Loss of miR-100 enhances migration, invasion, epithelial-mesenchymal transition and stemness properties in prostate cancer cells through targeting Argonaute 2

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Abstract. Evidence in literature has demonstrated that some microRNAs (miRNAs) play a pivotal role in most solid tumor metastasis. Previous studies have showed that miR-100 is downregulated in human prostate cancer tissue compared to normal prostate and also significantly decreased in bone metastatic prostate cancer samples compared with primary prostate cancer. Argonaute 2 (AGO2) is the core effector protein of the miRNA-induced silencing complex and overexpression of AGO2 might enhance tumor metastasis. However, it is unknown whether and how miR-100 and AGO2 regulates metastasis of prostate cancer. Here, we report that miR-100 negatively regulated migration, invasion, epithelial-mesenchymal transition (EMT), colony formation, spheroid formation and expression of the stemness factors c-Myc, Oct4 and Klf4 in PC-3 and DU145 cells. Furthermore, miR-100 expression was negatively correlated with bone metastasis of prostate cancer patients. Notably, luciferase assay showed that AGO2 was a direct target of miR-100. Downregulation of AGO2 repressed migration, invasion, EMT and stemness of prostate cancer cells, and reversed the effects seen with miR-100 downregulation. Downregulation of AGO2 enhanced expression of miR-34a and miR-125b which can suppress migration, invasion, EMT and stemness of cancer cells. Taken together, our findings indicate that loss of miR-100 promotes the metastatic ability of prostate cancer cells at least partially by upregulating AGO2 expression through modulating migration, invasion, EMT and stemness of cancer cells, and suggest that miR-100/AGO2 may play an important role in regulating the metastasis of prostate cancer and is a potential target of prevention and therapy.

Introduction

Prostate cancer (PCa) is the most frequently diagnosed malignant tumor in male and the second leading cause of cancer deaths in Western countries (1). The main problem arising from PCa is its propensity to metastasize to bone and raise bone relative events and death (2). Thus it is very important to understand the mechanism of metastasis progression for preventing and developing anti-metastatic therapies.

Substantial evidence has demonstrated that some microRNAs (miRNAs), which is a class of small non-coding regulatory RNAs (19-25 nucleotides), play a pivotal role in most solid tumor metastasis (3,4). In PCa, a series of miRNAs have been identified as suppressors of metastasis, such as miR-145, -143, -205, -34a, -203 and -200c (5-9). Recently, several studies also found that miR-100 expression is down-regulated in human PCa tumor tissue compared to normal prostate (10-12). Importantly, miR-100 level significantly decreases in the bone metastasis PCa samples compared with primary PCa (5). Moreover, it is also downregulated during the PCa progression (10,13,14) and its downregulation is related with hormone-refractory PCa (15). However, the importance of miR-100 in bone metastasis of PCa has not been elucidated to date.

Epithelial-mesenchymal transition (EMT) plays a key role in tumor cell metastasis (16) and also has been identified as an important step in bone metastasis of PCa (2,17). Furthermore, E-cadherin-mediated cell-adhesion system plays a critical role

in EMT which is regulated by various EMT-inducing transcription factors including Snail1/2, Twist1/2 and Zeb1/2 (18). Emerging evidence has demonstrated that cancer stem cells (CSCs) also are the critical drivers of tumor progression and metastasis (19). Importantly, certain miRNAs directly regulated EMT and the characteristics of CSCs (3,20,21). However, it is not known whether and how miR-100 regulates EMT and the characteristics of CSCs.

The bioinformatics (TargetScan) predicts that Argonaute 2 (AGO2 or Eif2c2) may be a putative target of miR-100. AGO2 is the core effector proteins of the miRNA-induced silencing complex (miRISC) (22) and plays a role in short interfering RNA-mediated gene silencing (23). Furthermore, AGO2 also is as a pivotal factor in some miRNA biosynthesis (24) and maturation (25). Notably, Ago2 was overexpressed in some malignant tumors, including PCa (24,26,27) and silencing of AGO2 arrested growth and promoted apoptosis of some malignant tumor cells (25,28). More importantly, overexpression of Ago2 promotes hepatocellular carcinoma (HCC) tumorigenesis and metastasis (23). Downregulation of AGO2 also retarded self-renewal in embryonic stem cells (29) and colony formation of HCC cells (23). These findings suggest that overexpression of AGO2 might enhance tumor metastasis. However, it is unknown whether and how AGO2 is involved in bone metastasis of PCa. Thus, we hypothesize that miR-100 directly targets AGO2 through which miR-100 negatively regulates metastatic abilities of PCa cells by modulating migration, invasion, EMT and stemness properties of cancer cells.

Materials and methods

Cell culture and generation of stable transfected cell lines. The PC-3 cell line and DU145 cell line of PCa were purchased from American Type Culture Collection (ATCC). The cells were grown in Ham's F-12 culture medium (Hyclone) and DMEM culture medium (Hyclone), respectively, supplemented with 10% fetal bovine serum (Hyclone). Cells were grown in a humidified atmosphere of 5% CO_2 at 37˚C. The sequence of pri-miR-100, anti-miR-100 and si-h-RNA-AGO2 (a small hairpin RNA targeting AGO2, 5'-CACGGAAGUCCAUCUGAA dTdT-3') were cloned into pMSCV-puromycin plasmid. Then, the plasmids were transfected into 293FT cells which were used as virus-generating host cells by calcium phosphate precipitation as described previously (30). After incubation at 37˚C for 6 h after transfection, the media were changed and the cells were incubated overnight. To produce new viruses, the media were collected three times a day until 293FT cells reached total confluence. Media containing viruses were used to infect PC-3 cells and DU145 cells. Twenty-four hours after the addition of viruses, infected cells were selected by adding puromycin to the growth medium. Stable cell lines were verified by qRT-PCR.

Plasmids, virus production and infection of target cells. The different regions of human miR-100 promoter were generated by PCR amplification from PC-3 cells and cloned into the *Kpn*I/*Hin*dIII sites of the pGL3-basic-luciferase reporter plasmid, respectively (Promega, Madison, WI, USA). The full length AGO2-3'-UTR region was generated by PCR amplifica-

tion from DNA of PC-3 cells, and subcloned into pEGFP-C3 and a modified pGL3-control-luciferase vector. To silence endogenous AGO2 expression, RNAi oligonucleotides were synthesizes by Invitrogen and cloned into the pSuper-retro-puro plasmid (Oligoengine, WA, USA). The sequences of the sense strand for AGO2 was 5'-CACGGAAGUCCAUCUGAA dTdT-3'. The miR-100 mimic and miR-100 inhibitor were purchased from RiboBio (RiboBio Co., Ltd., Guangzhou, Guangdong, China). Transient transfection was performed using the Lipofectamine 2000 reagent (Invitrogen) according to the manufacturer's instructions.

