P21-activated kinase 6 (PAK6) inhibits prostate cancer growth via phosphorylation of androgen receptor and tumorigenic E3 ligase murine double minute-2 (Mdm2)

Tong Liu, Yang Li, Hui Gu, Ge Zhu, Jiabin Li, Liu Cao, Feng Li*
Department of Cell Biology, Key Laboratory of Cell Biology, Ministry of Public Health, and Key Laboratory of Medical Cell Biology, Ministry of Education, China Medical University, Shenyang 110001, China

**Running title:** PAK6 inhibits prostate cancer growth by phosphorylation

*To whom correspondence should be addressed: Feng Li, Department of Cell Biology, Key Laboratory of Cell Biology, Ministry of Public Health, and Key Laboratory of Medical Cell Biology, Ministry of Education, China Medical University, Shenyang 110001, China Tel.: (86) 24 232 61056; Fax: (86) 24 232 61056; E-mail: fli@mail.cmu.edu.cn

**Key words:** PAK6; AR; Mdm2; phosphorylation; ubiquitin degradation; homeostasis

**Background:** Deregulation of Androgen-AR signaling may lead to prostate cancer.

**Results:** PAK6 promotes AR ubiquitin-mediated degradation through phosphorylation of AR and Mdm2, resulting in inhibition of prostate cancer growth in vivo.

**Conclusion:** PAK6 suppresses prostate cancer growth via regulating AR homeostasis.

**Significance:** Modulating PAK6 kinase activity is a therapeutic strategy for AR positive and hormone sensitive prostate cancer.

**SUMMARY**

The androgen receptor (AR) signaling pathway plays a crucial role in the development and growth of prostate malignancies. Regulation of AR homeostasis in prostate tumorigenesis has not yet been fully characterized. In the present study, we demonstrate that p21-activated kinase 6 (PAK6) inhibits prostate tumorigenesis by regulating AR homeostasis. First, we demonstrated that in normal prostate epithelium, AR co-localizes with PAK6 in cytoplasm and translocates into nucleus in malignant prostate. Furthermore, AR phosphorylation at Ser578 by PAK6 promotes AR-E3 ligase murine double minute-2 (Mdm2) association, causing AR degradation upon subsequent androgen stimuli. We also showed that PAK6 phosphorylates Mdm2 on Thr158 and Ser186, which is critical for AR ubiquitin-mediated degradation. Moreover, we found that Thr158 collaborates with Ser186 for AR-Mdm2 association and AR ubiquitin-mediated degradation as it facilitates PAK6-mediated AR homeostasis. PAK6 knockdown promotes prostate tumor growth in vivo. Strikingly, we found a strong inverse correlation between PAK6 and AR expression in cytoplasm of prostate cancer cells. These observations indicate that PAK6 may be important for the maintenance of androgen-induced AR signaling homeostasis and in prostate malignancy, as well as being a possible new therapeutic target for AR-positive and hormone-sensitive prostate cancer.

**The androgen receptor (AR),** a well-known transcriptional factor, generally regulates gene expression through a ligand-dependent mechanism. The androgen-AR signaling pathway plays an essential role in the development, function...
and balance of androgen response system, especially for the growth and development of prostate normal and cancer cells, as well as in recurrent prostate cancer after androgen ablation therapy (1, 2, 3, 4). Moreover, animal and human models showed that AR expression positively correlated with progression of primary and metastatic prostate cancer (5, 6). AR signaling homeostasis needs to be tightly controlled to maintain diverse cell functions. Deregulation of this homeostasis promotes defects in androgen responses and promotes prostate cancer (7).

Androgen stimulation promotes AR translocation from cytoplasm to nucleus and initiates transcriptional regulation of downstream target genes associated with a distinct profile of cofactors. Also, it is well known that excessive AR expression in nucleus leads to prostate cancer (8). AR undergoes post-translational modifications, such as phosphorylation, sumoylation, acetylation and ubiquitination (9). Especially, AR is a substrate for many protein kinases, such as Aurora-A, PKC, and Akt (10, 11, 12), which modulate AR activity via phosphorylation. AR phosphorylation also leads to its ubiquitination and degradation by E3 ligases, such as Murine double minute 2 protein (Mdm2) (13, 14, 15) and Carboxyl-terminus of Hsc70-Interacting Protein (CHIP) (16).

As a well-known negative regulator of AR, PAK6 is of great concern for the inhibition of prostate cancer growth. As a serine/threonine kinase, PAK6 was initially cloned from prostate cancer cells as an AR-interacting protein (17, 18). It is a member of the p21-activated kinases (PAKs) family, which is the major effector of RhoGTPases Cdc42 and Rac (19). Upon upstream signals, PAK proteins are involved in a variety of cellular functions, including gene regulation, cell motility and cell survival (20, 21, 22). The PAK family contains six members, subdivided into two groups: PAK1 to 3 (group I) and PAK4 to 6 (group II) (22, 23, 24). PAK6 exists primarily in cytoplasm, no matter if AR is present or not, while AR down-regulation is dependent on PAK6's kinase activity, but is not related to AR cofactors (25). However, the mechanism of AR modulation by PAK6 remains unclear. Although PAK6 is a tumor suppressor, its expression was increased in primary and metastatic prostate cancer compared to normal prostate epithelium (26). Also, no correlation between PAK6 and AR was observed in normal prostatic tissue and prostate cancer. Understanding how PAK6 regulates AR is vital for understanding prostate tumorigenesis.

In the present study, we aimed to assess how PAK6 regulates AR ubiquitin-mediated degradation through phosphorylation of AR and Mdm2, and its implication in the inhibition of prostate cancer growth in vivo.

