

Wild-type p53 suppresses the epithelial-mesenchymal transition and stemness in PC-3 prostate cancer cells by modulating miR-145

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Abstract. The principal problem arising from prostate cancer (PCa) is its propensity to metastasize to bone and the mechanism(s) need to be further elucidated. The tumor suppressor p53 plays an important role in regulating the epithelial-mesenchymal transition (EMT) and cancer cell stemness, which have been proposed to play critical roles in cancer metastasis. MiR-145, a direct target of p53, represses bone metastasis of PCa and is involved in regulating EMT and cancer cell stemness. However, it is unknown whether wild-type p53 (WT-p53) plays a role in regulating invasion, EMT and cancer cell stemness of PCa cells and whether miR-145 mediates the function of WT-p53. In the present study, we found that ectopic expression of WT-p53 inhibited the migration and invasion, and enhanced the adhesion of p53-null PC-3 cells derived from PCa bone metastasis. Furthermore, WT-p53 suppressed the expression of the mesenchymal markers fibronectin, vimentin, N-cadherin, ZEB2 and upregulated the expression of the epithelial marker E-cadherin in PC-3 cells. Moreover, WT-p53 also suppressed colony formation, tumor sphere formation and expression of CSC markers and stemness factors including CD44, Oct4, c-Myc and Klf4 in PC-3 cells. Importantly, WT-p53 upregulated the expression of miR-145, and the inhibitory

effects of WT-p53 on migration, invasion, EMT and stemness of PC-3 cells were reversed by anti-miR-145. Together, our findings demonstrate that WT-p53 suppresses migration, invasion, EMT and stemness in PC-3 cells at least partially through modulating miR-145. These results suggest that loss of WT-p53 may promote the bone metastasis of PCa at least partially through repressing miR-145 to elevate EMT and stemness of cancer cells.

Introduction

The principal issue derived from prostate cancer (PCa) is its inclination to metastasize to bone, which occurred in as many as 90% of patients with advanced PCa (1). However, the exact mechanisms of bone metastasis of PCa need further to be elucidated.

As a transcription factor, the tumor suppressor p53 mediates changes in gene expression that promote apoptosis, senescence or a reversible and protective cell cycle arrest (2,3). In about half of all human cancers, p53 is either lost or mutated. Loss of wild-type p53 (WT-p53) function is well known to influence cell cycle checkpoint controls and apoptosis (4) and gain of function of mutant p53 is involved in development and progression of many cancers (4-6). Importantly, emerging evidence has shown that WT-p53 also plays a role in regulating key stages of metastatic progression (4,6), but how it functions as metastasis/invasion suppressor is just beginning to be understood (7). In PCa, it also remains elusive whether and how WT-p53 regulates bone metastasis although mutant p53 may promote bone metastasis (5).

Recent studies have found that two important mechanisms by which p53 regulates metastasis, repression of migration and invasion of cancer cells through modulating epithelial-mesenchymal transition (EMT) and suppression of cancer stem cell (CSC) properties (4,8-11). EMT is a key step of the progression of tumor cell metastasis (12). It also has been identified as an important step in bone metastasis of PCa (13). E-cadherin plays a critical role in EMT which is regulated by transcription factors including Snail, Slug, Twist and Zeb1/2 (14). By targeting these transcription factors, p53

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Abbreviations: PCa, prostate cancer; miRNAs, microRNAs; EMT, epithelial-mesenchymal transition; CSCs, cancer stem cells; WT-p53, wild-type p53

Key words: prostate cancer, wild-type p53, microRNAs, epithelial-mesenchymal transition, cancer cell stemness

regulates EMT (6). Furthermore, the CSCs are the cells within tumors that possess the ability of self-renewal, immortalized proliferation and differentiate into the heterogeneous lineages of cancer cells which consist of the whole tumor (15,16). These capabilities of cancer stem cells have formed the basic definition of 'stemness' (17). Accumulating evidence suggests that cancer cell stemness is associated with the metastasis of tumors (16,17). Recent studies indicated that p53 has crucial influence on cancer cell stemness by regulating key stemness genes (8,11).

Emerging evidence has demonstrated that miRNAs are components of the cellular signaling circuitry that regulates the EMT program (18), such as miR-200 family (19,20), miR-34 family (21,22) and miR-205 (19). These miRNAs directly target transcription factors Snail, Slug and Zeb1/2, and regulate the EMT of cancer cells. Furthermore, miRNAs also played a pivotal role in regulating the properties of CSCs by negatively regulating the expression of certain key genes, such as CD44, Oct4, Sox2, c-Myc and Klf4 (23). Moreover, some miRNAs are transcriptionally regulated by p53 (24). Importantly, several miRNAs, such as miR-200c and miR-34 family mediate p53 regulation of EMT (8-11) and stem cell properties in cancers (21).

Our previous studies have demonstrated that miR-145 is associated with bone metastasis of PCa by suppressing EMT and stemness of cancer cells (25,26). Also microRNA-145 is directly regulated by WT-p53 (27-30), and the loss of WT-p53 function occurs in many PCa, therefore, we reasoned that WT-p53 may play a role in regulating EMT and cancer cell stemness of PCa cells and miR-145 may mediate the function of WT-p53.

