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microRNA-183 is an oncogene targeting Dkk-3 and SMAD4 in prostate cancer

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Background: The purpose of this study was to identify prostate cancer (PC) oncogenic microRNAs (miRs) based on miR microarray and to investigate whether these oncogenic miRs may be useful as PC biomarkers.

Methods: Initially, we carried out miR microarray and real-time PCR using RWPE-1, PC-3, DU-145 and LNCaP cells. To investigate the function of miR-183, we used a miR-183 knockdown inhibitor in cell growth and wound-healing assays. We used several algorithms and confirmed that they are directly regulated by miR-183.

Results: We identified three potential oncogenic miRs (miR-146a, miR-183 and miR-767-5P). The expression of miR-183 in PC cells (PC-3, DU-145 and LNCaP) was upregulated compared with RWPE-1 cells. MiR-183 expression was also significantly higher in PC tissues compared with that in matched normal prostate tissues. Additionally, miR-183 expression was correlated with higher prostate-specific antigen, higher pT and shorter overall survival. MiR-183 knockdown decreased cell growth and motility in PC cells and significantly decreased prostate tumour growth in *in vivo* nude mice experiments. We identified Dkk-3 and SMAD4 as potential target genes of miR-183.

Conclusion: Our data suggest that oncogenic miR-183 may be useful as a new PC biomarker and that inhibition of miR-183 expression may be therapeutically beneficial as a PC treatment.

Prostate cancer (PC) is the second leading cause of cancer death among men, accounting for 11% of deaths in the United States (Jemal *et al*, 2010). The etiology of PC is largely unknown, although several risk factors, such as ethnicity, family history and age, are associated with the disease (Pienta and Esper, 1993; Roemeling *et al*, 2006). In addition, several dietary constituents have been linked to PC risk and prevention (Shukla and Gupta, 2005; Chan *et al*, 2006). As prostate-specific antigen (PSA) screening test has spread, the number of cured patients has tended to increase. Also a significant number of patients with lymph node metastasis are identified during radical prostatectomy (Cheng *et al*, 2001) and 50% of those patients diagnosed with metastasis die within 5 years (Pound *et al*, 1999). Therefore, new tumour markers and effective therapeutic strategies are needed for PC.

MicroRNAs are well-known examples of non-coding RNAs (Ying *et al*, 2008) and identified human miRs currently number

1424 according to miRBase (<http://www.mirbase.org/>). MicroRNAs bind to the 3'UTR of target gene mRNA and repress protein translation or induce mRNA cleavage (McManus and Sharp, 2002), thereby inhibiting protein expression. It has been reported that aberrant expression of miRs occurs in PC (Volinia *et al* 2006; Porkka *et al*, 2007; Ambs *et al*, 2008; Ozen *et al*, 2008; Schaefer *et al*, 2010). Decreased expression of tumour-suppressor miRs result in increased expression of target oncogenes, whereas increased expression of oncogenic miRs leads to loss or decreased expression of target tumour-suppressor genes.

Therefore, the aim of this study is to identify new oncogenic miRs that influence PC progression and validate their function. Another aim of this project is to identify new therapeutic methods for PC treatment. In this study, we identified PC oncogenic miRs based on miR microarray data. After validating the microarray results, we identified miR-183 as a potential PC oncogenic

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miR. We confirmed its expression in PC tissues and performed functional analyses. We also established miR-183 low-stable expressing PC cell lines using a lenti-viral system and tested whether miR-183 inhibitor decreased PC growth compared with control inhibitor. Finally, we used a target scan algorithm (microRNA.org) to identify Dkk-3 and SMAD4 as target genes of miR-183. We also performed 3'UTR luciferase assays and western blot analysis to look at target gene protein expression in miR-183 knockdown prostate cells. Finally, we showed that Dkk-3 and SMAD4 overexpression decreased the tumourigenicity of PC cells via WNT/ β -catenin signalling.

MATERIALS AND METHODS

Cell lines and cell cultures. RWPE-1 cells derived from normal prostate epithelial cells were used as controls (ATCC, Manassas, VA, USA). Prostate cancer cell lines (PC-3, DU-145 and LNCaP) were purchased from the ATCC. Permanent stocks of cells were prepared and all cells were stored at -80°C until use. Cells were used for experiments within 6 months. RWPE-1 cells were cultured in keratinocyte-SFM (GIBCO/Invitrogen, Carlsbad, CA, USA). Prostate cancer cell lines were cultured in RPMI-1640 medium (UCSF Cell Culture Facility, San Francisco, CA, USA) supplemented with 10% fetal bovine serum.

MicroRNA microarray. For miR microarray, total RNA was extracted from RWPE-1, PC-3 and LNCaP cells using a miRNeasy Mini Kit. MicroRNA microarray analysis was carried out and analysed by LC Science (Houston, TX, USA).

Clinical samples. A total of 31 patients with pathologically confirmed PC were enrolled in this study (Veterans Affairs Medical Center at San Francisco). Written informed consent was obtained from all patients and the study was approved by the UCSF Committee on Human Research (Approval number: H9058-35751-01). The detailed information of patients is shown in Supplementary Table 1.

Transfection and RNA extraction. Ambion Anti-miR miRNA inhibitors (Negative Control/hsa-miR-183, Ambion/Applied Biosystems, Foster City, CA, USA) were transfected with siPORT NeoFX transfection agent (Ambion). Plasmids and pmirGLO dual-luciferase miRNA target expression vector (Promega, Madison, WI, USA) were transiently transfected into cells with Lipofectamine 2000 (Invitrogen). RNA (miR and total RNA) was extracted from formalin-fixed, paraffin-embedded (FFPE) human PC ($n = 31$) and matched adjacent non-cancerous normal prostate tissues or benign prostate hyperplasia tissues using a miRNeasy FFPE kit (Qiagen, Valencia, CA, USA) after laser capture micro-

dissection based on pathologist reviews. MicroRNAs and total RNA were extracted from cell lines using a miRNeasy Mini and RNeasy Mini Kit (Qiagen).

Cell viability assay. Viability of PC cells was measured by MTS assay (CellTiter 96 Aqueous One Solution Cell Proliferation Assay, Promega) 4 days after transfection of anti-miR miRNA inhibitors and plasmids (Mock/Dkk-3/SMAD4). Cell viability was determined by absorbance measurements at 490 nm using SpectraMAX 190 (Molecular Devices, Sunnyvale, CA, USA).