**EXPERIMENTAL PROCEDURES**

**Cell culture and transfections** - HEK-293 and COS-7 cell lines were cultured in Dulbecco’s modified Eagle’s medium (DMEM) containing 10% fetal bovine serum (FBS). The prostate cell line CWR22Rv1 cells, were cultured in RPMI-1640 medium containing 10% FBS, 10% charcoal-stripped serum and CSS (Hyclone, Thermo Fisher Scientific, Waltham, MA, USA). Lipofectamine 2000 was used for transfection. Cells at approximately 60% confluence were transfected for 6 hours and incubated with phenol red-free medium containing 10% CSS for 16 hours. Cell extracts were prepared following another 16-hour treatment with 10nM dihydrotestosterone
Plasmids and materials - pcDNA-EGFP-PAK6 wild-type (WT)/K436A (kinase dead, KA) were gifts from Dr Bockoh GM (The Scripps Research Institute, USA). Wild-type Mdm2 (Mdm2 WT) and C464A were from Dr Jochemsen AG (Leiden University Medical Centre, The Netherlands) and Dr Burgering BM (UMCU, Molecular Cancer Research, The Netherlands). AR and ARE-luc were gifts from Dr Zhao Y (China Medical University). His/Myc/GST-tagged PAK6 and Mdm2 were constructed by PCR and subcloned into pcDNA3.1-Myc-HisA, pcDNA3.1-HisC (Invitrogen, Grand Island, NY, USA) and pGEX-5X-1/2 (GE Healthcare, Waukesha, WI, USA) vectors, respectively. AR/Mdm2 deletions were obtained using PCR and subcloned into pGEX-5X-2/1. Site mutagenesis was generated from AR/Mdm2 WT using the QuickChange kit (Strategene, Wilmington, DE, USA) according to manufacturer’s instruction. PKC inhibitor (sc-3007) was from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Cycloheximide, DHT and MG132 were from Sigma-Aldrich (St. Louis, MO, USA).

GST pull-down Assay - The GST pull-down assays were performed by incubating equal amounts of GST and GST-fusion proteins immobilized by GST-sepharose beads (GE Healthcare) with in vitro-translated protein prepared by TNT-coupled translation system (Promega Biotech Co., Madison, WI, USA). Bound proteins were incubated and washed with binding buffer [20mM Tris, pH7.5, 50mM NaCl, 10% Glycerol, 1% NP-40], and stained by Ponceau or Coomassie brilliant blue. Bound proteins were then visualized by western blot.

Western blot analysis - Cells were lysed in RIPA lysis buffer [50mM Tris/HCl (pH 7.4), 150mM NaCl, 1% Nonidet P-40, 0.25% Na-deoxycholate, 1mM EDTA and protease inhibitor cocktail]. Denatured protein were analyzed by SDS-PAGE and transferred to a PVDF membrane (Millipore, Billerica, MA, USA). Samples were incubated and detected with indicated antibodies. Anti-AR, anti-PSA and anti-PARP/Paxillin antibodies were from Neomarkers, Thermo Fisher Scientific. Anti-PAK6 antibody was from Abcam (Cambridge, MA, USA), anti-Flag antibody was from Shanghai Genomics, inc. (Shanghai, China), anti-GAPDH antibody was from Shanghai KangChen (Shanghai, China). Remaining antibodies were purchased from Santa Cruz Biotechnology.

Immunoprecipitation - Cells were washed with PBS prior to cell lysis in 500µl IP lysis buffer [25mM Tris (pH 7.4), 150mM NaCl, 1% Nonidet P-40, 1mM EDTA], supplemented with 1mM PMSF and Protease Inhibitor Cocktail (Roche, Basel, Switzerland). Cell lysates were collected, washed and incubated at 4°C. Then, 30µl of protein A-agarose slurry (GE Healthcare) preloaded with antibodies or normal IgG was added to equal amounts of cell extracts and incubated overnight at 4°C. Washed precipitated proteins were analyzed by western blot.

PAK6 kinase assay - Commercialized PAK6 Kinase (Invitrogen) or immunoprecipitated synthesized PAK6 kinase was used for kinase assay in kinase buffer [50 mM HEPES, pH 7.5, 10 mM MgCl2, 2 mM MnCl2 and 0.2 mM DTT] added with 10µCi of [γ-32P] ATP (5,000 Ci/mmol) and 2.5µM cold ATP for 30 mins at 30°C. Phosphorylated proteins were analyzed by SDS-PAGE followed by autoradiography. Histone H3/H4 (Invitrogen) was used as the positive control. Ponceau staining indicated the loading amounts of GST-fusion proteins.
Ubiquitination assay - COS7 cells were transfected using plasmids with or without Myc-Ubiquitin for 16 hours in CSS and treated with 10nM DHT and 5µM MG132 for 6 hours. Cells were harvested in immunoprecipitation lysis buffer and denatured with 1% SDS. AR proteins were immunoprecipitated with anti-Flag or anti-AR antibodies and subjected to SDS-PAGE, followed by immunoblotting with anti-Myc, anti-Flag or anti-AR antibodies after stripping.

Immunofluorescence staining - Cells were fixed in 4% paraformaldehyde and then blocked with normal goat serum. Cells were incubated with the primary antibody for 1 hour at 25°C, washed with PBS with 1% triton x-100 and subsequently incubated with secondary antibody conjugated with green or red dye. DNA was stained using To-PRO-3 (Molecular Probes, Invitrogen) or DAPI (Roche). Confocal scanning analysis was performed using a Leika/Olympus laser confocal scanning microscope.

Lentiviral Production and Infection - hu6-MCS-CMV-puromycin EGFP-tagged vector was used to construct RNAi lentivirus system. Silence RNA sequences targeting PAK6 was produced by the GENECHEM company (Shanghai, China). shRNA PAK6 sequences is 5'-GCAGGCUAUUCCGAAGCAUTT-3' and shRNA Control sequences is 5'-UUCUCCGAACGUGUCAGCU-3'. Commercial control silencing/shPAK6 virus was used to infect CWR22Rv1 cells in a 12-well plate with 3 mg/ml polybrene. Infected CWR22Rv1 cells were then subjected to sorting by Enhancing Green Fluorescence Protein (EGFP) expression.