To test the hypothesis, we upregulated expression of WT-p53 in p53-null PC-3 cells derived from PCa bone metastasis and found that ectopic expression of WT-p53 inhibited migration, invasion, EMT and stemness of PC-3 cells, and the inhibitory effects of WT-p53 on EMT and cancer cell stemness of PC-3 cells were reversed by anti-miR-145. Our findings demonstrate that WT-p53 represses EMT and stemness of PC-3 cells at least partially by mediating the miR-145.

Materials and methods

Cell culture. The bone metastatic PCa cell line PC-3 was purchased from American Type Culture Collection (ATCC, Manassas, VA, USA) and grown in Ham's F-12 culture medium (HyClone, Logan, UT, USA) supplemented with 10% fetal bovine serum (HyClone). Cells were grown at a humidified atmosphere of 5% CO₂ at 37°C.

Plasmids and transient transfection. Plasmids expressing WT-P53 and miR-145-antagomiR were purchased from RiboBio Co. Ltd (Ribo, China). The cloning sequence of WT-P53 was from 203 to 1,384 in the CDS region and miR-145 in pMSCV was constructed as described previously (25). Before transfection, 2x10⁵ cells were seeded into each well of 6-well plates. After 24 h incubation in growth medium, the cells were transiently transfected with using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. Transfection medium is the Opti-Mem medium which is used for transient transfection. The trans-

fection medium was removed after 4-6 h, and the cells were incubated for an additional 48 h in 10% fetal bovine serum medium (2 ml per well).

Quantitative reverse transcription-PCR. The procedure was carried out according to the manuscript of All-in-One™ miRNA qRT-PCR detection kit (GeneCopoeia, Rockville, MD, USA). Total RNAs were extracted from cells by using RNeasy kit (Qiagen). Total RNA was reverse transcribed by adding poly-A sequence, and real-time PCR analysis was performed with specific primer to WT-p53 and hsa-miR-145 (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 values from three independent experiments were calculated following the 2^{-ΔΔCt} method of Schmittgen and Livak (31).

Western blot analysis. For the analysis of expression of related proteins, western blot assay was performed. The cells were seeded in 6-well plates. After 24-48 h, cells were washed with pre-chilled PBS and at confluence of 60-70% harvested in sample buffer [62.5 mmol/l Tris-HCl (pH 6.8), 2% SDS, 10% glycerol and 5% β-mercaptoethanol]. Equal amounts of protein from the supernatant were loaded per lane and resolved by SDS-polyacrylamide electrophoresis. Then, protein was transferred onto PVDF membrane (Millipore), blocked by 5% non-fat milk for 1 h at room temperature, and probed with primary antibodies (1:1,000) overnight at 4°C, including rabbit anti-P53, Oct4, c-Myc, Klf4, CD44 and mouse anti-E-cadherin, vimentin (Cell Signaling Technology); rabbit anti-N-cadherin (Millipore); mouse anti-fibronectin (BD Biosciences) and mouse anti-ZEB2 (Sigma, St. Louis, MO, USA). Membranes were washed three times (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 (Cell Signaling Technology).

Wound healing assay. PC-3 cells were trypsinized and seeded equivalently into 6-well tissue culture plates 24 h before scratching, and grew to reach almost total confluence in 24 h. Non-serum starvation lasted for 24 h. After cell monolayers formed, a wound was scratched onto the monolayer with a sterile 100 μl tip (Axygen, Union City, CA, USA). After scratching, the cells were washed with PBS twice and cultured in 10% fetal bovine serum media. Images of PC-3 cells migrating into the wound were captured at time points of 0, 6 and 12 h by an inverted microscope (x40).

Invasion assay. The invasion assay was performed 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 1.5x10⁵ cells were added to the upper chamber, and lower chamber was filled with the medium with 10% FBS. After incubated for 24-48 h, cells passed through the coated

membrane to the lower surface, in which cells were fixed with 4% paraformaldehyde and stained with hematoxylin. The cell count was done under a microscope (x100).

Adhesion assay. Briefly, 96-well plates were coated with 50 μ l fibronectin (50 μ g/ml) at cell incubator for 1 h. After washed with warm PBS, the plates were blocked with 1% BSA at 37°C for 1 h and washed twice. After trypsinization, suspended cells were seeded to each well with serum-free media at a density of 1.5×10^4 cells per well. The cells were incubated for 30 min, non-adherent cells were removed and plates were gently washed twice with PBS. Adherent cells were fixed in 4% paraformaldehyde for 20 min at room temperature, then stained with hematoxylin and counted under an inverted microscope (x100).

Colony formation assay. The cells were trypsinized as single cells and suspended in serum-free medium. Cells (300 cells/ml) were seeded into each well of 6-well plates for 1-2 weeks, and colonies were dyed with crystal violet. Plating efficiency = number of colonies (≥ 50 cells per colony) per input cells $\times 100\%$. Colony morphology was determined, by scoring under a light microscope.

