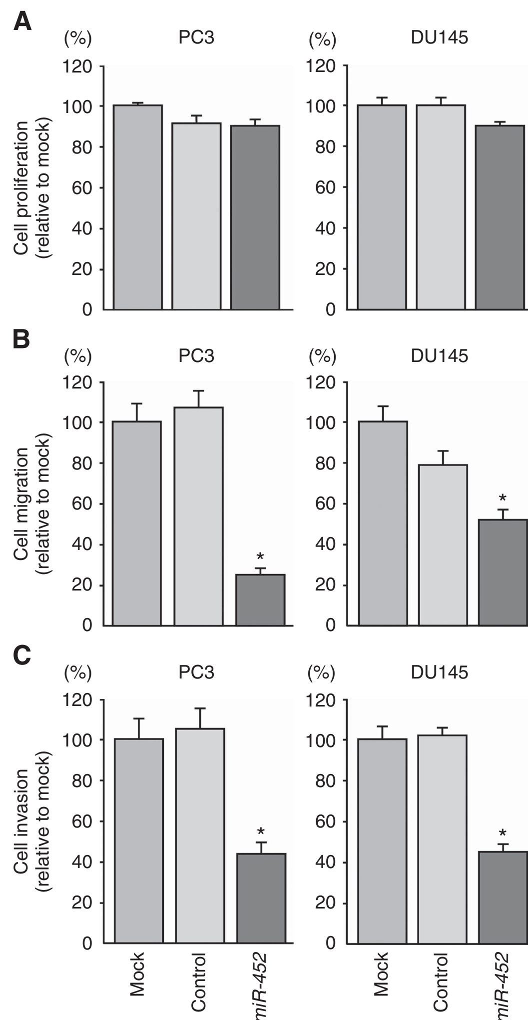

Figure 2. Functional analysis of *miR-452* transfection in PC3 and DU145 cells. (**A**) Cell proliferation was determined 72 h after transfection with *miR-452* using XTT assays. (**B**) Cell migration activity was determined 48 h after transfection with *miR-452* using uncoated Transwell polycarbonate membrane filters. (**C**) Effects of *miR-452* transfection on cell invasion in PC3 and DU145 cells. Cell invasion activity was determined 48 h after transfection with *miR-452* using Matrigel invasion assays. *$P<0.0001$. The bars indicate s.d.'s.

Identification of target genes regulated by *miR-452* in PCa. We performed *in silico* and microarray analysis to identify target genes of *miR-452*. First, the TargetScan programme showed that 3161 genes had putative target sites for *miR-452* in their 3′ UTRs. Next, we investigated the expression statuses of these genes in PCa clinical specimens and examined gene expression profiles in the GEO database (GEO accession number: GSE29079) to evaluate upregulated genes in PCa specimens. Among the 3161 putative target genes of the *miR-452*, 704 genes were significantly upregulated in PCa specimens compared with non-PCa tissues ($\log_2$ ratio >0.1). Finally, we performed genome-wide gene expression analysis using PC3 and DU145 cells (GEO accession number: GSE56243). Ten genes downregulated ($\log_2$ ratio <-0.2) by *miR-452* transfection were identified as putative target genes (Table 1). Methods for *miR-452* targets selection are shown in Supplementary Figure 3. Among these genes, *WWP1* was the most highly upregulated in PCa specimens; therefore, we selected *WWP1* for further studies.

***WWP1* was a direct target of *miR-452* in PCa cells.** To determine whether *miR-452* restoration influences WWP1 expression, real-time RT-qPCR and western blotting were performed using PC3 and DU145 cells. *WWP1* mRNA and WWP1 protein were significantly downregulated by *miR-452* transfection as compared with that in mock- or miR-control-transfected cells ($P<0.0001$; Figure 3A and B).