Tissue specimens and analysis - Among our tissue specimens, 24 specimens were from the No. PR481 tissue microarray purchased from Alenabio Co. (Beijing, China), and 13 specimens were procured from surgical specimens of patients with prostate cancer for which complete information on clinical metastatic status were available. All human tissues were collected and used according to protocols approved by the Ethics Committee of the China Medical University Health Science Center. After antigen retrieval, specimens were subjected to normal immunohistochemical and immunofluorescence staining. PAK6 and AR co-localization score or AR localization score were determined by intensity (0, 1, 2, or 3) and fraction of stained cells (0, 1, 2, 3, or 4). A total score (ranging from 0–12) was obtained by multiplying the staining intensity and fraction scores (27).

Tumor growth in human prostate carcinoma xenografts - 0.2 ml of CWR22Rv1 cells (2×10⁶) in Matrigel (BD Biosciences, San Jose, CA, USA) was inoculated into the dorsal side of 6- to 7-week-old NOD SCID nude male mice with an average weight of 20 to 25 g. Tumor size was measured using a caliper and recorded twice a week. At the end of the treatment, mice were sacrificed and tumors were removed, photographed and weighed. Standard tumor was a subcutaneous nodule ≥ 0.5 cm.

Cell Counting Assay - 5×10⁵ shRNA control/PAK6 lentivirus infected CWR22Rv1 cells were plated in 12-well plate. Cell amounts in each sample was measured daily in triplicates.

Statistical Analysis - Student’s t-test and one-way analysis of variance (ANOVA) were performed to determine the statistical significance among values for in vitro experiments. Data derived from the immunostaining analysis of human prostate tissue specimens were analyzed using
unpaired t-test. Comparisons were performed using two-tailed paired t-test. All continuous data are presented as the mean values ± s.e.m (standard error of the mean).

RESULTS

PAK6 involvement in AR localization and expression - PAK6 and AR are highly expressed in prostate cancer (2, 8, 26). To investigate the relationship between PAK6 and AR in prostate and cancer tissues, 14 normal prostatic tissues and 23 prostatic adenocarcinoma samples were analyzed by immunofluorescence staining. There was a remarkable co-localization of PAK6 and AR in cytoplasm in normal prostate epithelium, while no co-localization in malignant prostate was observed. AR translocated into nucleus in malignant prostate cells (Figure 1A). Because nutrient depletion is a common event during tumor growth and PAK6 is increased in primary and metastatic prostate cancer (26), we treated CWR22Rv1 cells with serum starvation to mimic nutrient depletion and found that PAK6 was increased while AR was decreased (Figure 1B). This result indicates that nutrient deprivation induces PAK6 expression and inhibits AR expression. To further assess the relationship between PAK6 and AR, CWR22Rv1 cells were transfected with Myc-PAK6 and AR protein levels were reduced upon DHT stimulation (Figure 1C). In this regard, PAK6 might suppress AR expression in cytoplasm.

PAK6 regulates AR localization through phosphorylation of AR on Ser578 - To elucidate how PAK6 negatively modulates AR, we focused on PAK6 kinase activity. Multiple lines of evidences indicate PAK6 negatively regulates AR through phosphorylation (25 and Figure S1). It has been reported that PAK6 phosphorylates AR in its DNA binding domain (amino acids 505-676) (25). GST-AR deletion constructs were used to map the shorter phosphorylation domain, which is between amino acids 559 and 624 of AR, by in vitro kinase assay (Figure 2A). Six candidate phosphorylation sites in this short region were chosen for single-site mutation from serine/threonine to alanine, which is resistant to phosphorylation. Further in vitro kinase assay screened out Ser578 as a novel phosphorylation site for PAK6 (Figure 2B). As the Ser578 site is also a PKC phosphorylation site on AR (11), a PKC inhibitor (sc-3007) was used in subsequent experiments to avoid nonspecific results.

To test the function of PAK6-mediated AR phosphorylation, phospho-AR Ser578 antibody was used, and its quality and availability was verified (Figure S2). Since AR and PAK6 are highly expressed in prostate cancer, AR and PAK6 constructs were transfected into HEK293 cells, which have no endogenous expression of AR and PAK6. Because AR translocation from cytoplasm to nucleus is the key step to initiate transcription of its downstream target genes, western blot analysis was performed with cytoplasmic and nuclear protein separately. It is demonstrated that wild-type PAK6 (PAK6 WT) but not kinase-dead (PAK6 KA) PAK6 phosphorylated AR mainly in the cytoplasm and reduced nuclear AR translocation, leading to downregulation of the AR downstream target gene prostate specific antigen (PSA) (Figure 2C). These results were obtained upon androgen stimulation, indicating that PAK6-mediated AR regulation is androgen-dependent and that PAK6 might obstruct AR nuclear translocation. PAK6 and AR co-localization was further examined by immunofluorescence staining. DHT-induced AR was shown to be retained in the...
cytoplasm by PAK6 WT, but not by PAK6 KA (Figure 2D). Inhibition of AR nuclear translocation by PAK6 relied on Ser578 phosphorylation (Figure 2C and 2D). Since PAK6’s kinase activity inhibitory effect on AR is triggered by DHT, the following results were obtained in the presence of androgen. It was demonstrated that pcDNA3.1-His-PAK6 localized in the cytoplasm, regardless of the presence of androgen, avoiding the obstructive effect of GFP tag on PAK6 nuclear translocation (Figure S3).

These results reveal that PAK6 inhibits AR nuclear translocation through Ser578 phosphorylation.

