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 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.
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 lentiviral 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 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 into cells with Lipofectamine 2000 (Invitrogen) 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.

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′-AAACTAGGCGCGCTAGTaaTGATGTGTTTCAAGTGTCATgT-3 |
| Dkk-3 reverse    | 5′-CTAGAcatGACACTGAAAACATCattACTAGGCGCGCTAGTTT-3′ |
| Mutated Dkk-3 forward | 5′-AAACTAGGCGCGCTAGTaaCTCTGCTAGTCTTGCTgTgT-3 ′ |
| Mutated Dkk-3 reverse | 5′-CTAGAcatGGCAAGAAGCTAGAAGACTAGGCGCGCTAGTTTT-3′ |
| SMAD4 forward    | 5′-AAACTAGGCGCGCTAGTaaCTCTGCTAGTCTTGCTgTgT-3 ′ |
| SMAD4 reverse    | 5′-CTAGAcatGACACTGAAAACATCattACTAGGCGCGCTAGTTT-3′ |
| Mutated SMAD4 forward | 5′-AAACTAGGCGCGCTAGTaaCTCTGCTAGTCTTGCTgTgT-3 ′ |
| Mutated SMAD4 reverse | 5′-CTAGAcatGGCAAGAAGCTAGAAGACTAGGCGCGCTAGTTTT-3′ |

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

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 Nhel cloning forward primer, 5′-CCTAGCttgctgataagattatt-3′; SMAD4 Xhol cloning reverse primer, 5′-CTCGAgatttigctgatacctgataa-3′. Polymerase chain reaction products were cloned into the pTarget-T-Mammalian Expression Vector System (Promega). pCMV6-SMAD4 was obtained by subcloning a Nhel–Xhol fragment from pTarget-T-SMAD4 into the Nhel–Xhol site of pCMV6-Entry Vector.

Oncogenic microRNA-183 in prostate cancer
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 ± 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–μl reaction volume in triplicate. The mRNA and miR expression levels were determined using the 2−ΔΔCT 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 (GeneCopoeia, 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 pEZX-AM03 (CmiR-AN0001-AM03, GeneCopoeia) with Lenti-Pac HIV mix were transfected into 293Ta cells (GeneCopoeia) 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-μm PES filter (Whatman/Fisher Scientific). PC-3 cells were infected with lentiviral particles with Polybrene (8 μg 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 μg 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): x2y/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.
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
Somatic microRNA expression in prostate cancer. 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 24 h after transfection (Figure 4A). Cell motility was also decreased in miR-183 inhibitor transfectants compared with controls at 4 days after transfection (Figure 4B). Cell viability was also significantly decreased compared with control inhibitor-transfected cells normalised as 1 (Figure 4C). 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).

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 complimentary miR-183-binding site within its 3' UTR. SMAD4 mRNA also has three potential complimentary miR-183-binding site within its...
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-depending 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; Ambs 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
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 oncomiRNA in several cancers such as colon (Bandrés et al., 2006), lung (Cho et al., 2009), hepatocellular (Li et al., 2010) and synovial sarcoma (Serar 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 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 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 normalized to β-actin using ImageJ software.
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 in SMAD4- and/or Dkk-3-transfected PC-3 cells. At 48 h after transfection, cell lysates were measured for relative luciferase activities. Data are presented as the mean value ± s.e.m. 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.

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).

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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