Tissue samples and RNA extraction. Tissue samples were collected from 64 primary PCa patients. The methods of data collection and RNA extraction were as previously described (5). The Institutional Ethics Board (IRB) at The First Affiliated Hospital of Sun Yat-sen University approved the study.

Luciferase assay. Luciferase assays were carried out in 293FT cells. Cells were co-transfected with miRNAs and luciferase reporter plasmids in 6-well plates and cultured for 48 h before the cells were harvested and lysed for luminescence detection. The following procedure and detection were performed using a luciferase assay kit (Promega) according to the manufacturer's protocol. Renilla luciferase was activated to emitting the primary luminescence, and firefly luminescence was used for normalization. Each test was repeated in triplicate.

Quantitative reverse transcription-PCR. The procedure was performed according to the manual of All-in-One™ miRNA qRT-PCR Detection kit (GeneCopoeia, USA). Total RNAs were extracted from cultured cells by using RNeasy kit (Qiagen), and were reverse transcribed by adding poly-A sequence, and real-time PCR analysis was performed with specific primer to hsa-miRNAs which need to be checked (GeneCopoeia). Each sample was analyzed in triplicate. No template and no reverse transcription were included as negative controls. U6 snRNA was used as normalization control. Relative expression levels from three independent experiments were calculated following the $2^{-\Delta\Delta Ct}$ method of Livak and Schmittgen (31).

Western blotting. For the analysis of expression of related proteins, western blot assay was carried out. Equal amounts of proteins from the supernatant were loaded per lane and resolved by SDS-polyacrylamide electrophoresis. Then, the proteins was transferred onto PVDF membrane (Millipore), blocked by 5% non-fat milk for 1 h at room temperature, and probed with probed with primary antibodies (1:1,000) overnight at 4˚C, including mouse anti-AGO2 (Abcam); anti-ZEB1 (Sigma, USA); rabbit anti-Oct4, c-Myc, Klf4, CD44 and mouse anti-E-cadherin, vimentin (CST, Cell Signaling Technology); mouse anti-fibronectin, N-cadherin (BD Biosciences). Membranes were washed thrice (10 min each) in TBS-T buffer and incubated for 40 min at room temperature with horseradish peroxidase-conjugated anti-mouse or anti-rabbit secondary antibodies. Membranes were washed thrice (10 min each) in TBS-T and developed using the ECL system. Protein loading was normalized by reprobing the blots with rabbit anti-β-actin (CST, Cell Signaling Technology).
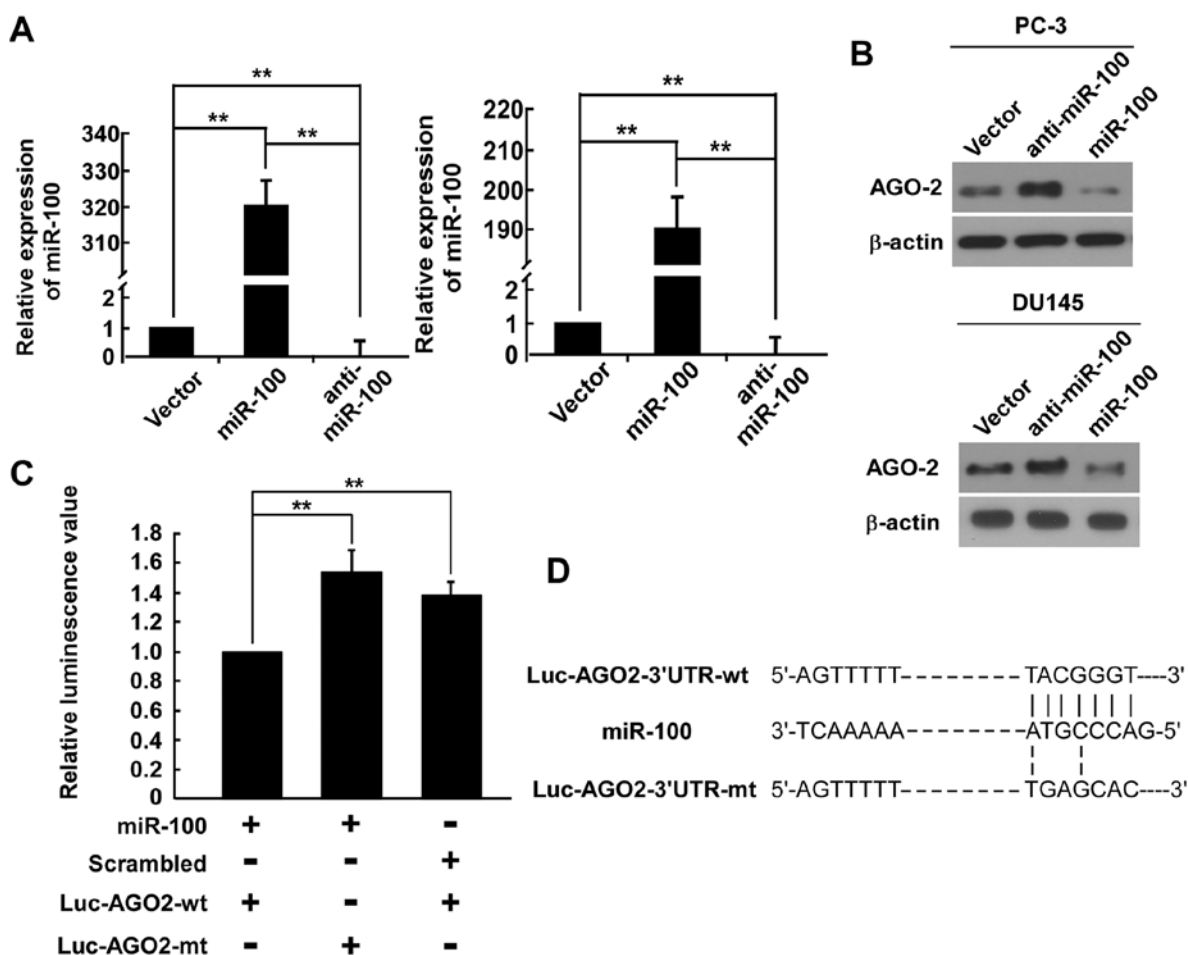
Figure 1. miR-100 negatively regulates expression of AGO2 by directly binding to its 3'-UTR. (A) miR-100 expression was detected by real-time PCR. (B) AGO2 expression was detected by western blotting. (C) Relative luminescence transfected with different factors in luciferase assay are presented as the mean ± SEM of each group (**p<0.01). (D) Wild-type 3'-UTR sequence or mutant 3'-UTR sequence of AGO2 was cloned into luciferase plasmids (Luc-AGO2-wt or Luc-AGO2-mt, respectively).

Wound healing assay. PCa cells were trypsinized and seeded into 6-well culture plates 24 h before scratching and grew to reach almost total confluence in 24 h, followed by non-serum starvation for 24 h. After cell monolayers formed, an artificial homogeneous wound was scratched onto the monolayer with a sterile tip (Axygen, USA). After scratching, the cells were washed with PBS and cultured in 10% fetal bovine serum media. Images of PC-3 cells migrating into the wound were captured at time-points 0, 6 and 12 h by inverted microscope (x40).