Next, we carried out luciferase reporter assays to demonstrate whether *miR-452* directly bound to the 3′ UTR of *WWP1*. The TargetScan database predicted that *miR-452* bound at position 150–156 in the 3′ UTR of *WWP1*. We used vectors encoding a partial wild-type sequence of the 3′ UTR of *WWP1* mRNA, including the predicted *miR-452* target site, or a vector lacking the *miR-452* target site. We found that the luminescence intensity was significantly reduced by cotransfection with *miR-452* and the vector carrying the wild-type 3′ UTR of *WWP1*. In contrast, the luminescence intensity was not repressed when the seed sequence of the target site was deleted from the vectors ($P<0.0001$; Figure 3C).

Effects of silencing *WWP1* on cell proliferation, migration, and invasion in PCa cell lines. To investigate the functional role of *WWP1*, we performed loss-of-function studies using *si-WWP1* transfectants. First, we evaluated the knockdown efficiency of *si-WWP1* transfection in PC3 and DU145 cells. RT-qPCR and

Table 1. Downregulated genes in *miR-452* transfectants and upregulated genes in GEO database

Entrez gene ID	Symbol	Gene name	Location	GEO fold change	PC3 *miR-452* transfectant	DU145 *miR-452* transfectant	Average
11 059	*WWP1*	WW domain-containing E3 ubiquitin protein ligase-1	8q21.3	0.7281096	− 0.3316	− 0.2049	− 0.2683
7464	*CORO2A*	Coronin, actin binding protein, 2A	9q22.33	0.608958	− 0.4396	− 0.2172	− 0.3284
2802	*GOLGA3*	Golgin A3	12q24.33	0.4781973	− 0.2149	− 0.2386	− 0.2268
9878	*TOX4*	TOX high mobility group box family member 4	14q11.2	0.44237	− 0.2266	− 0.2445	− 0.2355
7586	*ZKSCAN1*	Zinc finger with KRAB and SCAN domains 1	7q22.1	0.4397794	− 0.2062	− 0.2153	− 0.2107
80 195	*C10orf57*	Chromosome 10 open reading frame 57	10q22.3	0.3867658	− 0.3920	− 0.2061	− 0.2991
2768	*GNA12*	Guanine nucleotide binding protein (G protein) alpha 12	7p22.3	0.2904589	− 0.2326	− 0.2316	− 0.2321
3149	*HMGB3*	High mobility group box 3	Xq28	0.249856	− 0.4748	− 0.2121	− 0.3434
79 034	*C7orf26*	Chromosome 7 open reading frame 26	7p22.1	0.1855203	− 0.2318	− 0.2025	− 0.2172
8087	*FXR1*	Fragile X mental retardation, autosomal homologue 1	3q26.33	0.1841815	− 0.4090	− 0.2704	− 0.3397

Abbreviations: GEO = gene expression omnibus; ID = not applicable.