**PAK6-mediated AR phosphorylation promotes its ubiquitin-mediated degradation** - Because the inhibition of androgen-induced AR nuclear translocation by PAK6 contributes to accumulation of phosphorylated AR in the cytoplasm (Figure 2C), it became very interesting to further investigate AR fate in the cytoplasm. Amount of AR protein levels in cells is the result of equilibrium between synthesis and degradation. By using cycloheximide (CHX) to inhibit protein synthesis (28), PAK6 induced a reduction in AR protein levels upon DHT stimulation (Figure 3A). Because phosphorylation plays a crucial role in proteasome-mediated degradation of proteins, such as the cyclin E and p27<sup>Kip1</sup> protein (29, 30), we explored the possibility that PAK6-mediated phosphorylation regulates AR degradation. We showed that PAK6 induced a reduction of the wild-type AR (AR WT) rather than S578A mutant protein levels under DHT stimulation (Figure 3B).

To validate whether PAK6-dependent phosphorylation mediates AR proteasome-dependent degradation, we used MG132, a 26S proteasome inhibitor (31). Ubiquitination assay showed that PAK6 markedly enhanced AR WT but not AR S578A ubiquitination (Figure 3C). Because PAK6 regulates AR and ER (18), CWR22Rv1 cells were used, which express only AR but not ER (32). Stable knockdown of PAK6 by shRNA in CWR22Rv1 cells showed impairment of the endogenous ubiquitination of AR when treated with MG132 and DHT (7<sup>th</sup> lane from left, Figure 3D). Interestingly, PAK6-mediated AR ubiquitination is androgen dependent, indicating that PAK6 modulates endogenous ubiquitination of AR in vivo upon androgen stimulation.

Above data indicate that the accumulated AR in cytoplasm was further degraded in a proteasome pathway prompted by PAK6-mediated phosphorylation of AR.

**PAK6-mediated AR phosphorylation enhances AR-Mdm2 association** - E3 ligase is necessary for protein degradation, but PAK6 is not an E3 ligase. We examined several E3 ligases targeting AR for degradation, such as Mdm2 and CHIP, and Mdm2 seems effective in AR degradation. To test whether E3 ligase participation was affected by AR Ser578 phosphorylation, we compared Mdm2 interaction with AR WT and AR S578A mutant in vitro. As shown in Figure 4A, Mdm2 interacted strongly with AR WT, but much more weakly with AR S578A mutant. This result is also validated by immunoprecipitation in vivo (Figure 4B). Mdm2 associated stronger with AR WT than AR S578A mutant in the presence of PAK6 (Figure 4B), indicating that PAK6-mediated phosphorylation of AR is involved in the recruitment of Mdm2 to AR and further enhances the AR-Mdm2 association.

This finding led us to presume that Mdm2 may be associated with PAK6 and form a complex with AR, thus targeting AR
for ubiquitin-mediated degradation. GST pull-down assay showed that PAK6 and Mdm2 interact with each other in vitro (Figure 4C). Immunoprecipitation assay confirmed that PAK6 associates with Mdm2 in CWR22Rv1 cells (Figure 4D), which express endogenous PAK6, Mdm2 and AR. Since Mdm2 has been shown to interact with AR (14), our results indicate that PAK6, Mdm2 and AR form a complex. Therefore, these results suggest that PAK6 associate with Mdm2 to recruit AR for its ubiquitin-mediated degradation.

**PAK6 phosphorylates Mdm2 on Thr158 and Ser186** - As PAK6 is a protein kinase and interact with Mdm2, Mdm2 is a potential phosphorylated substrate of PAK6. The in vitro kinase assay verified this postulation and showed that amino acids 121-300 were the phosphorylation domain by PAK6 (Figure 5A and 5B). In this region, eight candidate mutants were constructed. Two novel sites in Mdm2, Thr158 and Ser186, were identified by in vitro kinase assay as the phosphorylation sites by PAK6 (Figure 5C).

**Functional roles of Mdm2 Thr158 and Ser186 in AR degradation** - Because PAK6-mediated AR phosphorylation was shown to enhance AR-Mdm2 interaction (Figure 4A and 4B), we postulated that PAK6 phosphorylates AR in the cytoplasm. Subsequently, PAK6 phosphorylates and recruits Mdm2 to the PAK6-AR complex, leading to AR ubiquitin-mediated degradation. To validate whether Mdm2 phosphorylation was related to AR proteasome degradation, ubiquitination assay was performed upon DHT stimulation in COS7 cells, which have no endogenous expression of PAK6 and AR, and low levels of Mdm2 expression. As expected, AR ubiquitination only occurred in the presence of Myc-Ub, (2nd lane from left, Figure 6A).

To study the function of Mdm2 T158 and S186, both sites were mutated into an alanine (A) to abolish the phosphorylation. We compared the effect of wild-type Mdm2 (Mdm2 WT) and mutants on AR ubiquitination and degradation. When treated with MG132, the coexistence of PAK6 and Mdm2 WT enhanced AR ubiquitination remarkably (4th lane from left, Figure 6A). However, the other three Mdm2 mutants, including Mdm2 T158A, S186A and T158A/S186A mutants, did not induce AR ubiquitination (Figure 6A). This result is consistent with Mdm2 C464A mutant, a RING finger mutant which failed to induce ubiquitination of substrate (33). The faint ubiquitylated AR bands in 6th, 7th and 8th lanes suggest that phosphorylation of Mdm2 by PAK6 be critical for AR ubiquitination and degradation.

