

Tumorigenesis and Neoplastic Progression

PIAS1 Is Increased in Human Prostate Cancer and Enhances Proliferation through Inhibition of p21

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Prostate cancer development and progression are associated with alterations in expression and function of elements of cytokine networks, some of which can activate multiple signaling pathways. Protein inhibitor of activated signal transducers and activators of transcription (PIAS)1, a regulator of cytokine signaling, may be implicated in the modulation of cellular events during carcinogenesis. This study was designed to investigate the functional significance of PIAS1 in models of human prostate cancer. We demonstrate for the first time that PIAS1 protein expression is significantly higher in malignant areas of clinical prostate cancer specimens than in normal tissues, thus suggesting a growth-promoting role for PIAS1. Expression of PIAS1 was observed in the majority of tested prostate cancer cell lines. In addition, we investigated the mechanism by which PIAS1 might promote prostate cancer and found that down-regulation of PIAS1 leads to decreased proliferation and colony formation ability of prostate cancer cell lines. This decrease correlates with cell cycle arrest in the G0/G1 phase, which is mediated by increased expression of p21^{CIP1/WAF1}. Furthermore, PIAS1 overexpression positively influences cell cycle progression and thereby stimulates proliferation, which can be mechanistically explained by a decrease in the levels of cellular p21. Taken together, our data reveal an important new role for PIAS1 in the regulation of cell proliferation in prostate cancer. (*Am J Pathol* 2012, 180: 2097–2107; DOI: 10.1016/j.ajpath.2012.01.026)

The development and progression of various human cancers are influenced by cytokines, some of which can

activate multiple signaling pathways. In human prostate, cytokines regulate cellular events in chronic inflammation, premalignant lesions such as inflammatory atrophy and high-grade intraepithelial neoplasia, and cancer.

Protein inhibitors of activated signal transducers and activators of transcription (PIAS) are a group of multifunctional proteins that play a role in the regulation of cytokines and other cellular pathways. The family consists of four members, PIAS1, PIASx (PIAS2), PIAS3, and PIASy (PIAS4), that exist in two isoforms, with the exception of PIAS1.¹ Besides their DNA and protein binding ability, which is mediated by the conserved SAP region, PIAS proteins also contain a RING (really interesting new gene) finger-like zinc-binding domain as well as a small ubiquitin like modifier (SUMO) interaction motif, thus functioning as SUMO-E3 ligases.² Therefore, PIAS can interact with and modulate the activity of various proteins and signaling cascades. PIAS1 and -3 were initially discovered as inhibitors of signal transducers and activators of transcription (STAT)1 and -3, respectively.^{3,4} STAT factors are a family of transcription factors which, on cytokine [interferon (IFN) γ , interleukin-6, and others] activation, become phosphorylated by Janus kinases (JAKs). Phosphorylated STATs dimerize and translocate to the nucleus, where they directly initiate gene activation.⁵ PIAS proteins can inhibit the DNA binding activity of STATs.² Furthermore, it has been shown that PIAS1 can SUMOylate STAT1 and thereby inhibits its IFN γ -mediated transactivation.⁶ Besides JAK/STAT signaling, PIAS proteins have also been implicated in the regulation of the nuclear factor (NF) κ B pathway, SMAD, and androgen receptor signaling.¹ Cytokine-induced responses are in addition controlled by suppressors of cytokine signaling (SOCS). On the basis of our previous findings demonstrating important roles of

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SOCS-3 and -1 in regulation of cellular events in prostate cancer, we initially hypothesized that selected members of the PIAS family are implicated in control of cell proliferation and apoptosis.⁷

Therefore, we investigated PIAS1 expression in human prostate cancer cells and uncovered its functional significance in this malignancy. In this study, we prove for the first time that PIAS1 protein expression is significantly increased in malignant tissues and demonstrate that down-regulation of PIAS1 leads to reduced cell proliferation and colony formation ability of prostate cancer cells. These growth-inhibitory effects are due to elevated expression of the cell cycle regulator protein p21^{CIP1/WAF1}, which leads to a G0/G1 arrest, whereas no significant effects on apoptosis are observed. These findings were confirmed by PIAS1 overexpression experiments which resulted in an increase in proliferation and reduced p21 expression.