Invasion assay. Invasion assay was conducted by using Transwell chamber consisting of 8-mm membrane filter inserts (Corning) coated with Matrigel (BD Biosciences). Briefly, the cells were trypsinized and suspended in serum-free medium. Then, 1.5×10^5 cells were added to the upper chamber, but lower chamber was filled with 1 ml medium with 10% FBS. After incubation for 24-48 h, cells passed through the coated membrane to the lower surface, in which cells were fixed, stained, photographed, and quantified by counting them in five random high power fields (x100).

Colony formation assay. The cells were trypsinized as single cells and suspended in 10% fetal bovine serum medium. Cells (300 cells) were seeded into each well of 6-well plates for 10-14 days, and colonies were dyed with crystal violet. Plating efficiency = number of colonies (≥50 cells per colony)/per input cells x 100%. To determine the morphology of the different colonies they were observed under a light microscope.

Self-renewing spheroid formation assay. Cells (500 cells/well) were seeded into 6-well Ultra Low Cluster plate (Corning) and were cultured in suspension in serum-free DMEM-F12 (BioWhittaker), supplemented with B27 (1:50, Invitrogen), 20 ng/ml EGF (BD Biosciences), 0.4% bovine serum albumin (Sigma), and 4 mg/ml insulin (Sigma) for 10-12 days. After 10-12 days, the number of PC-3 cell spheres (tight, spherical, non-adherent masses >50 μm in diameter) were counted, and images of the spheres were scored under an inverse microscope. Sphere formation efficiency = colonies/input cells x 100%.

Statistical analyses. All statistical analyses were carried out using the SPSS 17.0 statistical software package. Means ± SD was calculated and two-tailed Student's t-test or one-way ANOVA was performed using the data analysis tools provided by the software. The correlation coefficient (Spearman rank correlation test) was calculated to estimate the linear corre-
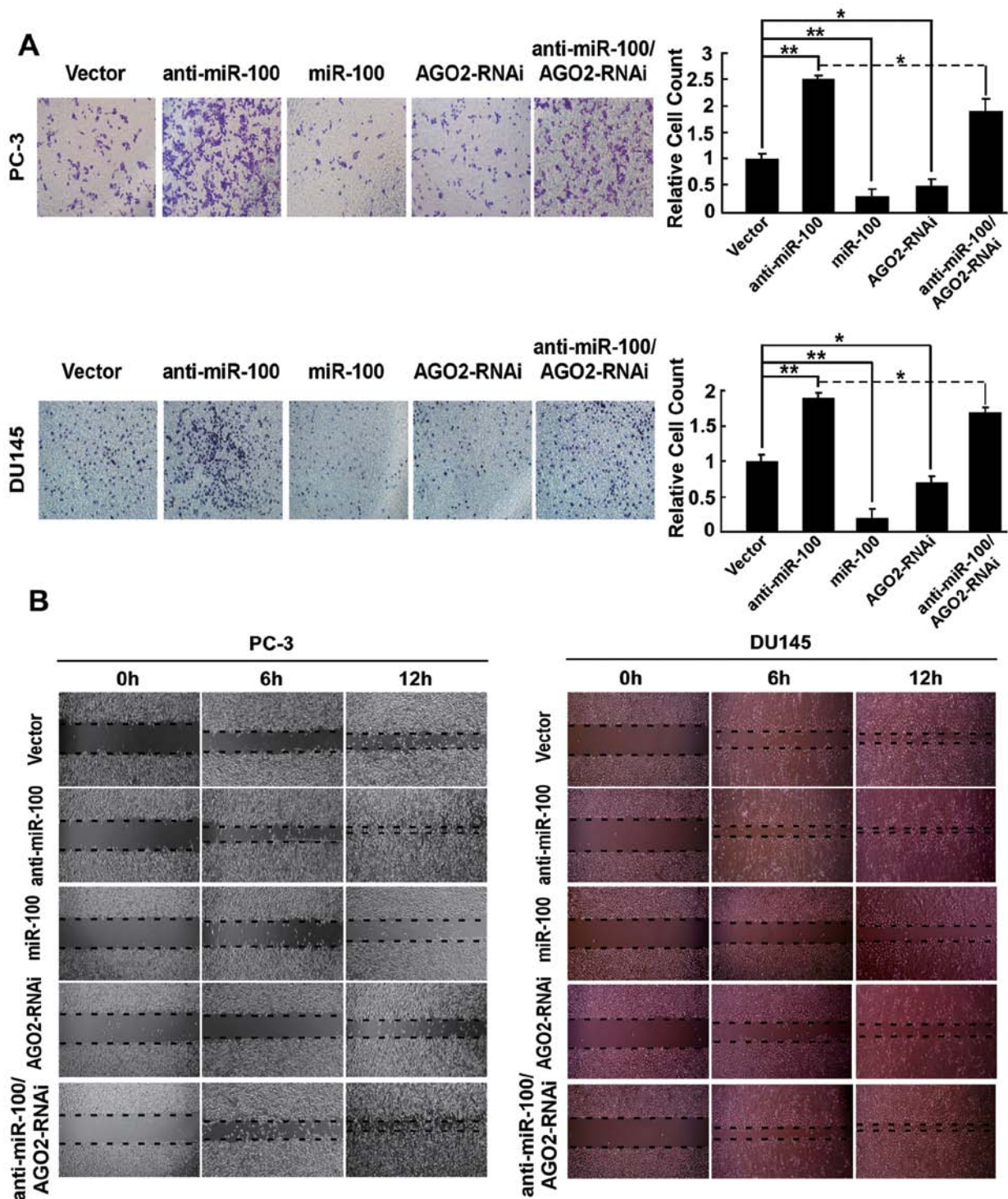
Figure 2. miR-100/AGO2 regulates migration and invasion of PCa cells. (A) miR-100 negatively regulated invasion, and downregulated AGO2 repressed invasion and reversed the effects of the downregulation of miR-100 on promoting invasion of PC-3 and DU145 cells. (B) miR-100 negatively regulated migration, and downregulated AGO2 repressed migration and reversed the effects of downregulation of miR-100 on promoting migration of PC-3 and DU145 cells (*p<0.05, **p<0.01).

lation between miR-100 and the clinicopathology in the specimens. p<0.05 was considered statistically significant.