Self-renewing spheroid formation assay. Cells (500 cells/well and 50-100 cells/well) were plated respectively onto 6-well and 24-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 10-14 days. After 10-14 days, the number of PC-3 cell spheres (tight, spherical, non-adherent masses $>50 \mu$ m in diameter) were counted, and image of the spheres were captured under an inverse microscope. Sphere formation efficiency = colonies/input cells $\times 100\%$.

Statistical analyses. Statistical significance of the studies was analyzed by Student's t-test or one-way ANOVA. Statistical significance was accepted at $p < 0.05$.

Results

WT-p53 repressed invasiveness of PC-3 cells and anti-miR-145 rescued the effect. To investigate the role of WT-p53 in the development and progression of PCa metastasis, we upregulated WT-p53 by transfecting the plasmid of WT-p53 expression in PC-3 cells, in which p53 is null. The expression of WT-p53 was confirmed by real-time PCR in mRNA level (Fig. 1A) and western blot analysis in protein level (Fig. 1B). We investigated whether WT-p53 was able to regulate invasion, migration and adhesion in PC-3 cells. By using Transwell Matrigel invasion assay to assess the invasive ability of cells, we found that WT-p53 repressed invasive ability to 45.7% of PC-3/vector (Fig. 1C). In adhesion assay, WT-p53 increased adhesive ability 2.87-fold that of PC-3/vector (Fig. 1D). As shown in Fig. 1E, in cell migration observed by wound healing assay, WT-p53 decreased the healing speed of the cell wound compared to the PC-3/vector.

Because miR-145 is transcriptionally regulated by WT-p53 (27-30), determined whether inhibiting miR-145 could rescue the effects of WT-p53. After upregulating WT-p53, we applied miR-145-antagomiR in PC-3 cells. The expression of miR-145 was confirmed by real-time PCR. We found that WT-p53 upregulated the expression of miR-145 31.0-fold that of the PC-3/vector (Fig. 1F). The anti-miR-145 repressed WT-p53 expression in mRNA and protein levels. It also completely counteracted expression of WT-p53 in PC-3 cells as anti-miR-145 increased the cell migration speed and invasive ability that was reduced by WT-p53 expression, and also reduced miR-145 expression and the adhesive ability that was increased by WT-p53 (Fig. 1). These results indicated that WT-p53 inhibited invasiveness of PC-3 cells through modulation of miR-145.

WT-p53 represses EMT and anti-miR-145 rescues the effect in PC-3 cells. To investigate whether miR-145 regulated invasiveness by repressing EMT, we examined the influence of ectopic expression of WT-p53 on expressions of E-cadherin, N-cadherin, fibronectin, vimentin and ZEB2 of PC-3 cells by western blot analysis. E-cadherin, which is one of key epithelial markers and supposed to be downregulated during EMT, was increased in PC-3 cells with WT-p53 expression. N-cadherin, fibronectin and vimentin, which are kind of mesenchymal markers and should be upregulated during EMT, were repressed in PC-3 cells with WT-p53 expression. ZEB2, which is a transcription factor upregulating EMT, was repressed in PC-3 cells with WT-p53 expression (Fig. 2A and B). Further, we detected the change of morphology of PC-3 cell with characteristics of EMT. The PC-3 cells with ectopic expression of WT-p53 converted the predominant mesenchymal phenotype to an evident epithelial phenotype i.e. from a stick-like or long spindle-shaped mesenchymal profile to a cobblestone-like or a short spindle-shaped epithelial morphology (Fig. 2C).

We determined whether inhibiting miR-145 would rescue the effects of expressing WT-p53 in PC-3 cell. As Fig. 2 shows, the anti-miR-145 completely counteracted the effects of WT-p53 expression in PC-3 cells as anti-miR-145 reduced the level of the epithelial cell marker E-cadherin that was increased by p53 expression, and restored expression levels of the mesenchymal cell marker N-cadherin, fibronectin, vimentin and ZEB2 that was reduced by WT-p53 expression. The anti-miR-145 also was able to convert the predominant epithelial phenotype that was changed by WT-p53 expression to an evident mesenchymal phenotype. These observations indicated that WT-p53 repressed EMT through modulation of miR-145 in PC-3 cells.

WT-p53 inhibits colony formation of PC-3 cells and anti-miR-145 rescues the effect. We determined efficiency of WT-p53 inhibiting colony-forming of PC-3 cells *in vitro*. The colony-forming assay was performed. The number of colonies (% plating efficiency) were 34.3% in PC-3/WT-p53 and 63.7% in PC-3/vector and significantly decreased in PC-3/WT-p53 compared with PC-3/vector ($p < 0.01$, respectively) (Fig. 3). Colonies with different morphologies *in vitro* are classified as holoclones, meroclones and paraclones (32). Holoclones are generally more round and tightly packed; paraclones are irregular in composition and often contain more elongated or