Wound-healing assay. Prostate cancer cells were seeded in six-well plates and transfected with anti-miR miRNA inhibitors. At 24 h after transfection, cells were transferred from 6-well plates to 12-well plates. After 24 h, a wound was formed by scraping the cells with a 200- μl pipette tip and washing twice with medium. Cells were observed at 0 and 48 h after scraping and photographed with a microscope (Nikon, Tokyo, Japan).

Plasmid. PrecisionShuttle pCMV6-Entry Vector and Myc-DDK-tagged ORF clone of homo sapiens dickkopf homolog-3 (*Xenopus laevis*, Dkk-3), transcript variant 1 as transfection-ready DNA NM_015881.5 were purchased from ORIGENE (Rockville, MD, USA). In order to make a SMAD4 overexpressing plasmid, SMAD4 was amplified with total RNA from RWPE-1 by reverse transcription-PCR (RT-PCR).

The following primers were used: SMAD4 NheI cloning forward primer, 5'-GCTAGCttgcttcagaaattggagacatt-3'; SMAD4 XhoI cloning reverse primer, 5'-CTCGAGattttgtagtcaccatcctgataa-3'. Polymerase chain reaction products were cloned into the pTarget-Mammalian Expression Vector System (Promega). pCMV6-SMAD4 was obtained by subcloning a NheI-XhoI fragment from pTarget-SMAD4 into the NheI-XhoI site of pCMV6-Entry Vector.

Luciferase reporter assay. A pmirGLO dual-luciferase miRNA target expression vector was used for 3'UTR luciferase assays (Promega). The 24 target-suppressor genes of miRNA-183 were selected based on a target scan algorithm (microRNA.org. (<http://www.microRNA.org/microRNA/home.do>)). Table 1 shows the primer sequences used for the 3'UTR plasmids of Dkk-3 and SMAD4. Supplementary Table 2 shows the primer sequences used for the 3'UTR plasmids of 22 genes that subsequent 3'UTR luciferase assays showed were not significantly affected. Plasmids for 3'-UTR luciferase assays were made as described previously (Ueno *et al*, 2011). For 3'-UTR luciferase assay, PC-3 cells were transfected with hsa-miR-183 precursor and pmirGLO Dual-Luciferase miRNA target expression vectors with wild-type or mutant target sequence using Lipofectamine 2000 (Invitrogen).

Table 1. Primer sequences

Primer name	Sequence
Dkk-3 forward	5'- AAAC TAGCGGCCGCTAGT ^{aa} TGATGTTTTCAGGTGTCAT ^g T-3'
Dkk-3 reverse	5'- CTAGA cATGACACCTGAAAACATCA ^{tt} ACTAGCGGCCGCTAGTTT-3'
Mutated Dkk-3 forward	5'- AAAC TAGCGGCCGCTAGT ^{aa} CTCTGCTCTGAGTCTTGCC ^g T-3'
Mutated Dkk-3 reverse	5'- CTAGA cGGCAAGACTCAGAGCAGAG ^{tt} ACTAGCGGCCGCTAGTTT-3'
SMAD4 forward	5'- AAAC TAGCGGCCGCTAGT ^{ct} TCAAAAAATAATGTGCCAT ^g T-3'
SMAD4 reverse	5'- CTAGA cATGGCACATTATTTTTGA ^{ag} ACTAGCGGCCGCTAGTTT-3'
Mutated SMAD4 forward	5'- AAAC TAGCGGCCGCTAGT ^{ct} CGCCCCCCCCCTGGCC ^g T-3'
Mutated SMAD4 reverse	5'- CTAGA cGGCCAGAGGGGGGGGGCG ^{ag} ACTAGCGGCCGCTAGTTT-3'

Bold shows PmeI (AAAC/GTTT) and XbaI (T/CTAGA) sites. Underline shows NotI internal site. Italics show the target sequence.

β -Catenin has an important role in Wnt- β -catenin signalling in cancer cells. Stabilised β -catenin translocates to the nucleus and complexes with Tcf regulating the expression of several oncogenes. To monitor Wnt/ β -catenin signal-transducing activity, we used TOPflash reporter plasmid containing Tcf-binding sites and measured Tcf transcriptional activity to observe the β -catenin-dependent pathway. For TOPflash luciferase assay, PC-3 cells were transiently co-transfected with pCMV6-Entry Vector, pCMV6-Dkk-3, pCMV6-SMAD4, TOPflash (Upstate, Lake Placid, NY, USA) and pRL-TK Vector (Promega) encoding Renilla luciferase as an internal control for transfection efficiency using FuGENE HD (Roche Diagnostics, Basel, Switzerland). Luciferase assay was performed using the Dual-Luciferase Reporter Assay System (Promega) at 48 h after transfection. Data are presented as the mean value \pm s.d. for triplicate experiments and the level of luciferase activity obtained with wild-type sequence or pCMV6-Dkk-3/pCMV6-SMAD4 vectors compared with mutant-type sequence or empty vectors in transfected cells that are normalised to 1.

TOPflash luciferase assays were performed to examine the effect of miR-183 on the Wnt/ β -catenin signalling pathway. Initially, anti-miR-183 inhibitor was transfected into PC-3 and DU-145 cells with siPORT NeoFX and after 48 h cells were trypsinised and re-suspended on 48-well plates. After 16 h, TOPflash and pRL-TK transfection were performed. Lysates were harvested after 48 h and TOPflash activity was measured as described above.

Quantitative RT-PCR. Extracted total RNA was reverse-transcribed into single-stranded cDNA with a TaqMan microRNA reverse transcription kit (Applied Biosystems). Real-time PCR was performed using first strand cDNA with TaqMan Fast Universal PCR Master Mix (Applied Biosystems). The assay numbers for the miR endogenous control (RNU48) and target miRs were as follows: RNU48 (001006), miR-183 (002269), miR-96 (000434), miR-182 (002334), β -actin (Hs99999903_m1), Dkk-3 (Hs00247426_m1) and p21 (Hs00355782_m1). Quantitative PCR was performed on an Applied Biosystems Prism 7500 Fast Sequence Detection System (Applied Biosystems). Quantitative PCR parameters for cycling were as follows: 95 °C for 20 s 40 cycles of PCR at 95 °C for 3 s and 60 °C for 30 s. All reactions were done in a 10- μ l reaction volume in triplicate. The mRNA and miR expression levels were determined using the $2^{-\Delta CT}$ method.