Results

MiR-100 directly targets AGO2 through interacting with the 3'-UTR. Since miRNAs regulate gene expression by sequence-specific binding to the 3'-UTR of targeted mRNA and the

analysis using publicly available algorithms (TargetScan, miRanda) indicated that the AGO2-3'-UTR region is the theoretical conserved target of miR-100, we examined whether miR-100 may directly target the AGO2-3'-UTR. Western blotting revealed that the expression level of AGO2 was significantly decreased in cells infected with miR-100 and increased in cells transfected with miR-100 inhibitor (Fig. 1A and B), suggesting that miR-100 may degrade the mRNA of
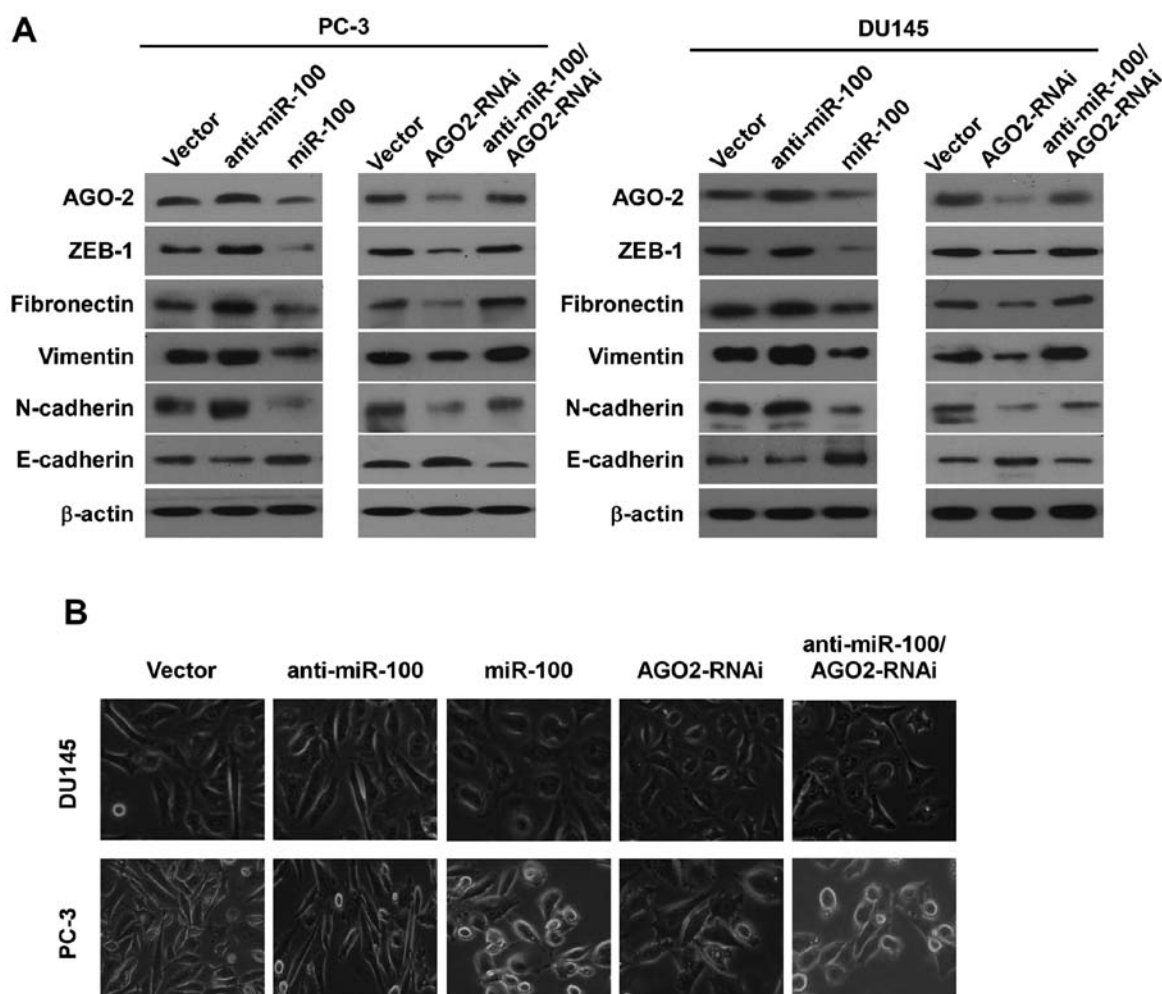
Figure 3. miR-100/AGO2 regulate EMT of PCa cells. (A) miR-100 positively regulated E-cadherin expression and negatively regulated N-cadherin, fibronectin, vimentin and ZEB1 expression; downregulated AGO2 increased E-cadherin expression and suppressed N-cadherin, fibronectin, vimentin and ZEB1 expression; downregulation of AGO2 reversed the effects of downregulation of miR-100 on repressing E-cadherin expression and promoting N-cadherin, fibronectin, vimentin and ZEB1 expression in PC-3 and DU145 cells. (B) Upregulation of miR-100 and downregulation of AGO2 converted the stick-like or long spindle-shaped mesenchymal profile to a cobblestone-like or a short spindle-shaped epithelial morphology, and downregulation of AGO2 reversed the effects of downregulation of miR-100 on cell morphological changes in PC-3 and DU145 cells.

AGO2. We carried out a dual-luciferase reporter gene assay. Luciferase reporter plasmids containing the wild-type 3'-UTR (Luc-AGO2-wt) or mutant 3'-UTR (Luc-AGO2-mt) of AGO2 (Fig. 1D) were constructed to determine the targeted region. As shown in Fig. 1C, luciferase activities were significantly higher in cells transfected with either Luc-AGO2-mt or scrambled miRNA, with 1.54- and 1.39-fold higher levels, respectively, than that in cells transfected with Luc-AGO2-wt. Moreover, the scrambled miRNA base sequence did not suppress Luc-AGO2-wt, and miR-100 could not bind to the mutant AGO2-3'-UTR. Thus, these observations indicated that miR-100 directly targeted AGO2 through interacting with the 3'-UTR.

MiR-100 negatively regulates migration, invasion and EMT in PCa cells. To investigate the role of miR-100 in the development and progression of PCa metastasis, overexpressing miR-100 (PC-3/miR-100 and DU145/miR-100) and downexpressing miR-100 (PC-3/anti-miR-100 and DU145/anti-miR-100) cell lines were established by retrovirus transfection. Blank plasmid transfected cells (PC-3/vector and

DU145/vector) were used as the control group. The expression levels of miR-100 in all cell lines were confirmed by real-time PCR (Fig. 1A). By using wound healing assay to assess cell migration and trans-well Matrigel invasion assay to assess the invasive ability of cells, we found that the invasive ability of PC-3/miR-100 and DU145/miR-100 and the healing speed of the cell wound were repressed, and inversely, the invasive ability and the healing speed of the cell wound of PC-3/anti-miR-100 DU145/anti-miR-100 were promoted compared to PC-3/vector and DU145/vector (Fig. 2).