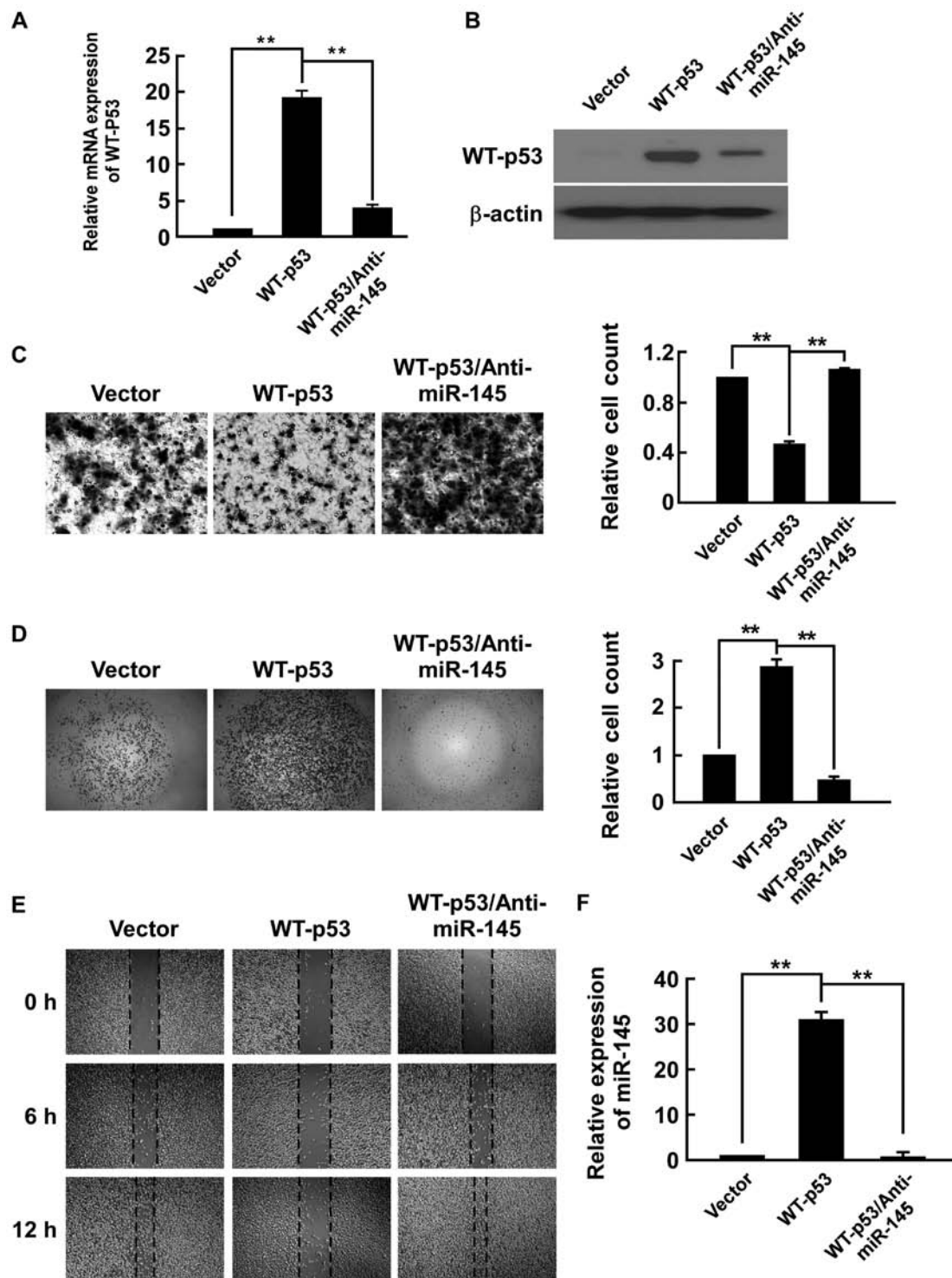


Figure 1. WT-p53 repressed invasiveness of PC-3 cells by modulating miR-145 in PC-3 cells. (A) The mRNA level of WT-p53 in PC-3/vector, PC-3/WT-p53 and PC-3/WT-p53/anti-miR-145. (B) WT-p53 protein level in PC-3/vector, PC-3/WT-p53 and PC-3/WT-p53/anti-miR-145. (C, D and E) WT-p53 efficiently suppressed migration and invasion; increased adhesion in PC-3 cells; and anti-miR-145 counteracted the effects of WT-p53 (** $p < 0.01$).

flattened cells; and microclones are an intermediate phenotype. We did not find typical holoclones in PC-3 cells. The proportion of microclones was 80.1% in PC-3/vector and 58.9% in PC-3/WT-p53, and WT-p53 significantly decreased the proportion of microclones of PC-3 cells ($p < 0.01$, respectively) (Fig. 3). Furthermore, we determined whether

WT-p53 regulates colony-forming by modulating miR-145 in PC-3 cells. As shown in Fig. 3, the anti-miR-145 completely counteracted the effect of expressing WT-p53 in PC-3 cells as anti-miR-145 restored colony-forming capability that was diminished by WT-p53 expression. The number of colonies was 71.4% and the proportion of microclones was 81.4%