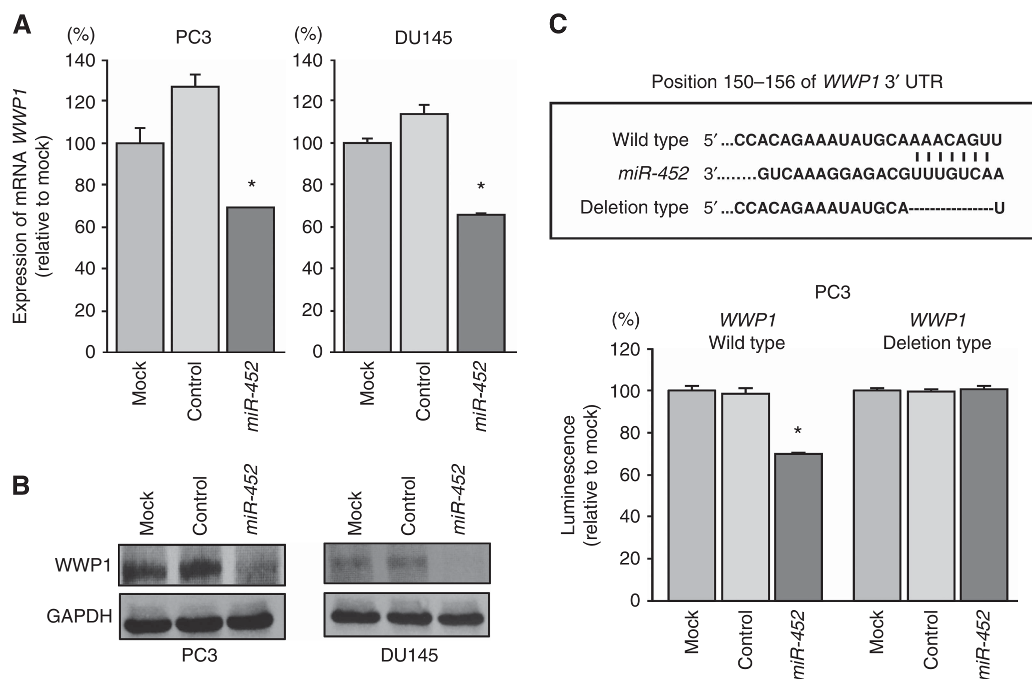

Figure 3. Downregulation of *WWP1* expression by *miR-452* in PC3 and DU145 cells. (**A**) *WWP1* mRNA expression 72 h after transfection with *miR-452*. GUSB was used as an internal control. (**B**) WWP1 protein expression 72 h after transfection with *miR-452*. GAPDH was used as a loading control. (**C**) *miR-452* binding sites in *WWP1* mRNA. Luciferase reporter assays were carried out using a vector encoding the putative *miR-452* target site in the *WWP1* 3′-UTR (position 150–156) for wild-type and deletion constructs. *$P<0.0001$. The bars indicate s.d.'s.

western blotting indicated that si-*WWP1* transfection effectively downregulated *WWP1* mRNA and WWP1 protein expression in PC3 and DU145 cells (Supplementary Figure 4A and B).

In functional assays, cell proliferation was not inhibited by transfection with si-*WWP1* in comparison with mock- or si-control-transfected cells (Figure 4A). However, cell migration and invasion assays demonstrated that cancer cell migration and invasion activity were significantly inhibited by si-*WWP1* transfection in comparison with mock- or si-control-transfected PC3 and DU145 cells ($P<0.0001$; Figure 4B and C). The representative micrographs of migration and invasion assays are shown in Supplementary Figure 1.

Pathways modulated by knockdown of *WWP1* in PCa cells. To further investigate which genes and pathways are modulated by *miR-452–WWP1* signalling, we performed genome-wide gene expression analysis using *si-WWP1* in PC3. After transfection with si-WWP1 in PC3 cells, we selected significantly upregulated or downregulated genes by si-WWP1 transfection (Log2 [si-WWP1/mock] >0.5 or Log2 [si-WWP1/mock] <-1.0) and analysed by KEGG pathways using GeneCodis software (http://genecodis.cnb.csic.es/). Table 2 indicates significantly upregulated and downregulated pathways by knockdown of *WWP1*. A variety of signalling pathways, including the ErbB signalling pathway and transforming growth factor (TGF)-beta signalling pathway, were significantly upregulated by *si-WWP1*. Pathways related to cancer cell migration and invasion, such as 'ECM-receptor interaction' and 'cell adhesion molecules', were significantly downregulated by knockdown of *WWP1* in PC3 cells.