To investigate whether miR-100 inhibited invasiveness by repressing EMT, we examined the influence of miR-100 on expressions of E-cadherin, N-cadherin, fibronectin, vimentin and ZEB1 in all cell lines by western blotting. We found that E-cadherin expression increased in PC-3/miR-100 and DU145/miR-100, and decreased in PC-3/anti-miR-100 and DU145/anti-miR-100 compared with PC-3/vector and DU145/vector. Contrarily, N-cadherin, fibronectin, vimentin and ZEB1 expressions decreased in PC-3/miR-100 and DU145/miR-100, and increased in PC-3/anti-miR-100 and DU145/anti-miR-100 (Fig. 3A). We then examined the change of morphology of
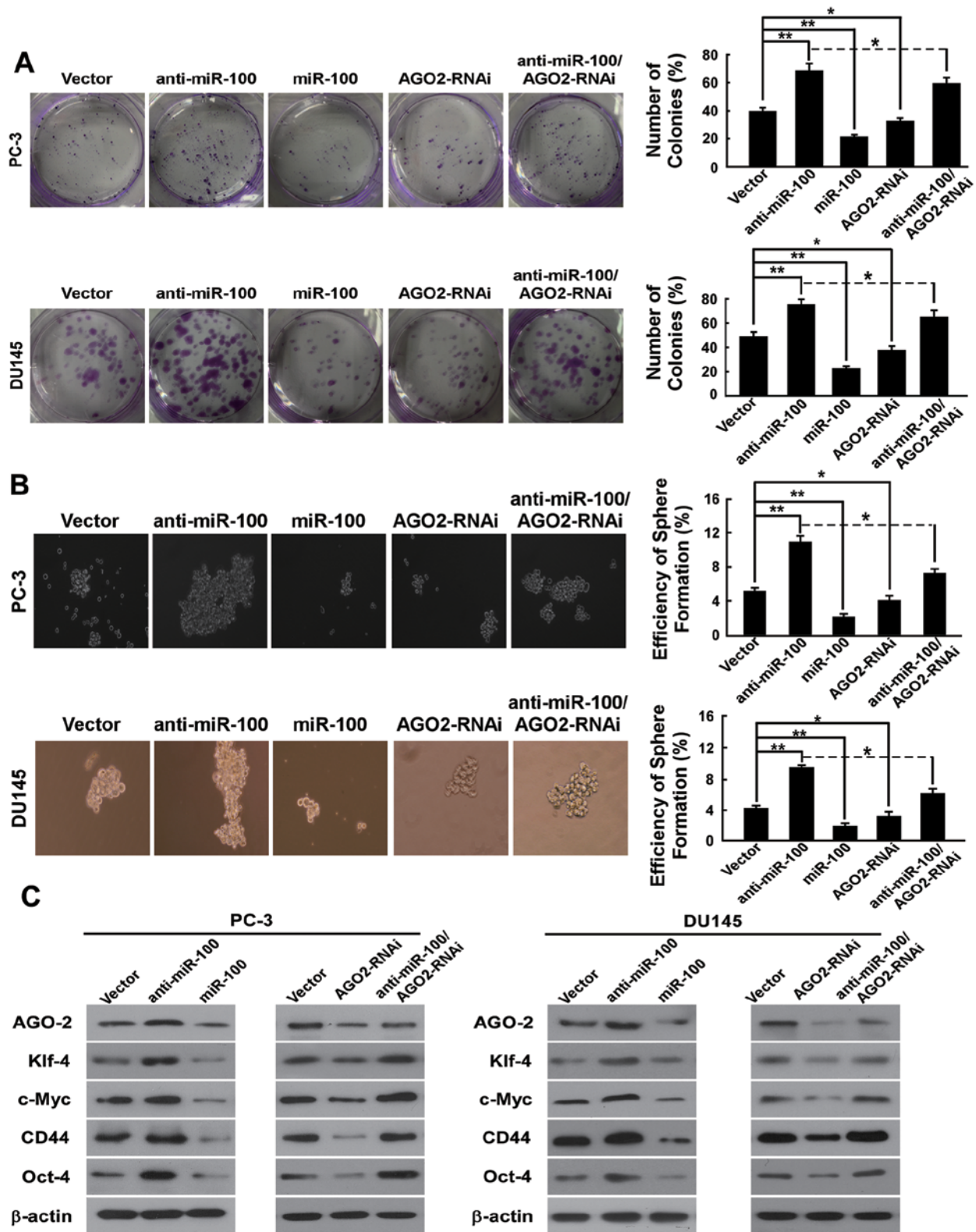
Figure 4. miR-100/AGO2 regulate stemness of PCa cells. (A) miR-100 negatively regulated colony formation, and downregulation of AGO2 repressed colony formation and reversed the effects of downregulation of miR-100 on promoting colony formation of PC-3 and DU145 cells. (B) miR-100 negatively regulated spheroid formation and downregulation of AGO2 repressed spheroid formation and reversed the effects of downregulation of miR-100 on spheroid formation of PC-3 and DU145 cells. (C) miR-100 negatively regulated CD44, C-Myc, Oct4 and Klf4 expression and downregulation of AGO2 repressed CD44, C-Myc, Oct4 and Klf4 expression and reversed the effects of downregulated miR-100 on promoting CD44, C-Myc, Oct4 and Klf4 expression (*p<0.05, **p<0.01).

all cell lines with specific reference to special characteristics during EMT. The results showed that overexpressing

miR-100 in PC-3 and DU145 cells converted the predominant mechenchymal phenotype to an evident epithelial phenotype

Table I. The clinical data of prostate cancer patients.

	No.of cases (%)
Age (years)	
≤75	32 (50.0)
>75	32 (50.0)
Total PSA	
≤85.5	32 (50.0)
>85.5	32 (50.0)
Free PSA	
≤5	33(51.6)
>5	31 (48.4)
Gleason score	
≤7	31 (48.4)
>7	33 (51.6)
Bone metastasis	
Yes	36 (56.3)
No	28 (43.7)

i.e., from the stick-like or long spindle-shaped mesenchymal profile to a cobblestone-like or a short spindle-shaped epithelial morphology and downregulation of miR-100 in PC-3 and DU145 cells increased the long spindle-shaped mesenchymal profile (Fig. 3B).

MiR-100 negatively regulates colony formation, tumor spheroid formation, CSC marker CD44 and 'stemness' factor expression in PCa cells. To determine the efficiency of miR-100 in regulating the stemness of PC-3 and DU145 cells, the colony-forming and spheroid formation assay were performed. In the colony-forming assay, we found that the number of colonies (% plating efficiency) were 22% in PC-3/miR-100, 69% in PC-3/anti-miR-100%, compared with 40% in PC-3/vector, and 23% in DU145/miR-100, 75% in DU145/anti-miR-100, compared with 49% in DU145/vector (Fig. 4A). These results indicated a dramatically repressing ability of miR-100 on colony formation. In spheroid formation assay, there were prostaspheres in all kinds of cells after culturing for 12 days under non-adherent conditions. As shown in Fig. 4B, the spheroid formation efficiency was 2.2% in PC-3/miR-100, 11% in PC-3/anti-miR-100, compared with 5.2% in PC-3/vector, and 1.9% in DU145/miR-100, 9.6% in DU145/anti-miR-100, and compared with 4.3% in DU145/vector.