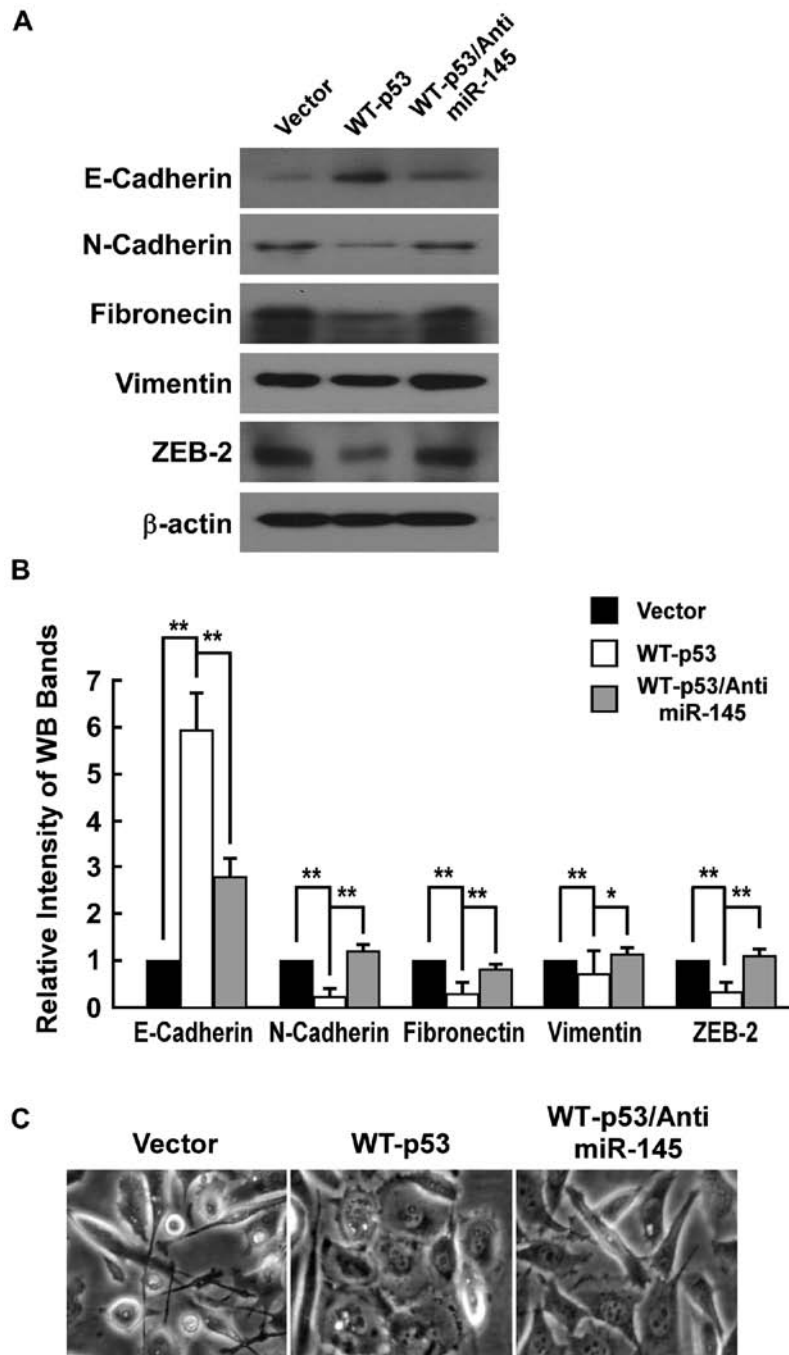


Figure 2. WT-p53 represses EMT by modulating miR-145 in PC-3 cells. (A and B) WT-p53 increased E-cadherin expression and suppressed N-cadherin, fibronectin, vimentin and ZEB2 expression and anti-miR-145 counteracted the effects of WT-p53 (* $p < 0.05$; ** $p < 0.01$). (C) WT-p53 converted a stick-like or long spindle-shaped mesenchymal profile to a cobblestone-like or a short spindle-shaped epithelial morphology, and anti-miR-145 counteracted the effects of WT-p53.

in PC-3/WT-p53/anti-miR-145. These data indicated that WT-p53 repressed cancer cell colony-forming by modulation of miR-145 in PC-3 cells.

WT-p53 inhibits tumor spheroid formation of PC-3 cells and anti-miR-145 rescues the effect. The ability to grow as non-adherent spheroids in the sphere medium has been widely used to assess the self-renewal capability of CSCs and is one of the properties of prostate CSCs (33). To confirm that WT-p53 can inhibit the self-renewal capability of PC-3 cells, prosta-sphere formation of PC-3 cells was studied. As shown in Fig. 4,

after culturing for 12 days under non-adherent conditions, there were prostaspheres in all the cell types. The spheroid formation efficiency was 4.8% in PC-3/vector and 2.3% in PC-3/WT-p53, confirming the presence of the self-renewal cell in PC-3/vector and PC-3/WT-p53, and WT-p53 suppressed significantly prostasphere formation ($p < 0.05$). Furthermore, we determined whether WT-p53 regulates tumor spheroid formation by modulating miR-145 in PC-3 cells. As shown in Fig. 4, the anti-miR-145 completely counteracted the effect of WT-p53 expression in PC-3 cells as anti-miR-145 restored the capability of tumor spheroid formation that was diminished