Expression of *WWP1* in clinical PCa specimens. To gain further insights into whether upregulation of WWP1 was correlated with cancerous or precancerous regions, we used tissue microarrays. Immunostaining was evaluated according to a previously described scoring method. Each case was scored on the basis of the intensity and area of staining. A total of 57 PCa samples, 10 prostatic

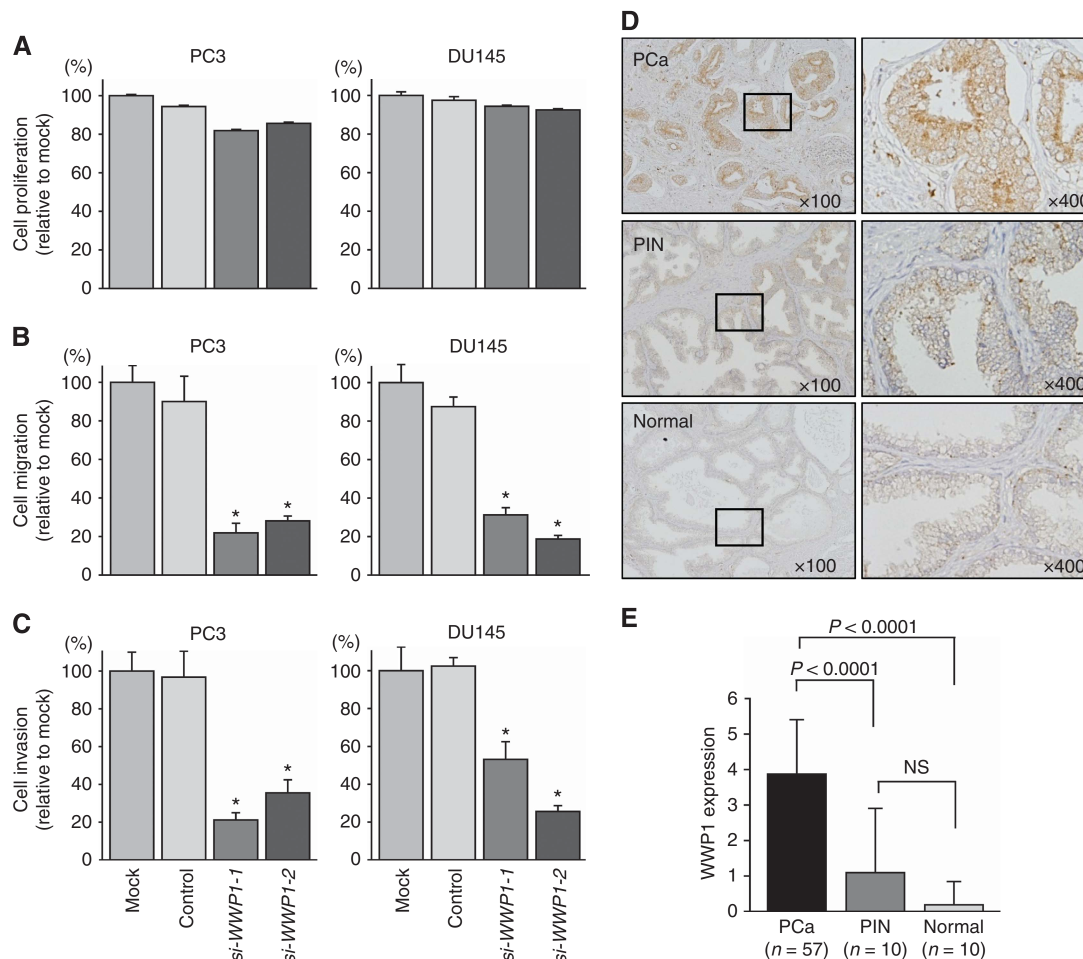

Figure 4. Effects of *WWP1* knockdown on cell proliferation, migration, and invasion in PCa cells and expression of WWP1 in clinical PCa specimens. (**A**) Cell proliferation was determined by XTT assays. (**B**) Cell migration activity was determined using uncoated Transwell polycarbonate membrane filters. (**C**) Cell invasion activity was determined by Matrigel invasion assays. (**D**) Representative image of IHC of WWP1 in the tissue microarray. (**E**) WWP1 was upregulated in PCa compared with PIN and normal tissue in the tissue microarray. *$P<0.0001$. The bars indicate s.d.'s.

intraepithelial neoplasia (PIN) samples, and 10 normal prostate samples were used to analyse WWP1 expression in this study (Supplementary Table 2).