To determine whether miR-100 also has an influence on CSC marker and stemness factor expression in PC-3 and DU145 cells, we detected the expression of stem cell properties-associated factor and marker including CD44, c-Myc, Oct4, and Klf4. As shown in Fig. 4C, compared with vector, overexpression of miR-100 in PC-3 and DU145 cells reduced the expression of CD44, which has been described as a prostate CSC marker based on clinical investigations and *in vitro* studies of PCa cell lines, and downregulated the expression of Oct4, c-Myc and Klf4, which are the key stemness factors, and are required for maintaining self-renewal

and pluripotency of stem cells. Downregulation of miR-100 in PC-3 and DU145 cells upregulated the expression of CD44, Oct4, c-Myc and Klf4.

MiR-100 is negatively correlated with bone metastasis, the Gleason score and serum PSA level in primary PCa. To determine whether the expression of miR-100 was related to clinicopathology in primary PCa patients, we collected 64 clinical paraffin-fixed PCa samples (the clinical data are showed in Table I). We detected expression level of miR-100 by using qRT-PCR. By comparing the mir-100 level of PCa specimens in 36 patients with bone metastasis with that in 28 patients without bone metastasis, we found that the mir-100 level in PCa specimens with bone metastases was significantly lower than that in PCa specimens without metastases (Fig. 5A). Next we assessed whether the mir-100 level was related with Gleason score, serum-free PSA and total PSA in primary PCa. The results showed significant inverse correlation between expression of miR-100 and Gleason score (Fig. 5C), the level of serum-free PSA (Fig. 5B) and the level of total PSA (Fig. 5D).

Downregulation of AGO2 represses invasion, migration and EMT of PCa cells. To demonstrate the effect of AGO2 on the development and progression of PCa metastasis, AGO2-downregulated cell lines were established in PC-3 cells (PC-3/AGO2-RNAi) and DU145 cells (DU145/AGO2-RNAi). The expression level of AGO2 was confirmed by western blotting (Fig. 3A). Transwell-Matrigel was used to assess the invasive ability of cells. We found that downregulating AGO2 decreased the invasive ability to 27.5% of PC-3/vector and 22.6% of DU145/vector (Fig. 2A). As shown in Fig. 2B, cell migration was observed by wound healing assay and the healing speed of the cell wound decreased in PC-3/AGO2-RNAi and DU145/AGO2-RNAi compared with PC-3/vector and DU145/vector (Fig. 3).

We then investigated whether AGO2 could regulate invasion and migration by repressing EMT. We examined the influence of downregulation of AGO2 on expression of E-cadherin, N-cadherin, fibronectin, vimentin and ZEB1 in PC-3 and DU145 cells by western blotting, and found that E-cadherin increased in PC-3/AGO2-RNAi and DU145/AGO2-RNAi, and N-cadherin, fibronectin, vimentin and ZEB1 decreased in PC-3/AGO2-RNAi and DU145/AGO2-RNAi compared with vector control cells (Fig. 3A). We also examined the change of morphology of PC-3 cells and DU145. The results showed that PC-3/AGO2-RNAi and DU145/AGO2-RNAi converted the predominant mechenchymal phenotype to an evident epithelial phenotype i.e., from the stick-like or long spindle-shaped mesenchymal profiles to a short spindle-shaped or a cobblestone-like epithelial morphology (Fig. 3B).

Downregulation of AGO2 inhibits colony formation, tumor spheroid formation, CSC marker CD44 and stemness factor expression in PCa cells. To demonstrate the effect of AGO2 on regulating stemness of PC-3 and DU145 cells, the colony formation and sphere formation assays were performed. In the colony-forming assay, we found that the number of colonies (% plating efficiency) was 33% in PC-3/AGO2-RNAi compared with 40% in PC-3/vector and 38% in DU145/AGO2-RNAi compared with 49% in DU145/vector (Fig. 4A). In the sphere
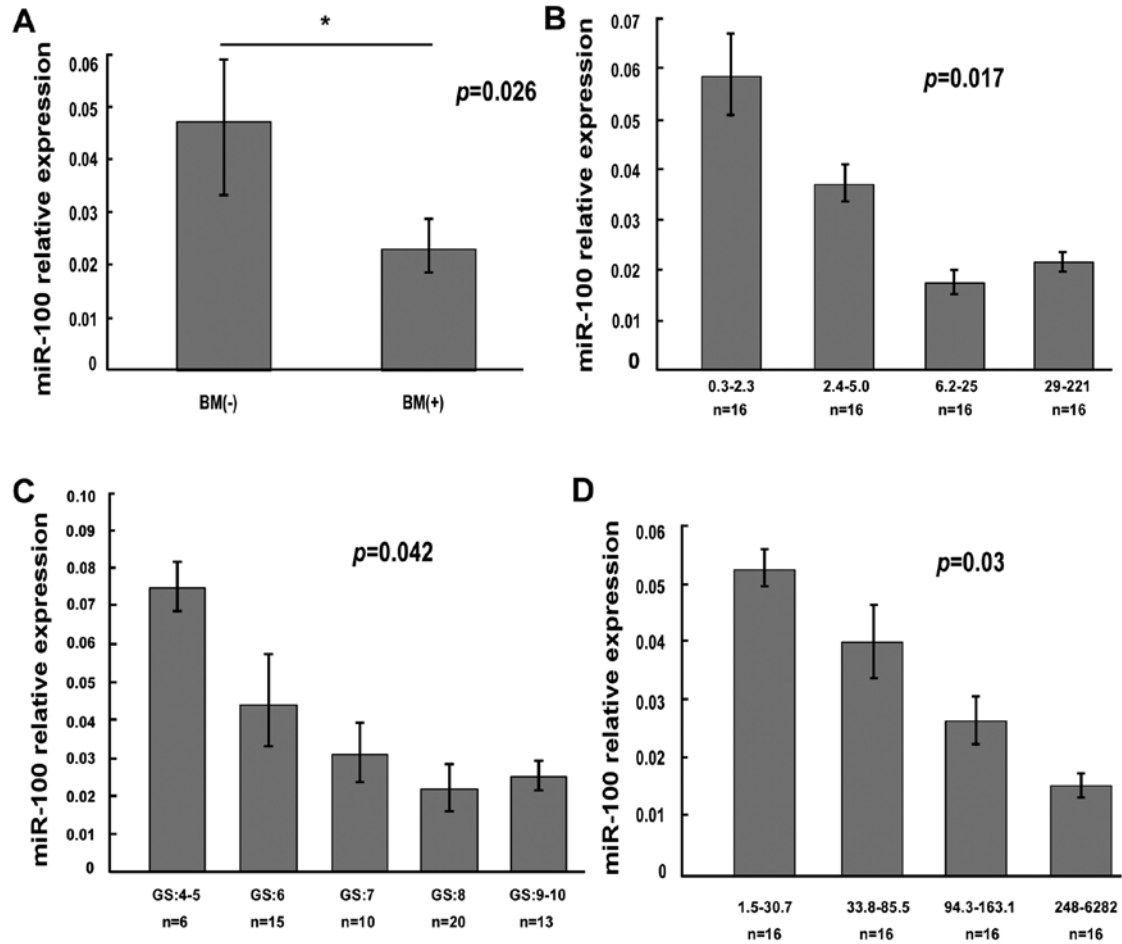
Figure 5. Downregulation of miRs-100 is associated with clinicopathological features of primary PCa. (A) The expression of miRs-100 in the patients with bone metastases was significantly lower than that without metastases (t-test, p=0.026). (C) miR-100 expression was negatively correlated with the Gleason scores (Spearman correlation -0.255; p=0.042). (B and D) Tumor samples were divided into four groups with equal sample size. miR-100 expression was negatively correlated with the level of free and total PSA of serum (Spearman correlation -0.298, p=0.017; Spearman correlation -0.371, p=0.003, respectively).