What is the function of Mdm2 T158 and S186? Is there any collaboration between them? Figure 6B showed that AR interacted strongly with Mdm2 WT and T158A, but much more weakly with Mdm2 S186A mutant, and did not interact at all with Mdm2 T158A/S186A mutant, indicating that phosphorylation of Mdm2 Ser186 by PAK6 is essential for AR-Mdm2 association. Furthermore, GST pull-down assay was used to judge the effect of PAK6-mediated phosphorylation of Mdm2 on AR-Mdm2 association. Increasing Mdm2 T158A mutant resulted in lowering S186A association with AR with equal amounts of S186A mutant. However, increasing Mdm2 S186A mutant did not diminish T158A association with AR with equal amounts of T158A mutant. Collectively, these results suggest that PAK6-mediated phosphorylation of Mdm2 Thr158 promotes Ser186 association with AR (Figure 6C). To elucidate the effect of PAK6-mediated phosphorylation of Mdm2 on PAK6-AR...
association, we showed (Figure 6D) that Mdm2 S186A but not T158A mutant weakened PAK6-AR association. Moreover, Mdm2 T158A/S186A mutant vigorously abolished PAK6-AR association with equal amounts of PAK6 protein. This further demonstrates that Mdm2 Thr158 acts on AR through modulating Mdm2 Ser186 association with AR as it facilitates PAK6-regulated AR ubiquitin degradation. Thus, phosphorylation of Mdm2 Thr158 by PAK6 collaborates with phosphorylated Ser186 for AR degradation.

**PAK6 inhibits prostate cancer growth in vivo** - To test the inhibitory effect of PAK6 on tumor growth, cell counting assay was firstly used. Stable knockdown of PAK6 by shRNA in CWR22Rv1 cells showed a significant enhancement of proliferation in vitro (Figure 7A). The proliferating marker gene cyclin D1 in AR signaling was upregulated in shRNA PAK6 group upon DHT stimuli (Figure 7B). Furthermore, the growth-inhibitory role of PAK6 was tested in vivo. NOD SCID nude male mice were subcutaneously injected with 2x10^6 CWR22Rv1 cells infected with lentiviruses harboring shRNA PAK6/Control. Four weeks after shRNA control/PAK6 CWR22Rv1 cells implantation, subcutaneous nodules became measurable. From the 4th to 7th week, shRNA PAK6-injected group on the right dorsal side developed rapidly and significantly larger tumors than controls on the left dorsal side (Figure 7C). The ratio of tumor development of shRNA PAK6 group was markedly higher than in shRNA control group. Meanwhile the final tumor weight of shRNA PAK6 was much heavier than shRNA control cells. These data indicate that PAK6 may suppress tumor growth in human prostate cancer.

**Relationship between PAK6 and AR in prostate cancer** - To further demonstrate the clinical relevance between PAK6 and AR, the correlation of PAK6 and AR in prostate cancer was investigated, and a significant decrease of co-localization of PAK6 and AR in the cytoplasm and an increased AR nuclear translocation associated with high grade tumor was observed, indicating a strong inverse correlation between expression of PAK6 and AR in cytoplasm (Figures 8A and S4). This may point out important implications for PAK6 in the maintenance of AR signaling homeostasis and in prostate malignancy, as well as in a therapeutic strategy for AR-positive and hormone-sensitive prostate cancer by modulating PAK6 activity.

**DISCUSSION**

PAK6, as a negative regulator of AR, received much attention for inhibiting prostate cancer, but the exact mechanisms had not been determined. In the present study, we investigated the mechanism of AR ubiquitin-mediated degradation prompted by PAK6 in the presence of androgen. Both PAK6 and AR are highly expressed in prostate cancer (2, 8, 26), while the correlation between PAK6 and AR in prostate cancer has not yet been reported. To the best of our knowledge, the present study is the first to show that AR co-localized with PAK6 in the cytoplasm of prostate epithelial cells, and translocated into the nucleus of malignant prostate cells (Figure 1A). Furthermore, a significant decrease of PAK6 and AR co-localization in the cytoplasm was accompanied by an increase of AR nuclear translocation associated with high-grade tumor. These data demonstrate that PAK6 plays an important role in regulating AR nuclear translocation. Androgen-induced AR nuclear translocation is the key step to
initiate the transcription of downstream genes involved in diverse cell functions, such as cell proliferation and survival (34). However, excessive AR activation would disrupt its homeostasis and causes disorders, such as prostate cancer. To maintain physiological status, cells will use strategies to balance AR expression. PAK6 suppressed AR expression and further impaired AR nuclear translocation in the presence of androgen.

Androgen ablation therapy is the main therapy in prostate cancer, is initially effective, and generally leads to disease remission (4). However, recurrent tumors arise within 2-3 years, on average (35). At the present, few therapeutic regimens have been described to effectively manage recurrent prostate cancers, which are currently considered as an incurable disease (35). Given the inhibitory effect of PAK6 on AR upon androgen stimuli, exploring the mechanisms of PAK6 downregulation of AR will provide insight into new eventual prostate cancer therapy. We established that PAK6 plays its inhibitory role through AR phosphorylation on Ser578, impairing AR translocation from cytoplasm to nucleus upon androgen stimulation. It was reported that PKC phosphorylates AR Ser578 in the presence of EGF, and impairs the Ku-70/80 and nuclear-cytoplasmic shuttling of AR (11), which partially supports our findings. Our results demonstrated that PAK6 kinase activity is involved in the inhibition of AR nuclear translocation.

As multiple evidences indicate that co-activators such as β-catenin, SRC1, p300, Tip60α and ARA55 and the SMRT co-repressor did not modify the relative inhibitory effect of wild-type or kinase-dead PAK6 on AR transactivation (25), we speculated that PAK6 might regulate AR through the ubiquitin-proteasome pathway instead of epigenetics. We have provided several lines of evidence to address this issue. On one hand, PAK6 efficiently promotes AR WT ubiquitination and degradation, but not AR S578A mutant. On the other hand, PAK6 knockdown failed to induce endogenous AR ubiquitin-mediated degradation upon DHT stimulation. These data suggest that PAK6 regulates AR ubiquitination and degradation. This may be viewed in another way that PAK6 expression may relapse after androgen deprivation therapy compared with tumors (26). Therefore, modulating PAK6 kinase activity may inhibit excessive AR nuclear translocation and promotes its proteolysis, and regulates AR expression homeostasis.