Expression of WWP1 was significantly higher in clinical PCa specimens than in normal prostate specimens ($P<0.0001$; Figure 4D and E). Furthermore, expression of WWP1 was significantly higher in clinical PCa specimens than in PIN specimens ($P<0.0001$; Figure 4D and E). GEO database analysis (accession number GDS2545) showed that *WWP1* expression in metastatic PCa was significantly higher than primary PCa (Supplementary Figure 5).

DISCUSSION

A substantial body of evidence suggests that aberrantly expressed miRNAs disrupt the tightly regulated RNA networks in cancer cells (Garzon *et al*, 2009; Iorio and Croce, 2012). Currently, these destructive events are thought to cause to cancer cell initiation, progression, and metastasis. Therefore, studies of differentially expressed miRNAs in cancer cells should provide important information regarding the molecular mechanisms underlying oncogenesis and metastasis. To date, 2578 human mature miRNAs have been annotated in the publicly available database (miRBase, release 21; http://www.mirbase.org/). MicroRNAs are often associated in clusters in the genome, and several studies have focused on the functional role of clustered miRNAs in human cancers (Mendell, 2008; Goto *et al*, 2015b). In the human genome, 429 human miRNAs have been found to be clustered at 144 sites, with inter-miRNA distances of <5000 bp (miRBase, release 21). The biological significance of clustered miRNAs in the human genome is still largely unknown. We have focused on down-regulated clustered miRNAs in cancer cells based on the miRNA expression signatures and investigated the functional significance of these miRNAs (Kojima *et al*, 2012; Kojima *et al*, 2014; Goto *et al*, 2014a; Goto *et al*, 2015a).

In this study, we focused on *miR-452* because *miR-224* and *miR-452* are located in close proximity on the human chromosome Xq28 region, representing a miRNA cluster. Our previous study showed that *miR-224* acts as a tumour suppressor by targeting oncogenic *TPD52* (Goto *et al*, 2014b). Our present data showed that restoration of *miR-452* significantly inhibited cancer cell migration and invasion, indicating that *miR-452* also acts as a tumour suppressor in PCa cells. The tumour-suppressive role of *miR-452* has been reported in gliomas, targeting stemness regulators, such as Bmi-1, *LEF1* and *TCF4*, and inhibiting stem-like traits (Liu *et al*, 2013). Furthermore, *miR-452* has been shown to function as a prognosis marker for overall survival in patients with glioma. It is well known that hypermethylation of promoter

Table 2. Significantly upregulated/downregulated pathways modulated by knockdown of *WWP1* in PC3