formation assay, there were prostaspheres in all the cell types after culturing for 12 days under non-adherent conditions. As shown in Fig. 4B, the spheroid formation efficiency was 4.1% in PC-3/AGO2-RNAi and 3.2% in DU145/AGO2-RNAi. Compared to vector (5.2% in PC-3, 4.3% in DU145), downregulation of AGO2 significantly decreased the proportion of self-renewal in PC-3 and DU145 cells.

Further, to demonstrate whether downregulation of AGO2 also has an impact on stemness factors of PCa cells, we measured the expression of CD44, c-Myc, Oct4, Klf4 and Sox2. As shown in Fig. 4C, PC-3/AGO2-RNAi and DU145/AGO2-RNAi reduced the expression of CD44, Oct4, c-Myc and Klf4 compared to vectors. These data indicate that downregulating AGO2 repressed stemness of PCa cells.

Downregulation of AGO2 reverses the effects of downregulated miR-100 promoting migration, invasion, EMT and stemness in PCa cells. Because AGO2 is directly regulated by miR-100, we determined whether AGO2 can reverse the effects of miR-100 on migration, invasion, EMT and stemness in PCa cells. AGO2 expression was downregulated by sequence AGO2-shRNA in PC-3/anti-miR-100. The expres-

sion of AGO2 was confirmed by real-time PCR and western blotting. We found that downregulation of AGO2 partially reversed the effects of miR-100 downregulation in migration speed and invasive ability in PC-3 and DU145 cells (Fig. 2). AGO2 downregulation also partially counteracted the effects of miR-100 downregulation of the expression of E-cadherin, N-cadherin, fibronectin, vimentin and ZEB1 and cell morphology (Fig. 3). As shown in Fig. 4, AGO2 downregulation in part counteracted effects of miR-100 downregulation on colony-forming capability, spheroid formation efficiency and the stemness factors expression in PC-3 and DU145 cells. These data indicate that inhibition of AGO2 at least to some extent reversed the effects of miR-100 downregulation on migration, invasion, EMT and stemness in PCa cells.

Downregulation of AGO2 promotes miR-125b and miR34a expression in PCa cells. Previous studies showed that Ago2 directly regulated certain miRNA biosynthesis (24) and maturation (25). We attempted to determine whether Ago2 promoted the metastatic ability of PCs cells by regulating miRNA expression. We evaluated the expression levels of 17 miRNAs, which were reported to play a role in development
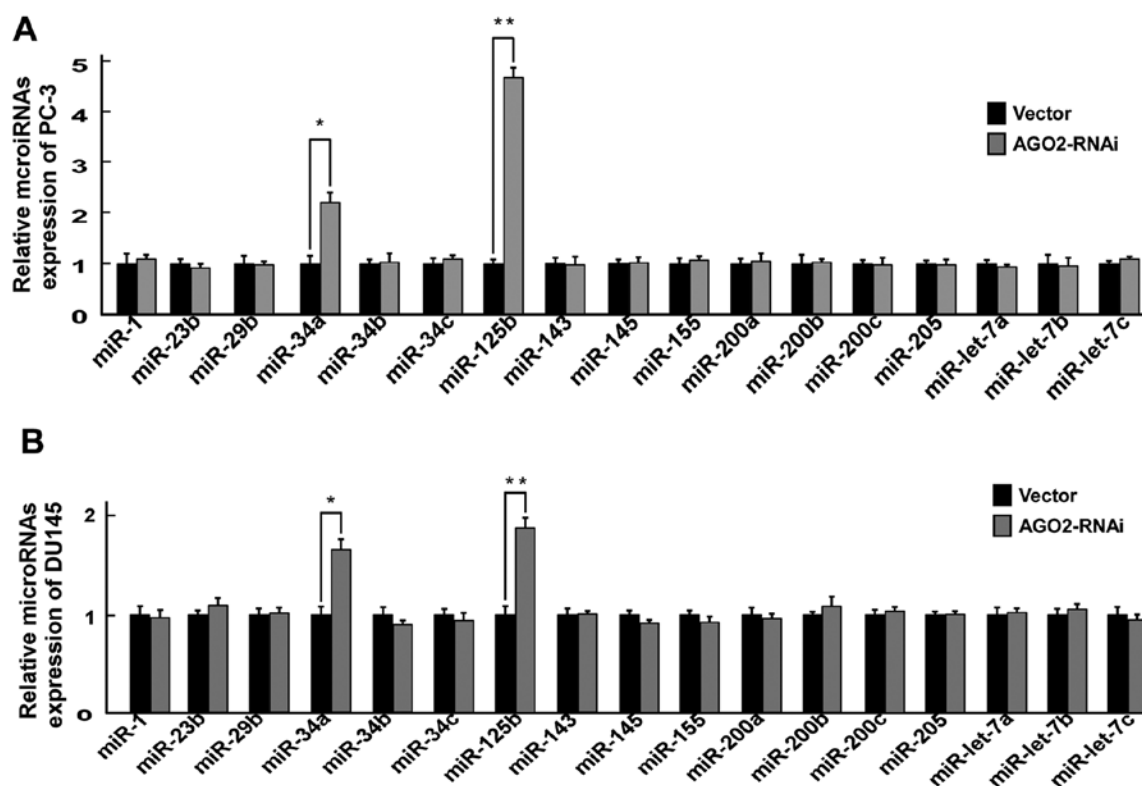
Figure 6. Downregulation of AGO2 promoted miR-125b and miR-34a expression in PCa cells (*p<0.05, **p<0.01).
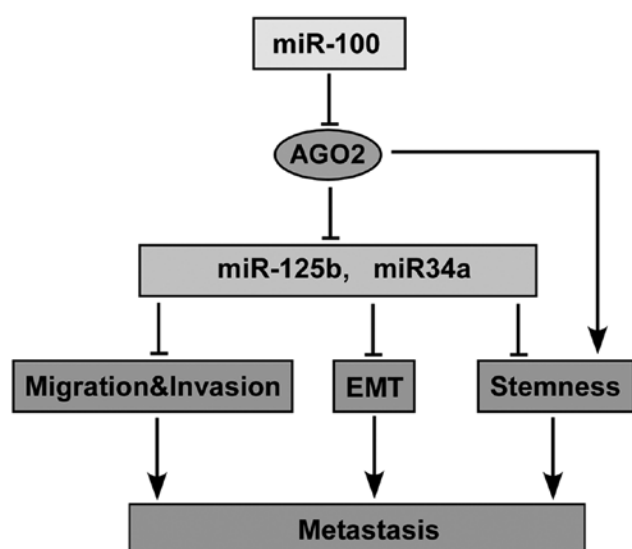

Figure 7. A diagram of the proposed interconnections among miR-100/AGO2, migration, invasion, EMT, stemness and metastasis.

and progression of PCa metastasis (5-9,32), in PC-3/AGO2-RNAi, PC-3/vector, DU145/AGO2-RNAi and DU145/vector by real-time PCR, and found that downregulation of AGO2 significantly upregulated miR-125b and miR34a expression (Fig. 6).