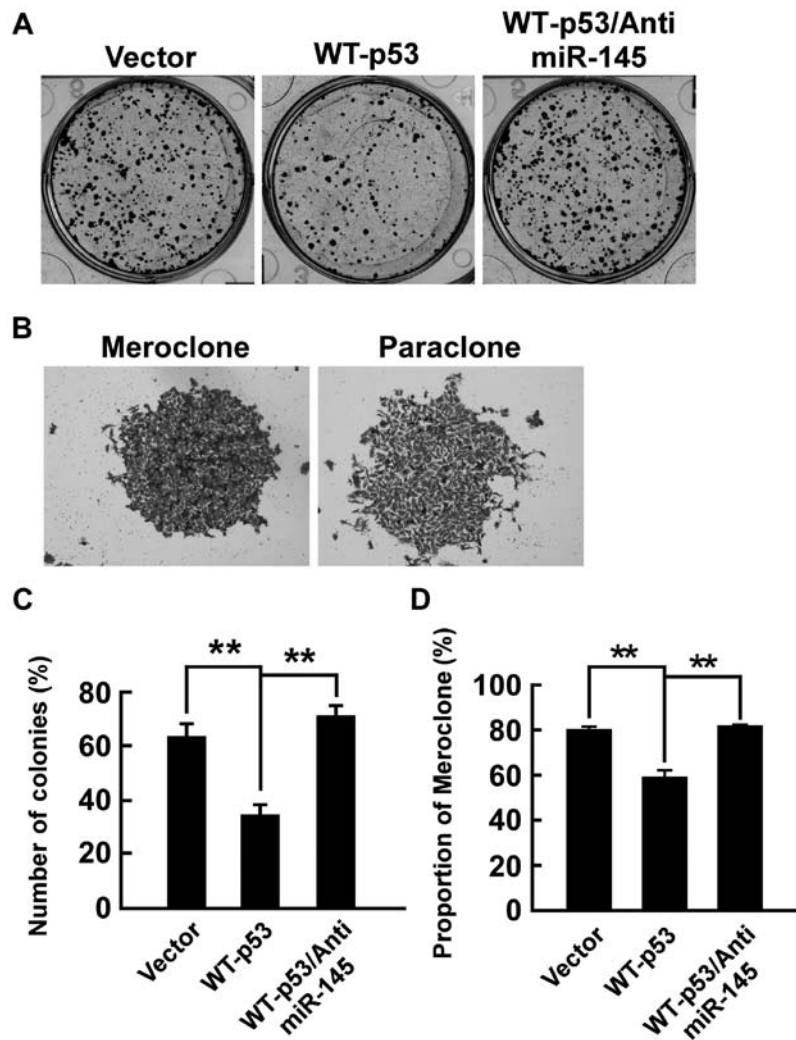
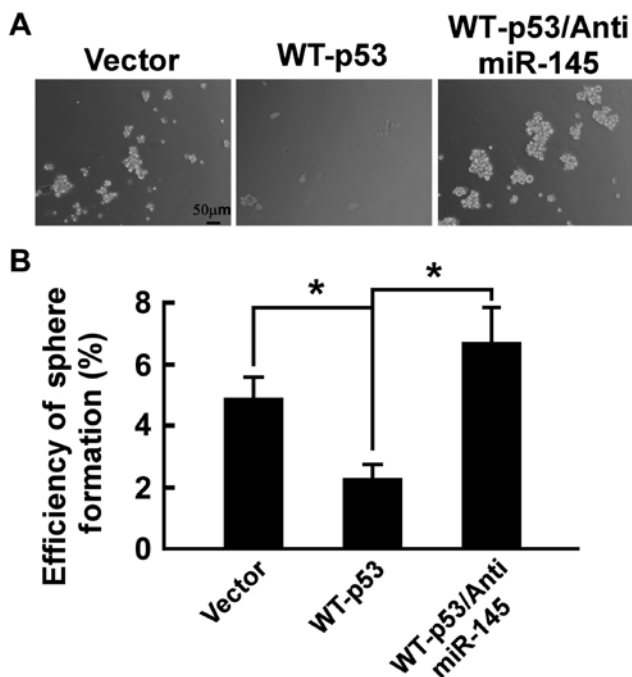


Figure 3. WT-p53 inhibits colony formation of PC-3 cells by modulating miR-145. (A) Colony-formation existed in PC-3/vector, PC-3/WT-p53 and PC-3/WT-p53/anti-miR-145. (B) A typical holoclone phenotype was not found, and only meroclonal and paraclonal colonies were observed. (C and D) WT-p53 significantly repressed the number of colonies formation and the proportion of meroclonal colonies. The miR-145 reversed the effect of WT-p53 (***p*<0.01).



by WT-p53 expression. The spheroid formation efficiency was 6.7% in PC-3/WT-p53/anti-miR-145. These data indicated that WT-p53 repressed the self-renewal capability of cancer cells by modulating miR-145 in PC-3 cells.