Western blot analysis. For total protein extraction, at 24 and 72 h after transfection, cells were washed in ice-cold phosphate-buffered saline and added to RIPA lysis and extraction buffer (Fisher Scientific, Pittsburgh, PA, USA) containing Protease Inhibitor Cocktail I (Millipore, Billerica, MA, USA). Dishes were incubated for 5 min on ice and cells were collected with a cell lifter and incubated for 30 min on ice followed by centrifugation at 12 000 g for 20 min at 4 °C. The supernatant as total protein was collected.

For nuclear and cytoplasmic fraction protein, at 48 h after transfection, cells were washed in ice-cold phosphate-buffered saline and re-suspended in cold buffer containing 10 mM HEPES, pH 7.3, 10 mM KCl, 1 mM EGTA, 0.1 mM EDTA and Protease Inhibitor Cocktail I were lysed in 0.5% IGEPAL CA-630 (Sigma, St Louis, MO, USA) for 15 min followed by centrifugation at 1000 g for 5 min at 4 °C. The supernatant as cytoplasmic fraction protein was collected. The pellet was re-suspended in cold buffer containing 20 mM HEPES, pH 7.3, 400 mM NaCl and Protease Inhibitor Cocktail I were lysed for 30 min followed by centrifugation at 17 000 g for 15 min at 4 °C. The supernatant as nuclear fraction protein was collected. Extracted protein was analysed using primary antibodies, followed by anti-mouse and -rabbit IgG HRP-conjugated secondary antibodies (Cell Signaling Technology, Beverly, MA, USA), and were visualised with LumiGLO Reagent and peroxide reagent (Cell Signaling Technology). The primary antibodies used were as follows: anti-Dkk-3 antibody 1:100

dilution (#10365-1-AP, Proteintech Group, Inc., Chicago, IL, USA), anti-SMAD4 antibody (1:200 dilution, #9515, Cell Signaling), anti- β -catenin antibody (1:1000 dilution, #9562, Cell Signaling), anti-CREB antibody (1:100 dilution, #9197, Cell Signaling) and anti- β -actin antibody (1:2000 dilution #3700, Cell Signaling Technology).

Establishment of stable miR-183 knockdown cells and inhibition of *in vivo* cell growth. Lentivirus system transfection was performed using Lenti-Pac HIV Expression Packaging Kit (Gene Copoeia, Rockville, MD, USA) according to the manufacturer's instructions. Hsa-miR-183 inhibitor vector (HmiR-AN0244-AM03, GeneCopoeia) or miRNA inhibitor scrambled control clone for pEZ-AM03 (CmiR-AN0001-AM03, GeneCopoeia) with Lenti-Pac HIV mix were transfected into 293Ta cells (Gene Copoeia) and medium was replaced with fresh medium containing 1/500 volume of the TiterBoost reagent 14 h after transfection. The supernatant containing lentiviral particles was collected in sterile tubes 48 h after medium replacement, centrifuged at 500 g for 10 min and filtered using a 0.45- μ m PES filter (Whatman/Fisher Scientific). PC-3 cells were infected with lentiviral particles with Polybrene (8 μ g ml⁻¹; Sigma-Aldrich), medium containing lentiviral particles was replaced with fresh medium after 24 h and then incubated for 72 h. Stably transfected cells were selected after transfection using Hygromycin (100 μ g ml⁻¹, Invitrogen, Carlsbad, CA, USA). Medium containing Hygromycin was replaced every 3 days. Stable miR-183 knockdown PC cells or control cells were injected subcutaneously into the right back side flanks of a 5-week-old nu/nu mice. Before injection, miR-183 expression was confirmed by real-time PCR to determine whether the miR-183 expression was significantly lower compared with that in control cells. Animals consisted of four female nude mice (strain BALB/c nu/nu; Charles River Laboratories, Inc., Wilmington, MA). Tumour size was measured with calipers once a week for 6 weeks, and tumour volume was calculated on the basis of width (x) and length (y): $x^2y/2$, where $x < y$. All animal care was in accordance with the guidelines of the San Francisco Veterans Affairs Medical Center and the study was approved by the San Francisco VA IACUC (Protocol number: 11-003-01).

Colony formation assay. Scramble control and miR-183 knockdown-stable transfectants were seeded in six-well microplates at a density of 100 cells per well. After 16 days, cells (Scramble and miR-183 knockdown-stable transfectants) were stained with HEMA 3 STAIN SET (Fisher Scientific). The stained area was measured by using ImageJ software.

Immunohistochemistry. Nine paraffin-embedded specimens were used for immunohistochemistry. Antigen retrieval was carried out by microwaving in citrate buffer (Thermo Scientific, Waltham, MA, USA). Slides were incubated at 4 °C overnight with anti-SMAD4 antibody (#ab40759, Abcam, Cambridge, MA, USA) and anti-Dkk-3 antibody (Proteintech Group, Inc.). The Thermo Scientific Lab Vision Ultra Vision Detection System (Thermo Scientific) was used as chromogen. Immunohistochemical staining was evaluated by visually assessing staining intensity (0–3). We used the Human Protein Atlas (<http://www.proteinatlas.org/>) as a reference for immunohistochemistry assessment. The criteria of intensity are as follows: 0, negative expression; 1+, weakly positive expression; 2+, moderate positive expression; 3+, strongly positive expression.

Statistical analysis. All statistical analyses were performed using GraphPad prism 5 software (GraphPad Software, San Diego, CA, USA). A P -value of <0.05 was regarded as statistically significant.