Materials and Methods

Tissue Microarray and Immunohistochemistry

To evaluate differences in PIAS1 expression between malignant and benign prostate tissue, we constructed a tissue microarray (TMA) of formalin-fixed, paraffin-embedded tissue blocks from 90 previously untreated prostate cancer patients who had undergone radical prostatectomy after tumor diagnosis in a screening program performed in the province of Tyrol by the Department of Urology, Innsbruck Medical University.⁸ The use of the archive samples was approved by the ethics committee of Innsbruck Medical University. The TMA was assembled using a manual tissue arrayer (Beecher Instruments, Sun Prairie, WI). Hematoxylin eosin and p63/ α -methylacyl-CoA racemase (AMACR) immunohistochemistry double staining to control the histological diagnosis, proliferating cell nuclear antigen (PCNA) and Ki-67 staining for proliferation status, and PIAS1 immunohistochemistry were performed on a Discovery-XT staining device (Ventana, Tucson, AZ). For PIAS1, Abcam (Cambridge, UK) antibody clone EPR281Y was used (dilution 1:10). Immunohistochemical evaluation was done by a uropathologist (G.S.) using the semiquantitative scoring system "quickscore" combining the proportion of positive cells and the average staining intensity.⁹

Cell Lines and Cell Culture

The human prostate cell lines LNCaP, PC3, DU-145, VCaP, CWR22RV1, and BPH-1 were obtained from the American Type Culture Collection (ATCC, Rockville, MD). The LNCaP subline LNCaP-IL-6+ was established in the presence of IL-6 as described previously.¹⁰ Benign prostate epithelial RWPE-1 cells were a gift from Dr. William Watson (University College Dublin, Ireland).¹¹ The identity of the used cell lines was confirmed by short tandem repeat analysis. PC3, DU-145, and CWR22RV1 cells were cultured in RPMI 1640 supplemented with 10% fetal calf serum and 20 mmol/L glutamine (both from PAA Laboratories, Pasching, Austria). LNCaP cells were grown in RPMI 1640 containing 10% fetal calf serum, 20 mmol/L glutamine, 20 mmol/L HEPES (Sigma, Vienna, Austria), 4.5 g/L D-glucose (PAA Laboratories), and

1% Na-Pyruvate (Lonza, Basel, Switzerland). VCaP cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum, 20 mmol/L glutamine, and 4.5g/L D-glucose. RWPE-1 were cultured as previously described.¹¹

Short Interfering RNA Transfection

The following short interfering RNA (siRNA) sequences were used for targeting human PIAS1: siPIAS1 A (sense: 5'-AAGGUCAUUCUAGAGCUUUAdTdT-3'; antisense 5'-UA-AAGCUCUAGAAUGACCUUdTdT-3'), siPIAS1 B (sense: 5'-CGAAUGAACUUGGCAGAAAAdTdT-3'; antisense: 5'-UUUCUGCCCAAGUUCUUCGdTdT-3'), sip21 (sense: 5'-CUUCGACUUUGUCACCGAGdTdT-3'; antisense: 5'-CUCGGUGACAAAGUCGAAGdTdT-3').¹² A non-targeting siRNA pool (Dharmacon, Epsom, UK) was used as a negative control. siRNA transfections were performed with Lipofectamine 2000 reagent (Invitrogen, Lofer, Austria). All cell lines were transfected with either 25 nmol/L siRNA against PIAS1 and/or p21 or control siRNA for 24 or 48 hours before mRNA and protein measurements, respectively. Transfection was performed twice in studies on proliferation and apoptosis.

PIAS1 Overexpression

Expression vector pEGFP-C1-PIAS1 and empty vector pEGFP-C1 were generated by Dr. Yaron Galanty (Gordon Institute, Cambridge, UK) as described.¹³ Cells were transfected with 3 μ g/mL of DNA using Fugene HD transfection reagent (Roche, Basel, Switzerland) for 48 or 72 hours following the manufacturer's instructions.

Cytoplasmic and Nuclear Fractionation

Fractions were obtained using NE-PER nuclear and cytoplasmic extraction kit (Pierce, Vienna, Austria) following the manufacturer's instructions.

Western Blot Analysis

Western blot analysis was performed as described previously.¹⁴ The following antibodies were used: anti-PIAS1 (dilution 1:750; Cell Signaling, Danvers, MA), anti-cleaved poly(ADP-ribose) polymerase (cPARP; dilution 1:1000; Promega, Madison, WI), anti-p21^{CIP1/WAF1} (dilution 1:1000; Cell Signaling), anti-GAPDH (dilution 1:100,000; Chemicon, Vienna), and anti-lamin A (1:5000; Abcam).