E3 ligase is necessary for ubiquitin degradation complex. Although Mdm2 and CHIP were reported to interact with AR and induce AR degradation (14, 16), we found that only Mdm2 interacted with the PAK6-AR complex. Moreover, PAK6-mediated AR phosphorylation on Ser578 and contributed to recruitment of Mdm2 to AR.

To gain further insight into the involvement of Mdm2 in PAK6-mediated AR ubiquitination, Mdm2 Thr158 and Ser186 were identified as the PAK6 phosphorylation sites. In this study, Mdm2 T158A, S186A and T158A/S186A mutants prevented accumulation of AR ubiquitination, indicating that phosphorylation of Mdm2 by PAK6 is indispensable for AR ubiquitin-mediated degradation. Akt mediates AR ubiquitination through phosphorylation of Mdm2 S166 and S186 (14). The impact of phosphorylation of Mdm2 T158 and S186 by PAK6 on AR-Mdm2 association was also investigated. It was demonstrated that phosphorylated Ser186 is essential for AR-Mdm2 association. Moreover, Mdm2
Thr158 collaborates with Ser186 for AR-Mdm2 association and AR ubiquitin degradation, as it facilitates PAK6-regulated AR homeostasis. Increasing PAK6 kinase activity not only suppresses AR nuclear translocation, but also recruits Mdm2 to AR ubiquitination complex.

We established that PAK6 inhibits prostate tumor growth via regulating AR proteolysis. Our in vivo study provided solid evidence in vivo. However, a previous study (36) described the ability of PAK6 to promote prostate tumor growth and invasive ability. We cannot rule out the reasons for this difference in the current study.

The novel model we described demonstrates how PAK6 targets AR for its degradation upon androgen stimulation, which stresses the necessary strategy to balance AR homeostasis to maintain cell physiological status. Interestingly, PAK6 is highly expressed in primary and metastatic prostate cancer (26). We suspect this is a stress-induced increment of PAK6 in response to increased AR in prostate cancer. Once AR homeostasis is disrupted, increasingly uncontrolled AR translocation will lead to prostate cancer. As shown by clinical results, the increase in AR nuclear translocation is associated with high grade tumor. In this regard, PAK6 expression is also increased to inhibit excessive AR expression along with the tumor aggressiveness. This may explain why PAK6 expression is enriched in prostate cancer. Thus, increasing PAK6 kinase activity might be a potential therapeutic strategy for AR-positive and hormone-sensitive prostate cancer.

Overall, this study demonstrated that PAK6 suppresses prostate carcinogenesis by impairing AR nuclear translocation and by promoting recruitment of E3 ligase Mdm2 to AR for its degradation via the ubiquitin-proteasome pathway. These results provide some light on the regulation machinery of AR homeostasis and inspire interventions on PAK6 kinase activity as a potential therapeutic strategy for prostate cancer.

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The abbreviations used are: AR, androgen receptor; PAK6, p21 activated kinase 6; DHT, dihydrotestosterone; PSA, prostate specific antigen; WT, wild-type; DMEM, Dulbecco’s modified Eagle medium; CHX, cycloheximide.

FIGURE LEGENDS

FIGURE 1 PAK6 involvement in AR localization and expression. (A) PAK6 and AR are co-localized in the cytoplasm of normal prostate cells, while AR is localized in nucleus in prostate cancer cells. Tissue specimens were subjected to immunohistochemical analysis as usual. And after antigen retrieval, specimens were fixed and incubated with anti-PAK6 antibody followed by Alexa Flour 594 (red) antibody and anti-AR antibody followed by Alexa Flour 488 (green) antibody. Nucleus was stained by DAPI (blue). The 2nd, 4th lines were the 25 folds enlarged pictures of 1st and 3rd lines, respectively. The 4th row was merged with red and green images, and 5th row was merged with red, green and blue images. The white arrows in 2nd line indicate AR colocalizes with PAK6 in cytoplasm, and arrows in 4th line indicate AR translocates into nucleus. (B) Starvation experiment showing that PAK6 expression is increased and AR decreased upon starvation. CW22Rv1 cells were starved with HBSS (Invitrogen) at different time points as indicated. Western blot analysis was performed and endogenous proteins were detected with indicated antibodies. (C) CWR22Rv1 cells transfected with Myc-PAK6 show decreased AR levels upon DHT activation. CWR22Rv1 cells were transfected with indicated amounts of pcDNA3.1-Myc-His-PAK6. Endogenous AR and exogenous PAK6 were detected with indicated antibodies.

FIGURE 2 PAK6 regulates AR localization through phosphorylation of AR on Ser578. (A) PAK6 phosphorylates AR between amino acids 559 and 624. HEK-293 cells were transfected with pcDNA3.1Myc-His-PAK6 and lysed for immunoprecipitation with anti-Myc antibody, the immunoprecipitated PAK6 kinase was incubated with GST-AR deletions (505-559aa, 559-624aa, 624-676aa and 505-676aa) for in vitro kinase assay. Histone H4 served as a positive control. (B) AR Ser578 is the phosphorylation site by PAK6. In vitro kinase assay was performed using commercialized PAK6 kinase and GST-AR WT (505-676) or GST-AR single-site mutations as indicated. Histone H3 (HH3) served as a positive control. (C) Wild-type PAK6 phosphorylated AR in the cytoplasm and reduced its nuclear translocation, leading to decreased PSA levels. HEK293 cells were co-transfected with pcDNA3.1-His -PAK6 WT/KA and pcDNA3.1-His-AR in the presence or absence of DHT, cytoplasmic and nuclear proteins were subjected to SDS-PAGE separately. Western blot analysis was performed as indicated, p-AR is phos-AR S578 antibody, PARP served as a nuclear loading control and Paxillin served as a cytoplasmic loading control. (D) Inhibition of AR nuclear translocation by PAK6 relied on Ser578 phosphorylation. HEK293 cells were co-transfected with pcDNA-EGFP-PAK6 WT/KA and pcDNA3.1-His-AR. Cells were starved with steroid hormone for 16hrs, stimulated with 10nM DHT for 0, 30 and 60 minutes as indicated, fixed and incubated with anti-AR antibody followed by Alexa Flour 546 (red) antibody. Nucleus was stained with Topro3 (blue). The white arrows in 4th rows indicate the
subcellular colocalization of PAK6 WT/KA and AR.