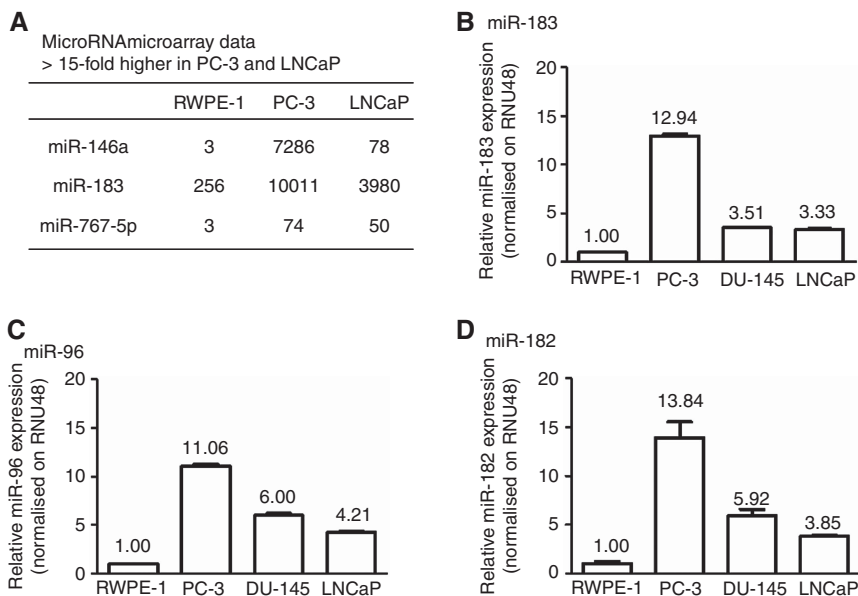


Figure 1. Oncogenic miRNAs in prostate cancer cells. (A) MicroRNA-microarray data (LC Science). Three microRNAs were found to have expression levels in prostate cancer cell lines (PC-3 and LNCaP) that are >15-fold higher than normal RWPE-1 cells. Real-time PCR results are shown as follows: (B) MiR-183, (C) miR-96 and (D) miR-182. Expression was normalised to RNU48. Data are presented as the mean value ± s.d. from three independent experiments and the level of miR-183, miR-96, miR-182 in each cell line was compared with that of RWPE-1 cells normalised as 1.

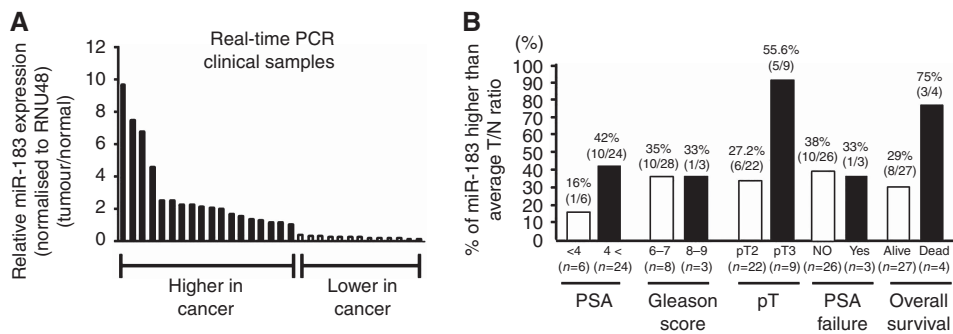


Figure 2. MiR-183 expression in human prostate tissues and association of miR-183 expression and clinicopathological parameters. (A) The expression levels of miR-183 in primary adjacent normal and prostate tumour tissues (n = 31) were examined by real-time PCR. MiR-183 expression levels in tumour tissues are presented and compared with the level in adjacent normal tissues normalised as 1. (B) Association of miR-183 expression and several clinicopathological parameters (PSA at diagnosis, Gleason score, pT, PSA failure, overall survival).

RESULTS

Expression of miR-183 in prostate cell lines and primary tissues and association of miR-183 with clinicopathological parameters.

To identify oncogenic miRs in PC, miRs were screened by a commercial miR microarray service using normal prostate epithelial cell (RWPE-1) and PC cell lines (PC-3 and LNCaP). Expression levels of three miRs (miR-146a, miR-183 and miR-767-5p) in PC-3 and LNCaP cells were 15-fold higher than that of RWPE-1 cells (Figure 1A). To validate miRNA expression, we performed real-time PCR and found that miR-183 expression in PC cells (PC-3, DU-145 and LNCaP) was significantly higher than RWPE-1 (Figure 1B). However, miR-146a and miR-767-5p expression in PC cells (PC-3, DU-145 and LNCaP) was not significantly different from RWPE-1 (data not shown). We also observed miR-182 and miR-96 expression level because miR-182, miR-183 and miR-96 are clustered genes. As expected, the expression of miR-182 and miR-96 were very similar to miR-183 expression as shown in Figure 1C and D.

To analyse miR-183 expression in clinical samples, miR-183 expression levels in clinical samples (31 samples) were investigated by real-time PCR. We compared miR-183 expression in prostate tumour (T) and adjacent normal tissues (N). Of 31 samples, the T/N ratio was more than 1.0 in 18 samples (58%), indicating that miR-183 was significantly higher in PC tissues compared with adjacent normal prostate tissues (Figure 2A). Interestingly, higher miR-183 expression was associated with higher PSA at diagnosis, higher pT and shorter overall survival after radical prostatectomy (Figure 2B). The data in Supplementary Figure 1 were from the public database GEO (accession #GSE21036; <http://www.ncbi.nlm.nih.gov/geo/>). MiR-183 expression was significantly higher in PC tissues compared with normal prostate tissues (Supplementary Figure 1).

Functional effects of miR-183 on normal prostate cells. To confirm the function of miR-183 in normal prostate cells, miR-183 precursor was transiently transfected into a normal prostate cell line (RWPE-1). The expression of miR-183 was significantly higher in miR-183 precursor-transfected RWPE-1 cells compared with

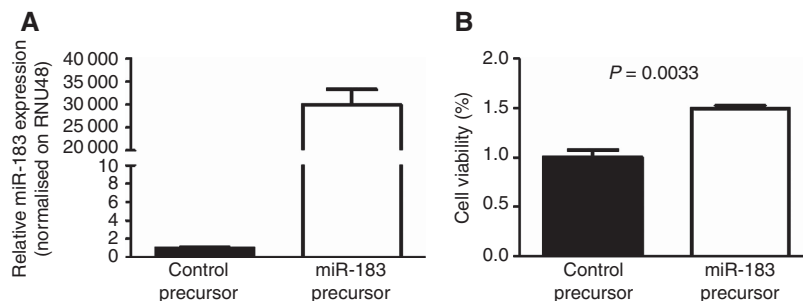


Figure 3. Functional effects of miR-183 overexpression in a normal prostate cell line (RWPE-1). (A) MiR-183 expression levels in RWPE-1 cell were measured by using real-time PCR at 24 h after transient transfection of miR-183 and control precursor. Data are presented as the mean value \pm s.d. for three independent experiments and compared with the level of control precursor-transfected cells normalised as 1. (B) Cell viability was analysed by the MTS assay 4 days after transient transfection.