KEGG entry number	Number of genes	Upregulated annotations	*P*-value	Genes
4630	11	Jak-STAT signalling pathway	2.25E-04	*SOS1, PIK3CA, PIK3R2, CCND1, CLCF1, IL6R, IL6ST, SPRED2, EP300, SPRY4, IL24*
4010	15	MAPK signalling pathway	2.27E-04	*MRAS, PTPRR, PPM1B, SOS1, PPM1A, RAPGEF2, MAPK9, PPP3R1, MKNK2, TRAF6, MECOM, CASP3, MAP4K3, TAOK2, PLA2G10*
5200	17	Pathways in cancer	2.51E-04	*PPARD, SOS1, KITLG, COL4A6, E2F3, FOXO1, PIK3CA, PIK3R2, CCND1, CDKN1B, MAPK9, TRAF6, EP300, MECOM, CASP3, FZD4, WNT5A*
4012	8	ErbB signalling pathway	3.09E-04	*SOS1, PIK3CA, PIK3R2, CDKN1B, HBEGF, MAPK9, RPS6KB2, ERBB3*
5215	8	Prostate cancer	3.34E-04	*SOS1, E2F3, FOXO1, PIK3CA, PIK3R2, CCND1, CDKN1B, EP300*
5220	7	Chronic myeloid leukaemia	5.65E-04	*SOS1, E2F3, PIK3CA, PIK3R2, CCND1, CDKN1B, MECOM*
5223	6	Non-small-cell lung cancer	5.75E-04	*SOS1, E2F3, PIK3CA, PIK3R2, CCND1, FOXO3*
4664	7	Fc epsilon RI signalling pathway	7.80E-04	*SOS1, PIK3CA, PIK3R2, SYK, MAPK9, PRKCE, PLA2G10*
5221	6	Acute myeloid leukaemia	8.51E-04	*PPARD, SOS1, PIK3CA, PIK3R2, CCND1, RPS6KB2*
4350	7	TGF-beta signalling pathway	1.13E-03	*INHBB, RBL2, NOG, ID1, EP300, RPS6KB2, ACVR2B*
4910	9	Insulin signalling pathway	1.26E-03	*SOS1, FOXO1, PIK3CA, PIK3R2, PTPN1, INSR, MAPK9, MKNK2, RPS6KB2*
5222	7	Small-cell lung cancer	1.31E-03	*COL4A6, E2F3, PIK3CA, PIK3R2, CCND1, CDKN1B, TRAF6*
4930	5	Type II diabetes mellitus	1.99E-03	*PIK3CA, PIK3R2, INSR, MAPK9, PRKCE*
4110	8	Cell cycle	2.96E-03	*FZR1, E2F3, CCND1, RBL2, CDKN1B, ORC4, EP300, CDKN1C*
4722	8	Neurotrophin signalling pathway	3.11E-03	*SOS1, PIK3CA, PIK3R2, FRS2, MAPK9, TRAF6, FOXO3, PSEN1*
4978	5	Mineral absorption	3.15E-03	*MT2A, SLC8A1, MT1M, MT1E, SLC40A1*
5213	5	Endometrial cancer	3.43E-03	*SOS1, PIK3CA, PIK3R2, CCND1, FOXO3*
4370	6	VEGF signalling pathway	3.52E-03	*PIK3CA, PIK3R2, KDR, PPP3R1, NOS3, PLA2G10*
5010	9	Alzheimer's disease	4.96E-03	*ADAM10, PPP3R1, ITPR2, CASP3, UQCR10, UQCRB, COX8C, CDK5R1, PSEN1*
KEGG entry number	**Number of genes**	**Downregulated annotations**	***P*-value**	**Genes**
4110	10	Cell cycle	6.51E-10	*CDK6, PTTG2, CCNB2, CCNA2, TFDP1, BUB1B, CDC45, SMAD4, BUB1, CDK1*
5146	6	Amoebiasis	1.25E-05	*IL1B, SERPINB3, CXCL1, SERPINB4, CSF2, FN1*
4115	5	p53 signalling pathway	2.15E-05	*CDK6, CCNB2, THBS1, GTSE1, CDK1*
5323	5	Rheumatoid arthritis	6.42E-05	*IL1B, CXCL6, CXCL1, LTB, CSF2*
4512	4	ECM-receptor interaction	8.34E-04	*THBS1, ITGB8, COL6A3, FN1*
4914	4	Progesterone-mediated oocyte maturation	9.10E-04	*CCNB2, CCNA2, BUB1, CDK1*
4060	6	Cytokine–cytokine receptor interaction	1.94E-03	*IL1B, CXCL6, CXCL3, CXCL1, LTB, CSF2*
4114	4	Oocyte meiosis	2.26E-03	*PTTG2, CCNB2, BUB1, CDK1*
4510	5	Focal adhesion	3.12E-03	*MYL10, THBS1, ITGB8, COL6A3, FN1*
4810	5	Regulation of actin cytoskeleton	4.01E-03	*DIAPH3, BDKRB2, MYL10, ITGB8, FN1*