RNA Isolation and Quantitative Real-Time PCR

Quantitative real-time (qPCR) was performed as described elsewhere.¹⁴ TaqMan gene expression assays for PIAS1 and p21 were used (Applied Biosystems, Brunn am Gebirge, Austria). HPRT1 was used as an endogenous control: forward primer, 5'GCTTTCCTTG-GTCAGGCAGTA3'; reverse primer; 5'GTCTGGCT-

TATATCCAACACTTCGT3'; probe, 5'FAMGTCTGGCT-TATATCCAACACTTCGTTAMRA3'. C_t values of PIAS1 or p21 and HPRT1, as assessed by ABI Sequence Detection Software, were used to calculate the ΔC_t .

[³H]thymidine Incorporation Assay

Cells were seeded in triplicates onto 96-well plates. For PIAS1 down-regulation, the cells were treated with 25 nmol/L of siRNA against PIAS1 or negative control siRNA for 48 hours followed by a medium change and second transfection for another 48 hours. For overexpression experiments, the cells were transfected with 3 μ g/mL of pEGFP-C1-PIAS1 or empty vector for 72 hours. Twenty-five microliters/well of [³H]thymidine (1 μ Ci/well) were added for the last 24 hours. DNA was harvested on 96-well filter plates (Perkin-Elmer, Brunn am Gebirge, Austria). Scintillation fluid (50 μ L) was added, and radioactivity was quantified using Chameleon 5025 liquid scintillation counter (HVD Life Sciences, Vienna, Austria).

Colony Formation Assay

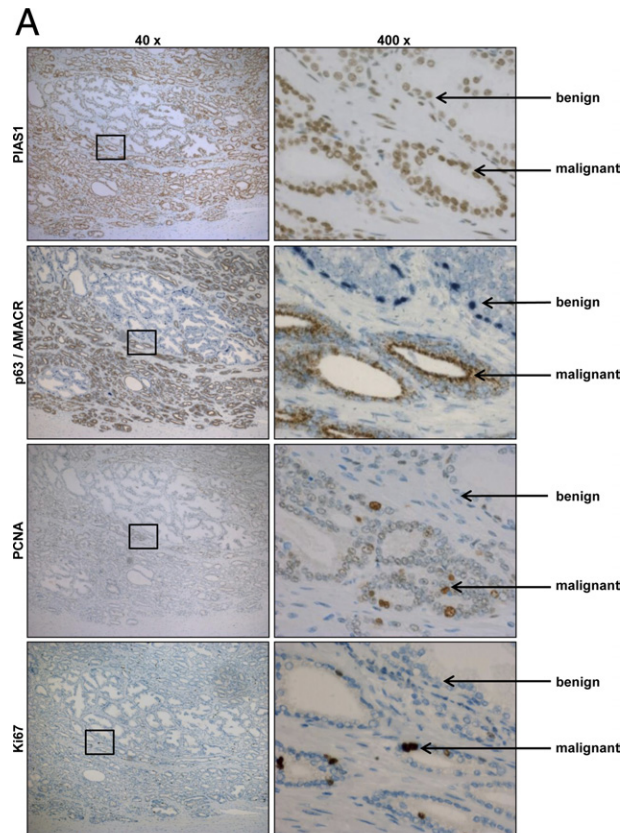
Cells were transfected with siRNA against PIAS1 or control siRNA twice. The number of viable cells was determined using CASY cell counter system (Schärfe System, Reutlingen, Germany). One thousand cells were seeded into a 75-cm² cell culture flask with 12 mL of medium and incubated for 12 days. Subsequently, the cells were fixed with 100% ice-cold methanol for 5 minutes and stained with PBS containing 20% methanol and 0.5% crystal violet (Sigma) for 2 minutes. Colony numbers were determined and quantified with a CCD camera with green electroluminescent transillumination.

Apoptosis Measurement

Cells were seeded onto six-well plates and transfected twice with siRNA against PIAS1 or control siRNA. The cells were trypsinized, and the pellets were resuspended in propidium iodide (PI) buffer (0.2% Triton-X-100, 2 ng/mL Na-Citrate, and 0.05 mg/mL PI) and kept light-protected at 4°C for 1 hour. To assess the percentage of apoptotic cells, the sub-G1 peak was measured using FACSCalibur (Becton Dickinson, Heidelberg, Germany).