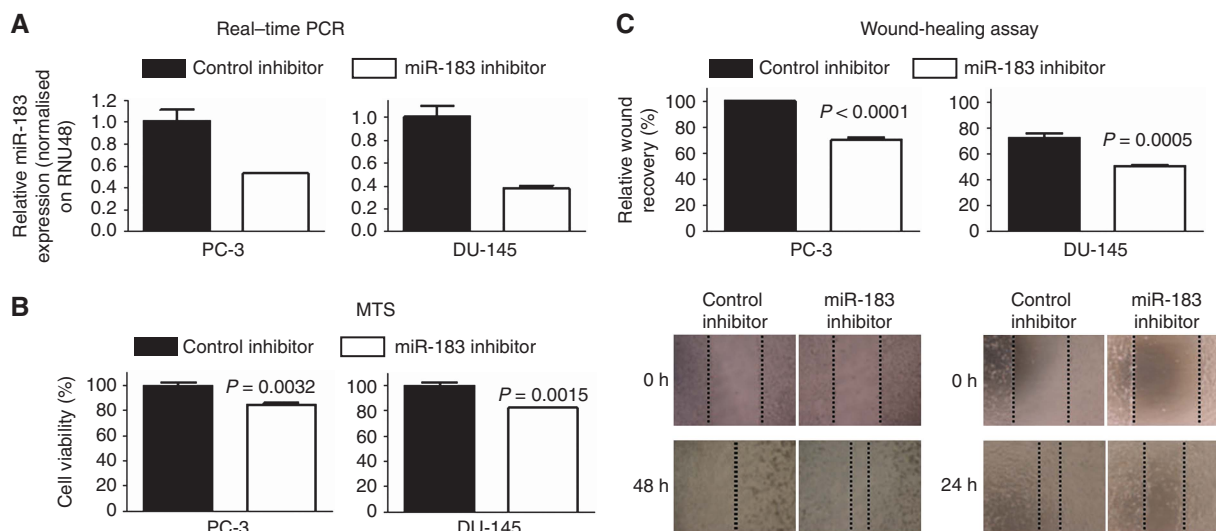


Figure 4. Functional effects of miR-183 knockdown in transfected PC-3 and DU-145 cells. (A) MiR-183 expression levels in prostate cancer cell lines (PC-3, DU-145) were measured by using real-time PCR at 48 h after transient transfection of miR-183 and control inhibitor. Data are presented as the mean value \pm s.d. for three independent experiments and compared with the level of control inhibitor-transfected cells normalised as 1. (B) Cell viability was analysed by the MTS assay 4 days after transient knockdown transfection. (C) Wound-healing assay with miR-183 inhibitor transfected cells. At 24 h after transient transfection, cells were transferred from 6-well to 12-well plates and further incubated for 24 h. A wound was formed by scraping and the width of the wound measured after 48 h (PC-3 cells) and 24 h (DU-145 cells).

control at 24 h after transfection (Figure 3A). Cell viability was also 1.5-fold higher in miR-183 precursor transfectants compared with controls at 4 days after transfection (Figure 3B).

Functional effects of miR-183 on PC cell lines. To analyse the function of miR-183 in PC cells, miR-183 and control inhibitors were transiently transfected into PC-3 and DU-145 cells. The expression level of miR-183 in miR-183 inhibitor-transfected PC cells was significantly decreased compared with control inhibitor at 48 h after transfection (Figure 4A). Cell viability was also decreased in miR-183 inhibitor transfectants compared with controls at 4 days after transfection (Figure 4B). Cell motility was also significantly decreased in miR-183 inhibitor-transfected cells (Figure 4C).

miR-183 knockdown inhibits *in vivo* cell growth. To analyse cell growth *in vivo* in a nude mouse xenograft model, lentivirus vectors expressing control and miR-183 inhibitors were transfected into PC-3 cells and stable transfectants were selected by Hygromycin resistance. To confirm expression of miR-183 in stable transfectants, real-time PCR was performed. The miR-183 expression level in miR-183 knockdown-stable transfectants was decreased to about 45% of that in control transfectants (Figure 5A). Colony

formation was significantly decreased in miR-183 knockdown-stable transfectants compared with scramble transfectants (Figure 5B). Control and miR-183 knockdown-stable transfectants were transplanted subcutaneously into the left and right back side flanks of nude mice, respectively. The average volume and weight of tumours were significantly reduced in mice injected with miR-183 knockdown-transfected cells (Figure 5C). The macroscopic appearance of tumour at 42 days after inoculation showed a larger mass in control transfectants than in miR-183 knockdown transfectants (Figure 5C). After extracting miR from xenograft tissues (control and miR-183 knockdown-stable transfectants), the relative expression of miR-183 was significantly lower in tumours of miR-183 knockdown-stable-transfected cells compared with control tumours (Figure 5D).

Target genes of miR-183. To identify the target genes of miR-183, we used target scan algorithms (microRNA.org), and Dkk-3 and SMAD4 were selected as potential target tumour-suppressor genes among 24 genes based on the 3'UTR luciferase assay results (Figure 6A and B). Dkk-3 mRNA has one potential complementary miR-183-binding site within its 3' UTR. SMAD4 mRNA also has three potential complementary miR-183-binding site within its