**FIGURE 3 PAK6-mediated AR phosphorylation promotes its ubiquitin-mediated degradation.** (A) PAK6 reduced AR levels under DHT stimulation. HEK293 cells were transfected with pcDNA3.1-Myc-His-PAK6 and pcDNA 3.1-His-AR, and treated with 10nM DHT and 10 µM cycloheximide (CHX) as indicated time. PAK6 and AR protein levels were measured by western blot analysis as indicated. (B) PAK6 reduced wild-type AR under DHT stimulation, while AR S578A mutant was unaffected. HEK293 cells were co-transfected with pcDNA-Flag-AR wild-type /S578A (505-919aa) (incuding DNA binding domain and ligand binding domain) and pcDNA3.1-Myc-His-PAK6 treated with 10nM DHT and 10 µM cycloheximide. (C) Ubiquitination assay showed that PAK6 markedly enhanced AR WT but not AR S578A ubiquitination. HEK293 cells were transfected with Myc-Ubiquitin, pcDNA-Flag-AR WT/S578A (505-919aa) in combination with pcDNA3.1-Myc-His PAK6/vector treated or untreated with 5 µM MG132 for 6hrs in the presence of 10nM DHT. 30 µg of total lysates were set aside as input, equal amounts lysates of protein were then harvested for ubiquitination assay. (D) Stable knockdown of PAK6 by shRNA in CWR22Rv1 cells showed impairment of the endogenous ubiquitination of AR when treated with MG132 and DHT (7th lane from left). CWR22Rv1 cells infected with lentiviruses harboring shRNA control/PAK6 in were treated with or without 5 µM MG132 for 6hrs in the presence/absence of 10nM DHT. Equal amounts lysate's of protein were then harvested for ubiquitination assay.

**FIGURE 4 PAK6-mediated phosphorylation of AR enhances AR association with Mdm2.** (A) Mdm2 interacted strongly with AR WT, but much more weakly with AR S578A mutant. In vitro translated pcDNA-Flag-AR WT/S578A (505-919aa) was incubated with GST-Mdm2 as indicated and analyzed with anti-Flag antibody. GST served as control. (B) Mdm2 associated stronger with AR WT than AR S578A mutant in the presence of PAK6. COS7 cells were co-transfected with pcDNA-Flag-AR WT/S578A (505-919aa) in combination with pcDNA-EGFP-PAK6 and Mdm2 as indicated, treated with 10nM DHT. 30 µg of total lysates were set aside as input, equal amounts lysates of protein were immunoprecipitated with anti-Flag antibody and immunoblotted with anti-Mdm2 antibody. (C) GST pull-down assay showed that PAK6 and Mdm2 interact with each other in vitro. In vitro translated pcDNA3.1-Myc-His-PAK6 was incubated with GST-Mdm2 and analyzed with anti-Myc antibody. GST served as control. (D) Immunoprecipitation assay confirmed that PAK6 associates with Mdm2 in CWR22Rv1 cells. Equal amounts of CWR22Rv1 cell lysates were immunoprecipitated with anti-PAK6 or IgG. Endogenous PAK6 and Mdm2 were immunoblotted with anti-PAK6 and anti-Mdm2 antibodies.

**FIGURE 5 PAK6 phosphorylates Mdm2 on Thr158 and Ser186.** (A) PAK6 phosphorylates Mdm2. HEK-293 cells were transfected with pcDNA3.1-Myc-His-PAK6 and lysed for immunoprecipitated with anti-Myc antibody, the immunoprecipitated PAK6 kinase was incubated with GST-Mdm2 and analyzed by autoradiography. (B) PAK6 phosphorylates Mdm2 between amino acids 121 and 300. In vitro kinase assay was performed using commercialized PAK6 kinase and GST-Mdm2 full-length and deletions as indicated and analyzed by autoradiography. (C) Mdm2 Thr158 and Ser186 are the phosphorylation sites by
PAK6. *In vitro* kinase assay was performed using commercialized PAK6 kinase and GST-Mdm2 (121-300) or GST-Mdm2 single-site mutations as indicated and analyzed by autoradiography. Histone H3 (H3) served as a positive control.

**FIGURE 6** Functional roles of Mdm2 Thr158 and Ser186 in AR degradation. (A) PAK6 induces AR ubiquitination dependent on Mdm2 phosphorylation. COS7 cells were transfected with pcDNA3.1-His-AR in combination with vector, pcDNA3.1-His-PAK6 or pcDNA3.1-His-Mdm2 mutants in the presence or absence of Myc-ub in 10% charcoal-stripped medium for 16hrs, followed by treatment with 10nM DHT for 16hrs. The cells were then harvested for ubiquitination assay. (B) AR interacted strongly with Mdm2 WT and T158A, but weakly with Mdm2 S186A mutant. *In vitro* translated pcDNA3.1-His-AR was incubated with GST-Mdm2 mutants as indicated and analyzed with anti-AR antibody. GST served as control. (C) PAK6-mediated phosphorylation of Mdm2 Thr158 promotes Ser186 association with AR. *In vitro* translated pcDNA3.1-His-AR was incubated with equal amounts of GST-Mdm2 S186A or T158A mutant. Meanwhile, amounts increasing of *in vitro* translated T158A or S186A (5, 10 and 20µl) were added and analyzed with anti-AR antibody. GST served as control. (D) Mdm2 S186A but not T158A mutant weakened PAK6-AR association. *In vitro* translated pcDNA3.1-His-AR was incubated with equal amounts of GST-PAK6, meanwhile amounts increasing of *in vitro* translated Mdm2 mutants (5, 10 and 20µl) were added and analyzed with anti-His and anti-AR antibodies. GST served as control.