WT-p53 inhibits CD44, c-Myc, Oct4 and Klf4 expression and anti-miR-145 restores their expression in PC-3 cells. To elucidate whether WT-p53 also have an influence on CSC marker and ‘stemness’ factor expression of PCa cells, we upregulated WT-p53 in PC-3 cells by transfecting the plasmid of WT-p53 expression and detected the expression of stem cell properties-associated factors and markers, including c-Myc, Oct4, Klf4 and CD44. As shown in Fig. 5, WT-p53 reduced the expression of CD44, which has been described as prostate CSC

Figure 4. WT-p53 suppresses the ability of tumor sphere formation in PC-3 cells by modulating miR-145. (A) Tumor sphere formation was observed by PC-3/vector, PC-3/WT-p53 and PC-3/WT-p53/anti-miR-145. (B) WT-p53 efficiently suppressed the ability of spheroid formation and miR-145 reversed the effect of WT-p53 (**p*<0.05).

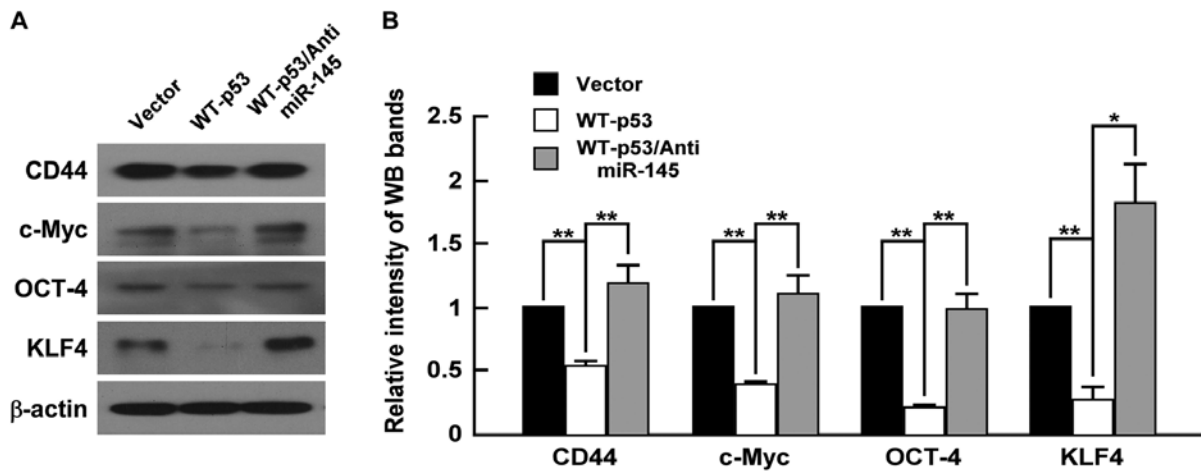


Figure 5. WT-p53 inhibits CD44, c-Myc, Oct4 and Klf4 expression in PC-3 cells by modulating miR-145. The overexpression of WT-p53 by transfecting the plasmids of WT-p53 significantly reduced the expression levels of CD44, C-Myc, Oct4 and Klf4, and anti-miR-145 counteracted the effects of WT-p53 (* $p < 0.05$; ** $p < 0.01$).

marker based on clinical investigations and *in vitro* studies of prostate cancer cell lines (21), 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 (34). Furthermore, we determine whether WT-p53 regulates the stemness factors by modulating miR-145 in PC-3 cells, and found that anti-miR-145 counteracted the effects of WT-p53 expression as anti-miR-145 restored expression levels of CD44, Oct4, c-Myc and Klf4 that was reduced by WT-p53 expression (Fig. 5). These data indicated that WT-p53 repressed 'stemness' factors through modulation of miR-145 in PC-3 cells.

Discussion

We have previously identified that miR-145 may repress bone metastasis of PCa and is involved in regulating EMT and stemness of PCa cells (25,26). In the present study, we found that the ectopic expression of WT-p53 inhibited migration, invasion, EMT, colony formation, tumor sphere formation and expression of CD44, Oct4, c-Myc and Klf4 in PC-3 PCa cells, and enhanced miR-145 expression in PC-3 cells. Importantly, the anti-miR-145 was able to reverse the above-mentioned inhibitory effects of WT-p53. Our findings demonstrate that WT-p53 repressed EMT and cancer cell stemness of PC-3 cells at least partially through regulation of miR-145.

Many studies have demonstrated that p53 plays a key role in the function of cell cycle, apoptosis, senescence, DNA-repair mechanisms and autophagy (3,4). However, the emerging evidence demonstrates that p53 also plays a crucial role in regulating key stages of the metastatic progression (4,6). In PCa, although some studies have showed that mutant p53 gain of function promoted cancer development and metastasis (5,35-38), there are hardly any studies on the role of WT-p53 in PCa metastasis. We found that WT-p53 repressed invasion and migration, and suppressed EMT, which is a key step of the progression of tumor cell metastasis, and cancer stem cells properties in PC-3 cells, which might be the critical drivers of tumor progression and metastasis (16,17). Zhou *et al* (39,40) have found that the combined deficiency for p53 and Rb in prostate epithelium

results in invasive and highly metastatic prostate carcinogenesis, and preferential malignant transformation of prostate stem cell compartment by combined deficiency by p53 and Rb indicates a critical role of these genes in the regulation of prostate stem cells during ontogenesis. Moreover, the loss of Pten/TP53 in prostate epithelium led to transformation of multipotential progenitors and EMT (41). These findings provide evidences that loss of WT-p53 may promote the metastasis of PCa by elevating migration, invasion, EMT and stemness of cancer cells.