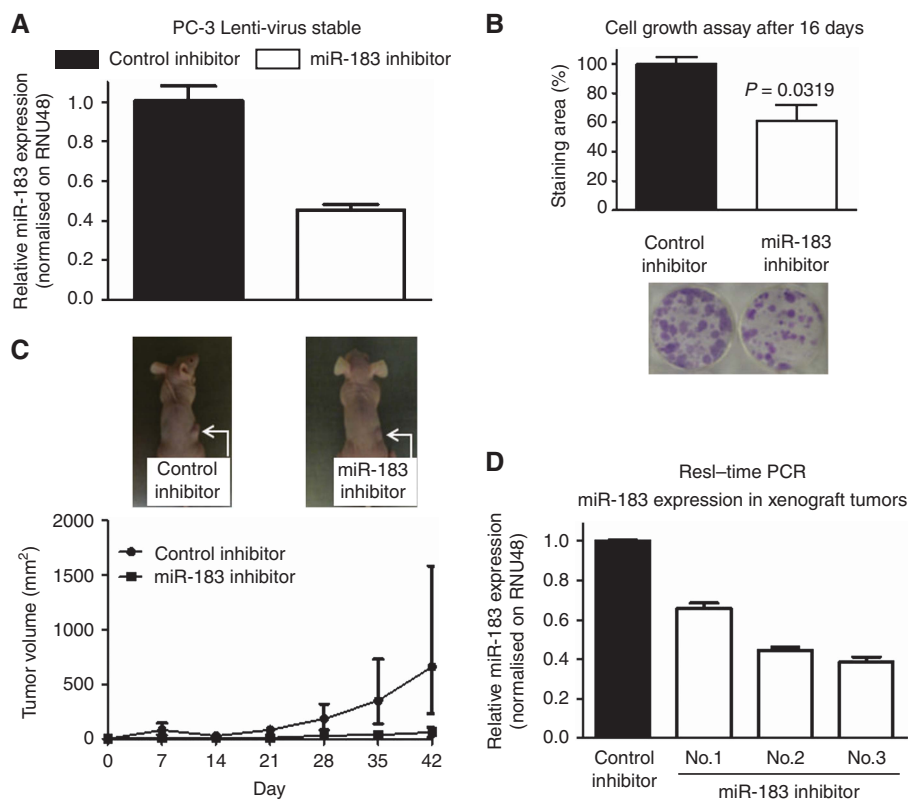


Figure 5. *In vivo* assessment of tumour growth with control and miR-183 inhibitor stably transfected PC-3 cells. (A) The level of miR-183 expression in miR-183 knockdown PC-3-stable transfectants and controls was observed using real-time PCR. (B) Colony formation assay with controls and miR-183 knockdown-stable transfectants. 16 days after seeding, cells were stained and measured using ImageJ software. (C) Macroscopic appearance of tumours on day 42 after subcutaneous injection of PC-3 control (left panel) and PC-3-miR-183 inhibitor (right panel) cells. (D) MiR-183 expression levels in tumours were confirmed by real-time reverse transcription-PCR after extracting microRNA from xenograft tissues 42 days after subcutaneous injection.

3'UTR. To determine the inhibitory effect of miR-183 on Dkk-3 and SMAD4 translation, 3'UTR luciferase assay was performed with PC-3 cells. The luciferase activity of Dkk-3 wild-type 3'UTR vector in miR-183 precursor-transfected cells was significantly decreased compared with Dkk-3 mutated-type 3'UTR vector (Figure 6A). The luciferase activity of SMAD4-position 449 wild-type 3'UTR vector in miR-183 precursor-transfected cells was also significantly decreased compared with SMAD4-position 449 mutated-type 3'UTR vector, but there were no difference in SMAD4-position 1149 and position 2982 (Figure 6B). To examine the inhibitory effect of miR-183 on protein levels, western blot analysis was carried out at 72 h after miR-183 inhibitor transfection into PC cells. We observed that the protein levels of Dkk-3 and SMAD4 in miR-183 inhibitor-transfected cells were increased compared with control inhibitor (Figure 6C and D).

We performed immunohistochemistry and observed an inverse association between miR-183 and target gene protein expression (Dkk-3 and SMAD4; Supplementary Figure 2).

Effect of Dkk-3 and SMAD4 overexpression on PC-3 cells. To confirm whether Dkk-3 and SMAD4 inhibit PC-3 cell growth, Dkk-3/SMAD4-expressing and control vectors were transfected into PC-3 cells. Dkk-3 and SMAD4 mRNAs were analysed by real-time PCR at 24 h after transfection and were significantly increased in transfected PC-3 cells (data not shown). Overexpression of Dkk-3 and SMAD4 protein was also confirmed by western blot analysis at 48 h after transfection (Figure 7A). Cell growth was significantly decreased in Dkk-3 and SMAD4 transfectants compared with control (Figure 7B). Dkk-3 and SMAD4 have an important role in Wnt signalling pathways in cancer progression.

To investigate the direct effect of these genes on the Wnt- β -catenin canonical pathway, we looked at expression of β -catenin in nuclear and cytoplasmic fractions in Dkk-3- and SMAD4-transfected PC-3 cells. Although there was no difference in the expression of cytoplasmic β -catenin (data not shown), the expression level of nuclear β -catenin was decreased in Dkk-3- and SMAD4-transfected PC-3 cells compared with mock cells (Figure 7C). In addition, Tcf transcriptional activity was decreased to 70% in Dkk-3- or/and SMAD4-transfected PC-3 cells compared with control, indicating that Dkk-3 and SMAD4 inhibits the β -catenin-dependent pathway in PC cells (Figure 7D).

Effect of miR-183 on Wnt- β -catenin pathway. As TOPflash luciferase activity was significantly decreased with Dkk-3 and SMAD4 overexpression, we also performed the same assay using miR-183 inhibitor. As shown in Supplementary Figure 3, TOPflash luciferase activity was significantly decreased with miR-183 inhibitor transfection.

DISCUSSION

There have been several miR studies related to clinical PC and most have shown that aberrant expression of miRNAs occurs in PC, some of which function as tumour-suppressor genes or oncogenes (Volinia *et al*, 2006; Porkka *et al*, 2007; Ambis *et al*, 2008; Ozen *et al*, 2008; Schaefer *et al*, 2010). Based on the literature, the level of miR-183 expression in primary PC has previously been described to be higher than adjacent normal tissues (Schaefer *et al*, 2010). MiR-183 is located on human chromosome 7 and forms a