Discussion

Our previous study found that miR-100 significantly decreased in bone metastasis tissue compared with primary PCa (5). In the present study, we found that the expression of miR-100 was significantly lower in PCa specimens with bone metastases than that without metastases. Leite et al (13,14) also reported that miR-100 was downregulated during the PCa progression, from high grade prostate intraepithelial neoplasia through metastasis. Moreover, Sun et al (10) found that miR-100 was downregulated in the more advanced PCa C4-2 cells relative to the parental LNCaP cells. Importantly, our results showed that mir-100 negatively regulated migration, invasion EMT and stemness properties of PCa cells. Therefore, these findings indicate that mir-100 is a metastatic suppressor in PCa.

However, mir-100 appears to be cancer specific in regulating tumor metastasis. Huang et al (33) found that miR-100 expression increased in metastatic pancreatic cancer cells. Furthermore, Wang et al (34) reported that miR-100 overexpression strongly associates with advanced tumor progression in renal cell carcinoma. Moreover, miR-100 was transiently introduced into A549 cells, NCI-H727 and NCI-H1437 cells, resulting in a significant increase of cell migration activity (35). The above results suggest that miR-100 may be a promoter of tumor metastasis. On the contrary, in clear cell renal cell carcinomas, miR-100 was downregulated in metastatic tissue samples compared with normal tissue and was downregulated in metastatic tissue in comparison with primary tumor tissue (36). Furthermore, downregulation of miR-100 was significantly associated with lymph node metastasis and reduced survival in Small cell carcinoma of the cervix (37). Thus, these findings supported that miR-100 was a suppressor of metastasis.

Some previous studies have demonstrated that Ago2 is a promoter of metastasis in different solid tumors. In hepato-

cellular carcinoma (HCC), Ago2 overexpression promoted migration and metastasis of HCC cells (23). AGO2 was over-expressed in colon cancer relative to adjacent non-cancer tissue and the expression of AGO2 appeared to increase in advanced tumors with distant metastasis, suggesting it may promote tumor invasion (38). In human breast cancer cells, high level of Ago2 was correlated with an enhanced proliferation and migration ability (39). In serous ovarian carcinoma, AGO2 mRNAs were overexpressed in solid metastases compared with primary carcinomas and higher levels of AGO2 mRNA was significantly associated with high-grade histology (40). In head and neck squamous cell carcinoma cell lines and primary tumors, AGO2 was overexpressed and was functionally significant in cell lines (41). In the present study, the results showed that downregulation of AGO2 repressed migration, invasion, EMT and stemness of PCa cells. These findings indicated that AGO2 was a promoter of tumor progression and metastasis. Furthermore, our results showed that AGO2 is a direct target of miR-100. Thus, the critical mechanism of miR-100 suppression of metastasis in PCa cells may be to downregulate AGO2 expression. However, it remains to be determined how AGO2 regulates metastasis.

Recent studies have shown that Ago2 directly regulates miRNA biosynthesis (24). For example, the silencing of Ago2 upregulated miR-125b expression in myeloblastic HL60 cells (42). In the present study, we showed that repressing AGO2 in PCa cells enhanced expression of miR-125b and miR-34a, which were downregulated in human PCa tissues compared with normal tissues and in bone metastatic PCa tissues compared to primary tumors (5,7,10,12). Many studies have demonstrated that miR-100 and miR-125b were concurrently downregulated in human PCa tissues (10,12) and in bone metastatic PCa tissue (5). These findings suggested that downregulation of miR-125b and miR-34a in human primary and metastatic PCa tissues might be related to upregulation of Ago2, which was directly regulated by miR-100.

Previous studies have demonstrated that miR-34a and miR-125b are mainly metastatic suppressors, and play an important role in inhibiting metastasis of cancer cells, including PCa cells (7,12,43-48). The miR-34a was able to directly inhibit EMT regulators Snail1, ZEB1 and ZEB2 (43,44), and the stemness factor CD44 in PCa cells (7). Downregulation of miR-125b was able to promote metastatic oncoprotein expression and enhance migration, invasion and metastasis (45-48). ERBB2, a target of miR-125b (49), played an important role in promoting stemness properties and EMT (50-52). Therefore, an important mechanism of AGO2 promoting metastasis of PCa cells may be to enhance metastasis-promoting proteins, EMT and stemness by repressing expression of miR-34a and miR-125b.

Recent studies have demonstrated that Ago2 can directly regulate expression of stemness factors Oct4, Sox2, Nanog, Klf4 and c-myc following its binding to the regulatory regions of functional genes (53). Moreover, in Ago2-knockdown embryos at the blastocyst stage, transcription levels of Oct3/4, Nanog and Sox2 were decreased (54). In this study, our results showed that downregulation of AGO2 repressed expression of stemness factors Oct4, Klf4 and c-myc of PCa cells. Thus another mechanism of Ago2 regulating metastasis of PCa may be to directly upregulate stemness gene expression. In addition, Ago2 might have regulatory functions in CSC self-renewal

through the RNA-mediated gene silencing mechanism as a component of RISC (55).

Our results showed that AGO2 was a direct target of miR-100, and played an opposite role to miR-100 in regulating migration, invasion, EMT and stemness of PCa cells. Furthermore, downregulation of AGO2 was able to partially reverse the effects seen with miR-100 downregulation in PCa cells. Importantly, downregulation of AGO2 enhanced expression of miR-34a and -125b which can suppress migration, invasion, EMT and stemness of cancer cells. Thus, a proposed model of the close interconnections among miR-100, AGO2 and metastasis is shown in Fig. 7.

In conclusion, our findings indicated that miR-100 negatively regulates the metastatic ability of PCa cells at least partially by targeting AGO2 which repressed the metastatic suppressor miR-34a and miR-125b expression, and modulated migration, invasion, EMT and stemness of cancer cells. Our results suggest that miR-100/AGO2 may play an important role in regulating metastasis of PCa and is a potential novel target for prevention and therapy.

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