Abbreviations: ECM = extracellular matrix; KEGG = Kyoto Encyclopedia of Genes and Genomes; VEGF = Vascular endothelial growth factor; *WWP1* = WW domain-containing E3 ubiquitin protein ligase-1.

regions containing CpG islands is closely linked with gene silencing in cancer cells. Recent study showed that *miR-452-miR-224* locus was downregulated in PCa compared with nonmalignant prostate tissue specimens. Downregulation of these miRNAs were associated with frequent aberrant promoter hypermethylation (Kristensen *et al*, 2014). Furthermore, *GABRE* as *miR-452* and *miR-224* host gene was silenced by aberrant promoter hypermethylation, and methylation status of this region was a useful biomarker for biochemical recurrence after radical prostatectomy (Kristensen *et al*, 2014). However, another group demonstrated the oncogenic function of *miR-452* in hepatocellular carcinoma targeting *CDKN1B* (Zheng *et al*, 2014). Likewise, *miR-452* has been shown to be upregulated in advanced melanoma, thereby promoting the epithelial-mesenchymal transition (Knoll *et al*, 2014). One of the mechanisms of *miR-452* upregulation in melanoma is induction by E2F1, which has been shown to directly activate the *miR-224/452* cluster (Knoll *et al*, 2014). Therefore, investigation of the molecular mechanisms of transcriptional control of the *miR-224/452* cluster is necessary in various types of cancer.

According to prior studies, the function of *miR-452* varies greatly depending on the type of cancer. *miR-452* may have opposing roles in different types of cancer by targeting different pathways or cancer-associated genes. We narrowed down putative candidate genes of *miR-452* regulation to 10 genes in this study, and we focused on *WWP1* and performed further analysis. As for other candidate genes of *miR-452* regulation, the functional significance of PCa is still unclear. Among them, *GNA12* as a member of G-proteins is reported to contribute to cancer cell invasiveness (Rasheed *et al*, 2013). Aberrant G-protein-coupled

receptors (GPCRs)-mediated signal enhanced cancer cell progression and aggressiveness, and GPCR signal primarily through heterotrimeric G-proteins (Gα, Gβ, and Gγ). Upregulation of *GNA12/13* were involved in aggressiveness and observed in advanced cancer tissues (Kelly *et al*, 2006; Rasheed *et al*, 2013). *HMGB3* contains one or more high mobility group DNA-binding motifs, and overexpression of *HMGB3* was reported in several cancers (Li *et al*, 2015). Interestingly, *HMGB3* was direct regulation of *miR-205* in breast cancer (Elgamal *et al*, 2013). Our recent study demonstrated that *miR-205* act as a tumour-suppressive miRNA in PCa cells through targeting centromere protein-F (Nishikawa *et al*, 2015). These facts suggest that the putative target gene list for *miR-452* contains important oncogenic genes involved in PCa pathogenesis. In this study, we showed that *WWP1* was a direct target of *miR-452* and that knockdown of *WWP1* significantly inhibited cancer cell proliferation, migration, and invasion in PCa cells. WWP1 is highly conserved among different animals and is ubiquitously expressed in many tissues (Zhi and Chen, 2012). Accumulating evidence suggests that E3 ubiquitin ligases play important roles in cancer development (Chen and Matesic, 2007; Zou *et al*, 2015). Overexpression of WWP1 was observed in breast and PCa (Chen *et al*, 2007; Zhou *et al*, 2012). Furthermore, significantly higher expression of *WWP1* in PCa bone metastasis has been reported (Wang *et al*, 2014). Previous studies have indicated that overexpression of the mRNA and protein levels of WWP1 is significantly correlated with gene copy number gains in both types of cancers (Chen *et al*, 2007; Zhou *et al*, 2012), suggesting that WWP1 acts as an oncogene in these cancers. Interestingly, *WWP1* was located on the human chromosome 8q21 region, which frequently displays gain of copy numbers in human cancers, including breast cancer and PCa (Byrne *et al*, 2012). Our previous study showed that tumour protein D52 (TPD52) located on chromosome 8q21 region was direct regulation of tumour-suppressive *miR-224* in PCa cells (Goto *et al*, 2014b). A number of studies have reported that human chromosome 8q21.11–8q21.3 regions were frequently amplified in various cancers, including PCa (Byrne *et al*, 2012). Previous studies of PCa cells showed that *TPD52* containing region was amplified and expression of *TPD52* was highly elevated in cancer tissues (Rubin *et al*, 2004). Amplified genome regions are increasingly considered as targets of cancer therapy. Interestingly, *TPD52* and *WWP1* were located in this region, indicating that novel cancer pathways mediated by these responsible genes might be highlighted as PCa therapeutic targets.