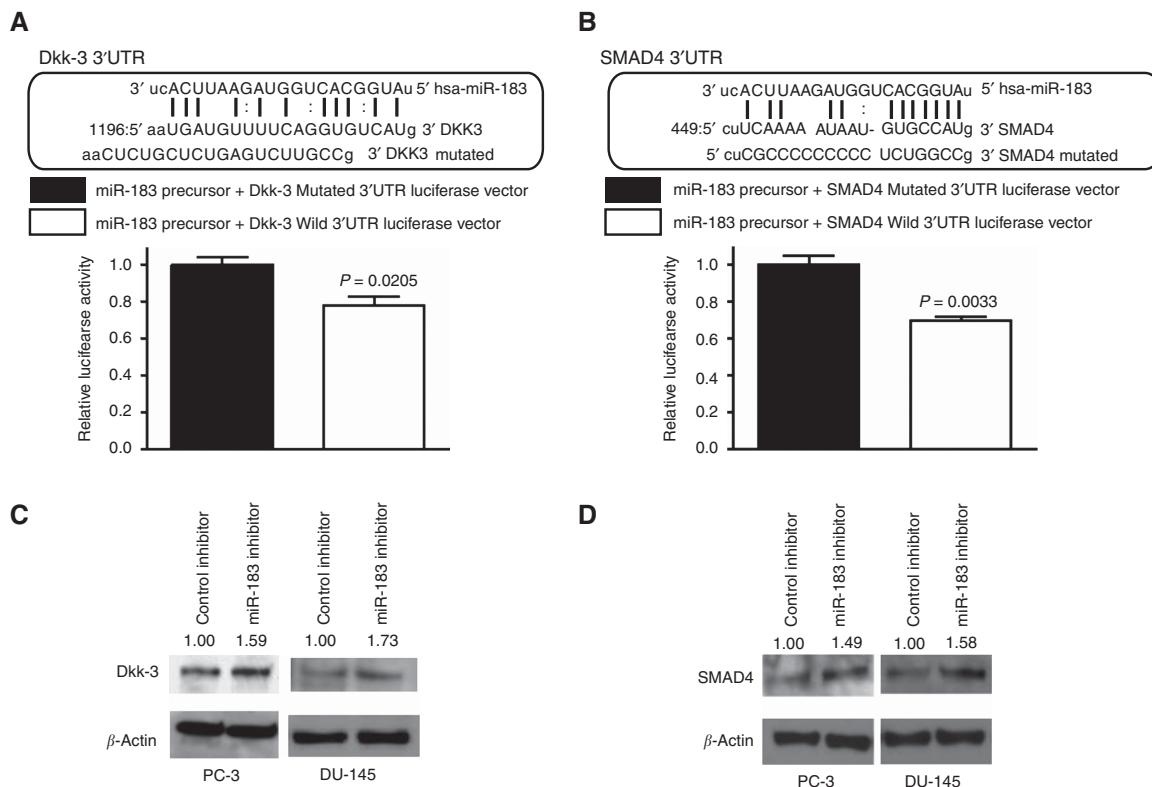


Figure 6. MiR-183 targets *Dkk-3* and *SMAD4* genes. **(A)** and **(B)** *Dkk-3* (left) and *SMAD4* (right) 3'UTR sequence and complementary miR-183-binding sequences. MiR-183-binding sites in the *Dkk-3*/*SMAD4* 3'UTR predicted by microRNA.org. Upper sequence is miR-183, middle is the target wild type, the bottom is the target mutated sequence. MiR-183 precursor was transfected into PC-3 cells and after 24 h, the 3'UTR vectors with wild-type or mutated-type sequence were transfected into the miR-183 precursor-transfected PC-3 cells. Cell lysates were used to measure relative luciferase activities 48 h after 3'UTR vector transfection. Levels of luciferase activity were compared with those of cells transfected with 3'UTR vector with mutated-type sequence. **(C)** and **(D)** *Dkk-3* and *SMAD4* protein levels after miR-183 knockdown. At 72 h after transfection, total protein was extracted and used for western blot analysis. β -Actin was used as a loading control. *Dkk-3* and *SMAD4* protein expression was normalised to β -actin using ImageJ software.

cluster with miR-96 and miR-182. MiR-183-96-182 cluster expression has been found to be upregulated in primary PC (Mihelich *et al*, 2011). We also observed that the expression of three miRNAs (miR-183, miR-96, miR-182) was higher in PC cell lines and their expression pattern was very similar. In this study, we focused on miR-183 as its expression was highest of the miRNA cluster (miR-96, miR-182, miR-183) in the microarray results from PC cell lines. MiR-183 has also been suggested to be an onco-miRNA in several cancers such as colon (Bandrés *et al*, 2006), lung (Cho *et al*, 2009), hepatocellular (Li *et al*, 2010) and synovial sarcoma (Sarver *et al*, 2010).

To confirm that miR-183 expression was higher in PC tissues, we performed real-time PCR to determine the miR-183 expression in laser capture microdissected clinical samples and also found that miR-183 expression to be significantly higher in PC compared with matched adjacent normal prostate tissues and higher miR-183 expression was associated with higher PSA at diagnosis, higher pT and shorter overall survival after radical prostatectomy. These results are consistent with previous results (Schaefer *et al*, 2010) and accession #GSE21036 from GEO (Supplementary Figure 1) and may suggest that miR-183 may be a tumour marker in PC.

The expression of miR-183 was also higher in three PC cell lines (PC-3, DU-145 and LNCaP) compared with the normal RWPE-1 cells consistent with the miR microarray results. There are few reports regarding the functional role of miR-183 in PC, thus we overexpressed miR-183 in a normal prostate cell line (RWPE-1) with miR-183 precursor. As expected, normal prostate cell proliferation was significantly increased with miR-183 precursor

compared with control. We next performed functional analyses (MTS, wound healing, colony formation and *in vivo* growth) using a miR-183 knockdown technique in PC cell lines. MiR-183 knockdown inhibited PC cell proliferation and decreased PC cell motility. We also performed an *in vivo* study and observed that miR-183 knockdown reduced tumour growth. These results suggest that miR-183 functions as an oncogene in nature and has an important role in cell growth and motility of PC cells and the miR-183 inhibitor may be noble treatment tool for PC.

We next used several algorithms to identify potential target tumour-suppressor genes of miR-183. The microRNA.org algorithm identified that the 3'UTR of *Dkk-3* mRNA has one putative miR-183-binding site and the 3'UTR of *SMAD4* mRNA has three putative miR-183-binding sites. We performed 3'UTR luciferase assay, and observed that luciferase activity was decreased after transfection of miR-183 precursor and a 3'UTR vector containing either *Dkk-3* or *SMAD4*. *Dkk-3* and *SMAD4* protein expression were also significantly upregulated in miR-183 inhibitor-transfected PC-3 and DU-145 cells, indicating that *Dkk-3* and *SMAD4* are direct targets of miR-183. We also performed immunohistochemistry and observed an inverse correlation of miR-183 and target gene protein expression (*SMAD4* and *Dkk-3*). As shown in Supplementary Figure 2, miR-183 expression was significantly higher in lower *SMAD4* and lower *Dkk-3* protein expression samples (Supplementary Figure 2).