Recently studies have found that miRNAs contribute to downregulation of mRNA and protein expression observed after p53 activation (24) and several miRNAs, which were the direct transcriptional targets of p53, played a critical role in mediating p53 regulation of EMT in cancer progression (8-11). The miR-34a, a tumor suppressor which directly targets Snail, was decreased due to the absence of WT-p53 function, thus Snail-dependent EMT was activated in colon, breast and lung carcinoma cells (22). The p53 also played a role in regulating EMT through transcriptional activation of miR-200c (8). Inhibition or overexpression of the miR-200 family affected p53-regulated EMT by altering ZEB1 and ZEB2 expression (10). Mutant p53 gain-of-function induces EMT through modulation of the miR-130b-ZEB1 axis in endometrial cancer (42). Previous studies have demonstrated that miR-145 is transcriptionally and post-transcriptionally regulated by WT-p53 (27-30), and miR-145 augments the effects of p53 by suppressing the inhibitors of p53 in cervical cancer cells (29). In this study, we found that WT-p53 enhanced miR-145 expression in PC-3 cells and anti-miR-145 reversed EMT features of PC-3 cells which were inhibited by ectopic expression of WT-p53. Importantly, miR-145 is upregulated by WT-p53, but not with mutant p53 in PC-3 cells (30), the anti-miR-145 also repressed the expression of WT-p53, and miR-145 itself also represses EMT in PC-3 cells (25). Collectively, these findings indicate that miR-145 is a mediator of WT-p53-regulated EMT.

Although miR-34a and miR-200c mediated p53-regulated EMT of cancer cells mainly through targeting EMT regulators Snail1, ZEB1 and ZEB2 (8,10,42), our results suggested that miR-145 might mediate p53-regulated EMT of PC-3 cells by

targeting several pathways. We found that WT-p53 repressed expression of the mesenchymal markers N-cadherin, which was one of miR-145 targets (43), and ZEB2, which was speculated as one of miR-145 targets and a transcription factor promoting EMT, and the inhibitory effects of WT-p53 on expression of N-cadherin and ZEB2 in PC-3 cells were reversed by anti-miR-145. We also found that metastasis-promoting protein HEF1 was a direct target of miR-145 and it promoted EMT of PC-3 cells (the results not shown). Therefore, miR-145 may mediate WT-p53-regulated EMT of PCa cells by targeting N-cadherin, ZEB2 and HEF1.

Besides regulating EMT, miRNAs which are the direct transcriptional targets of p53 played a role in mediating p53 regulation of cancer cell stemness in cancer progression. Liu *et al* (21) found that miR-34a inhibited prostate cancer stem cells and metastasis by directly repressing CD44. Furthermore, p53 regulated stem cell properties through modulating miR-200c by regulating KLF4 and BMI1 (8). Our previous results showed that miR-145 repressed stemness of PC-3 cells by suppressing CD44, Oct4, c-Myc and Klf4 (26). In present study, we showed that anti-miR-145 reversed colony formation and tumor sphere formation of PC-3 cells which were inhibited by ectopic expression of WT-p53. Moreover, WT-p53 also suppressed Oct4, c-Myc and Klf4 in PC-3 cells, which were directly targeted by miR-145 (27,31), CD44, which was speculated as one of miR-145 putative targets (miRWalk), and the inhibitory effect of WT-p53 on the above-mentioned 'stemness' factors is reversed by anti-miR-145. All these findings indicate that miR-145 also is a mediator of WT-p53 regulation of cancer cell stemness and suggest that miR-145 may target CD44, Oct4, c-Myc and Klf4 in PC-3 cells.

Recent evidence has demonstrated that the EMT can generate cancer cells with properties of stem cells (44,45). This important finding implies a direct link between EMT and cancer stem cells. A previous study found that miR-200 played a critical role in linking EMT phenotype with stem cell signatures by regulating the expression of Lin28B and Notch1 in PCa cells (45). We found that p53/miR-145 pathway regulated both EMT and stemness of PC-3 cells. These finding suggested that the p53/miR-145 pathway might be the new link between EMT and cancer cell stemness in PC-3 cells. Thus, the discovery of molecular targets which are regulated by p53/miR-145 axis and linked to EMT and CSC properties are required.

In conclusion, our findings demonstrate that WT-p53 suppresses migration, invasion, EMT and stemness of PC-3 PCa cells at least partially through modulating miR-145. These results suggest that loss of WT-p53 might promote bone metastasis of PCa at least partially through repressing miR-145 to elevate EMT and stemness of cancer cells. Therefore, the activation of the p53/miR-145 regulatory axis may function as a therapeutic alternative for bone metastasis of PCa.

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