**FIGURE 7** PAK6 inhibits prostate cancer growth *in vivo*. (A) Stable knockdown of PAK6 by shRNA in CWR22Rv1 cells showed a significant enhancement of proliferation *in vitro*. CWR22Rv1 cells infected with lentiviruses harboring shRNA control/PAK6 were plated in 12-well plates with equal amounts and counted every day for one week. (B) The proliferating marker gene cyclin D1 in AR signaling was upregulated in shRNA PAK6 group upon DHT stimuli. shRNA control/PAK6 in CWR22Rv1 cells were treated with or without DHT for 16hrs. Equal amounts lysates of protein were then harvested for western blot with antibodies as indicated. (C) Effect of shRNA control (left dorsal side) or shRNA PAK6 (right dorsal side) on the growth of CWR22rv1 cells inoculated into nude mice. NOD SCID nude male mice were subcutaneously injected with 2x10⁶ CWR22Rv1 cells infected with lentiviruses harboring shRNA PAK6/Control. Tumor volume was monitored over time as indicated, and the tumor was excised and weighed after 49 days. PAK6 depletion causes a high ratio of tumor development (Table) and an increase in tumor volume and weight (Graphs).

**FIGURE 8** (A) Relationship between PAK6 and AR in prostate cancer. 14 normal prostate, 6 well-differentiated, 9 moderately-differentiated and 8 poorly-differentiated prostate tumor specimens were collected and subjected to immunofluorescence analysis. The score of immunostaining colocalization of PAK6 and AR in cytoplasm or localization of AR in nucleus was counted from 30 independent visions and determined by intensity (0, 1, 2, or 3) and fraction of stained cells (0, 1, 2, 3, or 4). A total score (ranging from 0–12) was obtained by multiplying the staining intensity and fraction scores. ** means P-value less or equal to
0.001 was considered statistically significant according to upaired t-test. (B) **Proposed mechanism for the relation between PAK6, AR, Mdm2 and ubiquitin-proteasome degradation.** Upon DHT stimulation, PAK6 obstructs AR nuclear translocation through phosphorylation on AR Ser578, this phosphorylation enhances the binding between AR and Mdm2. The further phosphorylation of Mdm2 by PAK6 promotes proteasome to recognize the polyubiquitylated AR, leading to AR degradation.
FIGURE 1

A

Normal
Prostatic Tissue

Prostatic
Adenocarcinoma

PAK6  AR  DAPI  Merge

B

Starvation

0  0.5  1  2  4 hrs

PAK6  AR  GAPDH

C

+DHT

0  0.5  1  2 μg

Myc-PAK6  AR  GAPDH
FIGURE 4

A

Input GST Mdm2
AR WT AR S578A

Flag-AR

-Mdm2 GST fusion proteins

B

Input

IP:Flag
IB:Mdm2
Flag-AR
Mdm2
GFP-PAK6
GAPDH

Flag-AR WT
Flag-AR S578A
Mdm2
PAK6

+ + + + +

C

Input Mdm2 GST

Myc-PAK6

GST fusion proteins

D

Input IP-PAK6 IP-IgG

Mdm2
PAK6
FIGURE 5

A
Mdm2 GST H3

- $^{32}$P-Mdm2
- $^{32}$P-H3

Mdm2 GST fusion proteins

B

GST-Mdm2 deletions

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C

GST-Mdm2 Mutations

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- GST-Mdm2 mutations
FIGURE 6

A

Mdm2 WT
Mdm2 C464A
Mdm2 T188A
Mdm2 S186A
Mdm2 T188A S186A
Myc-Ub
AR
PAK6
MG132

IP:AR
IB:My-ubquitin
IB:AR

AR
Mdm2
PAK6
GAPDH

B

Input
GST
Mdm2 WT
Mdm2 T158A
Mdm2 S186A
Mdm2 T158A S186A

AR
GST-Mdm2
mutations

C

Input
T158A
S186A

AR
Mdm2
S186A
T158A

AR
Mdm2 GST
fusion proteins

D

Input
T158A
S186A

AR
His-AR
His-GST

His-AR
His-Mdm2

GST-PAK6
fusion proteins
FIGURE 7

A Cell Counting Assay

- shRNA Control
- shRNA PAK6

P<0.001

B

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<th>Groups</th>
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<td>shRNA PAK6</td>
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C means shRNA control
P means shRNA PAK6

Cyclin D1
PAK6
GAPDH

Tumor volume (mm$^3$)

Final tumor weight (mg)

P=0.0031

P=0.011
FIGURE 8

A

The subcellular localization of PAK6 and AR in high grade tumor

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<td>Well-differentiated</td>
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<td>Prostatic adenocarcinoma</td>
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B

Diagram showing the interaction between DHT, PAK6, Mdm2, and AR, leading to AR degradation.
P21-activated kinase 6 (PAK6) inhibits prostate cancer growth via phosphorylation of androgen receptor and tumorigenic E3 ligase murine double minute-2 (Mdm2)

Tong Liu, Yang Li, Hui Gu, Ge Zhu, Jiabin Li, Liu Cao and Feng Li

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