Cell Cycle Distribution

PC3 and DU-145 cells were seeded onto six-well plates and transfected with siRNA against PIAS1 or pEGFP-C1-PIAS1 as well as respective controls for 48 hours. The cells were trypsinized and centrifuged. Pellets were fixed in 70% ice-cold ethanol for 30 minutes on ice. Afterward, the cells were washed in PBS containing RNase A (100 μ g/mL) for 10 minutes, centrifuged, resuspended in PI buffer, and kept light-protected at 4°C for 1 hour. Cell cycle distribution was analyzed using FACSCalibur (Becton Dickinson). Cell cycle analysis was also performed after thymidine block as described previously.¹²



B

intensity	no staining (0-0.5)	weak (0.5-1.5)	intermediate (1.5-2)	strong (2-3)	N	significance
benign	31 (39.2%)	34 (43.0%)	11 (13.8%)	3 (3.8%)	79	***
malignant	11 (12.2%)	37 (41.1%)	28 (31.1%)	14 (15.6%)	90	P ≤ 0.0003

Figure 1. Increased expression of PIAS1 in human prostate cancer tissue. **A:** Immunohistochemical staining of one representative core of a prostate cancer patient for PIAS1, p63/AMACR, PCNA, and Ki-67. Original magnification: $\times 40$ (left) or $\times 400$ (right). **B:** Quantification of the prostate cancer TMA data.

Immunofluorescence

Cells were seeded onto glass coverslips and allowed to attach for 24 hours. Depending on the assay, they were grown for 2 days without treatment (for localization studies) or transfected with siRNA against PIAS1 or control siRNA (for siRNA efficiency studies and p21 expression experiments). Subsequently, the cells were washed with PBS and fixed with 4% paraformaldehyde for 10 minutes. The cells were washed with PBS and permeabilized with PBS/1% bovine serum albumin/0.2% Triton X100 for 5 minutes. After a 30-minute blocking step with PBS/1% bovine serum albumin, coverslips were incubated for 1 hour with primary antibodies against PIAS1 (dilution 1:50; Cell Signaling), p21 (WAF1/CIP1; dilution 1:50; Cell Signaling), or cytokeratin 8/18 (dilution 1:500; Sigma) or combinations of these. After washing, coverslips were incubated with the following fluorescence-labeled secondary antibodies: goat anti-rabbit 555, donkey anti-mouse 488, or goat anti-chicken 488 (all Invitrogen). Coverslips were finally washed and mounted with