It is known that *Dkk-3* expression in primary PC is low (Kawano *et al*, 2006) and overexpression of *Dkk-3* inhibits cell proliferation in PC-3 cells through the Wnt/JNK signalling

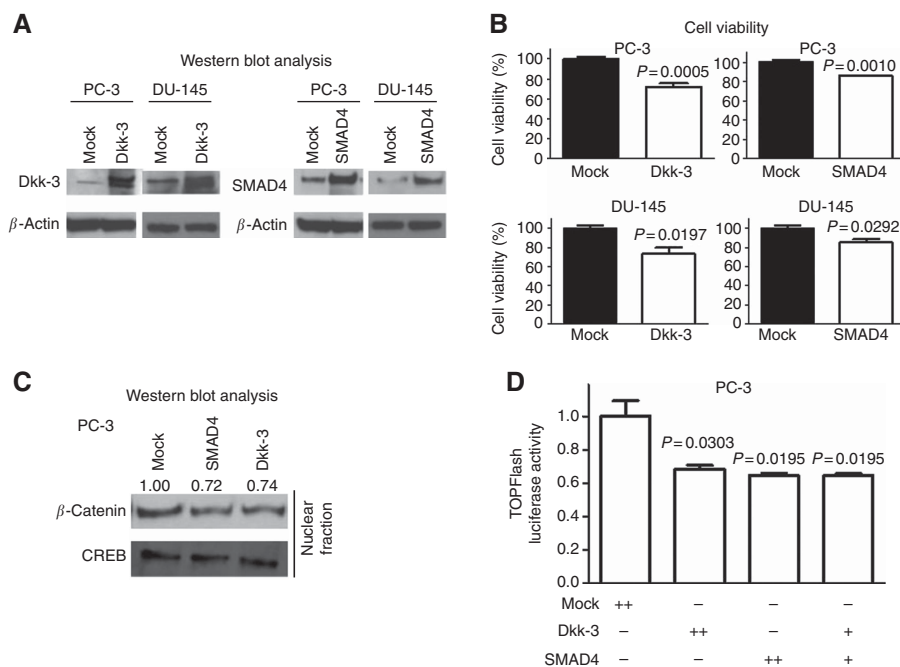


Figure 7. Functional effects of Dkk-3 and SMAD4 overexpression on PC-3 cells. (A) Expression of Dkk-3 and SMAD4 protein in Mock, Dkk-3 or SMAD4 overexpressing transiently transfected PC-3 and DU-145 cells. At 48 h after transfection, total protein was extracted and analysed by western blot analysis. β -Actin was used as a loading control. (B) Cell viability was analysed by MTS assay 4 days after transient transfection. (C) β -Catenin protein levels in Dkk-3 and SMAD4 overexpressing PC-3 cells. Either Mock, Dkk-3- or SMAD4-expressing plasmids were transiently transfected into PC-3 cells. After transfection (48 h), nuclear and cytoplasmic proteins were extracted and analysed by western blot analysis. Nuclear β -catenin expression was normalised to CREB by using ImageJ software. (D) Tcf-reporter transcriptional activity in Dkk-3- or/and SMAD4-transfected PC-3 cells. At 48 h after transfection, cell lysates were measured for relative luciferase activities. Data are presented as the mean value \pm s.d. for triplicate experiments and the level of luciferase activity obtained with Dkk-3 or/and SMAD4 vectors compared with empty vectors in transfected cells that are normalised to 1. + Shows the amount of plasmid vectors.

pathway (Abarzua *et al*, 2005). In our study, we also examined the effect Dkk-3 overexpression on PC cells and our results showed that Dkk-3 inhibited cell proliferation. This result is similar to a previous report (Abarzua *et al*, 2005).

Regarding the role of Dkk-3 in the Wnt/ β -catenin canonical pathway, there have been conflicting reports. Kawano *et al* (2006) reported that Dkk-3 did not affect on the Wnt/ β -catenin signalling pathway in LNCaP cells because Dkk-3 did not alter cellular distribution of β -catenin and had no significant effect on β -catenin/TCF-dependent transcription. However, Lu *et al* (2009) reported that the activity of the Wnt/ β -catenin signalling pathway in PC-3 cells is higher among PC cells (PC-3, DU-145 and LNCaP). In our study, we used PC-3 cells to look at the role of Dkk-3 on Wnt/ β -catenin signalling and we found that β -catenin expression in the nuclear fraction and Tcf transcriptional activity were significantly decreased with ectopic Dkk-3 expression, indicating that overexpression of Dkk-3 in PC-3 cells inhibits Wnt/ β -catenin signalling.

We also identified SMAD4 as a target gene of miR-183. SMAD4 has an important role as a central intracellular signal transduction component of the transforming growth factor- β family and is regarded as a tumour-suppressor gene in several cancers including PC (Hahn *et al*, 1996; de Winter *et al*, 1997; MacGrogan *et al*, 1997). It has also been reported that nuclear expression levels of SMAD4 in primary PC is lower than that in benign prostate hyperplasia tissues and is significantly reversely correlated with Gleason score (Horvath *et al*, 2004). SMAD4 knockdown was found to increase the frequency of metastasis to the lung in PC-3 cells, indicating that SMAD4 is involved in PC progression and metastasis (Ding *et al*, 2011). In our study, overexpression of SMAD4 in PC-3 cells decreased cell growth *in vitro*. Our results are similar to those of previous reports, suggesting that SMAD4 is a

crucial tumour-suppressor gene in PC. As the SMAD complex directly activates the p21 gene promoter in cooperation with the transcription factor Sp1 (Ijichi *et al*, 2004), and Liu *et al* (2011) showed that SMAD4 knockdown decreased c-Myc and p21 protein in PC-3 cells, we measured p21 expression in SMAD4-transfected PC-3 cells and observed that the level of p21 was 1.4-fold higher compared with controls 48 h after transfection (data not shown).

Evidence of cross-talk between Smad signalling and the Wnt pathway has been reported (Labbé *et al*, 2007), so we examined the relationship between SMAD4 and β -catenin in PC. In our study, β -catenin expression in the nuclear fraction and Tcf transcriptional activity were significantly decreased in SMAD4- and/or Dkk-3-transfected PC-3 cells and miR-183 knockdown decreased Wnt/ β -catenin signal-transducing activity (Supplementary Figure 3). Our results suggest that Dkk-3 and SMAD4 may have an inhibitory effect on the Wnt/ β -catenin pathway regulated by miR-183.

In conclusion, this is the first report to show that the oncogenic miR-183 activates the Wnt/ β -catenin pathway by directly inhibiting tumour suppressors Dkk-3 and SMAD4 in PC. Our results indicate that increased levels of miR-183 may be an important biomarker for PC and a therapeutic target for treatment. However, additional studies with increased number of clinical samples will be needed to firmly establish the role of miR-183 in PC and its use in clinical applications.

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