WWP1 targets and ubiquitinates a variety of cancer-related proteins, including p53, p63, and Smad4, in several cancers (Moren *et al*, 2005; Laine and Ronai, 2007; Li *et al*, 2008). In hepatocellular carcinoma cells, silencing of *WWP1* expression promotes cleavage of caspase3 protein and expression of p53; these events suppress cell growth and promote apoptosis in cancer cells (Cheng *et al*, 2014). Another study showed that WWP1 increases the stabilisation of p53 protein in the cytoplasm and decreases p53 transcriptional activity (Laine and Ronai, 2007).

Interestingly, several studies have indicated that WWP1 acts as a regulator of receptor signalling in cancer cells. For example, WWP1 enhances ErbB2 and EGF receptor signalling through regulating ring finger protein 11 (RNF11), a negative regulator of these receptors (Chen *et al*, 2008). TGF-b receptor type 1 is degraded by WWP1 ubiquitination, and these events cause inhibition of TGF-b signalling (Komuro *et al*, 2004; Chen *et al*, 2007). Moreover, other studies have suggested that WWP1 interacts with ezrin and is involved in MET signalling. The MET receptor and its ligand, hepatocyte growth factor, have important effects on normal epithelial cells and cancer cells (Karamouzis *et al*, 2009; Gherardi *et al*, 2012). Ezrin is thought to be involved in several signalling pathways, such as cell adhesion to the extracellular matrix and receptor tyrosine-kinase signalling (Geissler *et al*, 2013; Goni *et al*, 2014; Oneyama *et al*, 2015). Overexpression of ezrin has been observed in several types of cancers (Li *et al*, 2015; Ren *et al*, 2015; Singh *et al*, 2016). Thus, interaction of WWP1 with ezrin activates MET signalling, playing a pivotal role in cancer cell progression and metastasis.

In this study, we identified WWP1-mediated cancer pathways by using genome-wide gene expression analysis of *si-WWP1* transfected cells. Our data showed that several pathways were involved in WWP1 downstream pathways, such as the 'TGF-beta signalling pathway', 'ECM-receptor interaction', 'cell adhesion molecules', 'focal adhesion', and 'regulation of actin cytoskeleton'. The identification of these novel molecular pathways and targets mediated by the *miR-452/WWP1* axis may lead to a better understanding of PCa progression and metastasis.

CONCLUSIONS

Downregulation of *miR-452* was validated in PCa clinical specimens, and this miRNA was shown to function as a tumour suppressor in PCa cells. Expression of *miR-452* predicted a short duration of progression to CRPC. To the best of our knowledge, this is the first report demonstrating that tumour-suppressive *miR-452* directly targeted WWP1. Moreover, WWP1 was upregulated in PCa clinical specimens and contributed to cancer cell invasion, indicating that this target functioned as an oncogene. The identification of novel molecular pathways and targets regulated by the *miR-452/WWP1* axis may lead to a better understanding of PCa progression and metastasis.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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