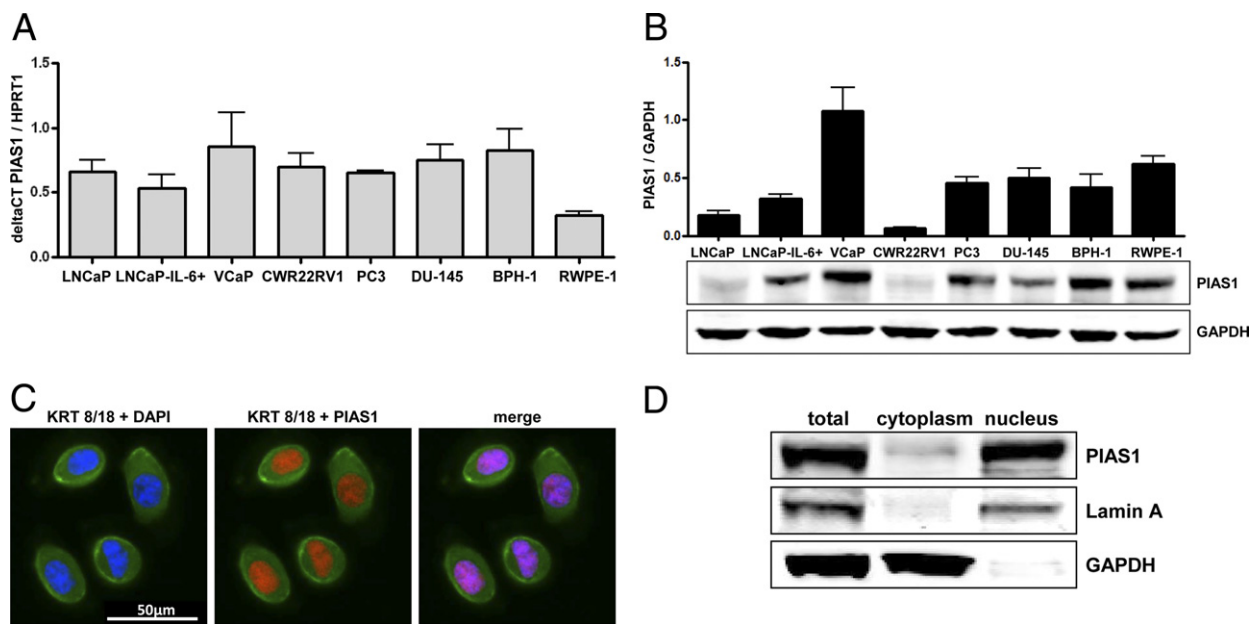


Figure 2. PIAS1 is expressed in the nucleus of prostate cell lines. PIAS1 (A) mRNA and (B) protein expression in prostate cell lines was assessed by qPCR and Western blot analysis, respectively. Data represent mean value \pm SEM of three independent experiments. C: Localization of PIAS1 (red) was determined in PC3 cells by immunofluorescence. Counterstainings of nuclei (blue) and cytokeratin 8/18 (KRT 8/18; green) were performed to determine the localization of PIAS1. Original magnification, $\times 400$. D: Confirmation of PIAS1 nuclear localization by cytoplasmatic and nuclear fractionation of PC3 cell lysates. GAPDH and lamin A were used as cytoplasmic and nuclear markers, respectively.

Vectashield Hard Set mounting medium containing DAPI (Vector Laboratories, Burlingame, CA) on glass slides. The cells were visualized using fluorescent microscopy on a Zeiss Axio Imager M1 microscope.

Results

PIAS1 Expression Is Increased in Malignant Human Prostate Tissue

To investigate PIAS1 expression patterns in benign and malignant human prostate tissue, we performed a TMA after immunohistochemical staining of 90 malignant and 79 benign areas of prostate cancer specimens. Immunohistochemistry of representative cores revealed that PIAS1 staining is stronger in malignant areas in comparison to benign tissue (Figure 1A). Staining for p63 and AMACR determined benign and malignant areas, respectively (Figure 1A). Stainings for the commonly used markers PCNA and Ki-67 were performed to determine the proliferative potential of the cells (Figure 1A).¹⁵ Interestingly, the staining pattern of PIAS1 correlated with that of PCNA and Ki-67, thus suggesting a pro-proliferative role for PIAS1. Statistical analysis of the TMA confirmed that the average staining intensity of PIAS1 in tumors compared to benign tissue was significantly increased ($P < 0.0003$) (Figure 1B). The specificity of the used PIAS1 antibody was proven by immunohistochemical staining of siRNA-treated or PIAS-1 overexpressing PC3 cells (see Supplemental Figure S1A at <http://ajp.amjpathol.org>).

PIAS1 Is Expressed in Benign and Malignant Prostate Cell Lines and Shows Nuclear Localization

To determine PIAS1 expression in human prostate cell lines, we measured mRNA levels by qPCR and protein expression by Western blot. We detected PIAS1 mRNA in all benign and malignant cell lines (Figure 2A). Nevertheless, in comparison to mRNA expression, PIAS1 protein expression was more heterogenous. VCaP cells showed the highest expression, whereas in CWR22RV1 cells, expression of PIAS1 protein was almost below the detection limit (Figure 2B). Furthermore, immunofluorescence and cell fractionation methods were applied to determine the subcellular localization of PIAS1. Both methods confirmed PIAS1 expression predominantly in the nuclei of PC3 cells (Figure 2, C and D). The same results were obtained with other prostate cell lines (data not shown). The specificity of the PIAS1 antibody used for immunofluorescence was controlled by PIAS1 down-regulation and overexpression (see Supplemental Figure S1B at <http://ajp.amjpathol.org>).

PIAS1 Down-Regulation Decreases Cell Proliferation and Colony Formation Ability

PIAS1 is implicated in modulation of NF κ B, STAT1, and androgen receptor signaling, which are known regulators of cellular proliferation and apoptosis in prostate cancer.^{2,16–19} Furthermore, the TMA results suggested an impact of PIAS1 on cellular proliferation. To address

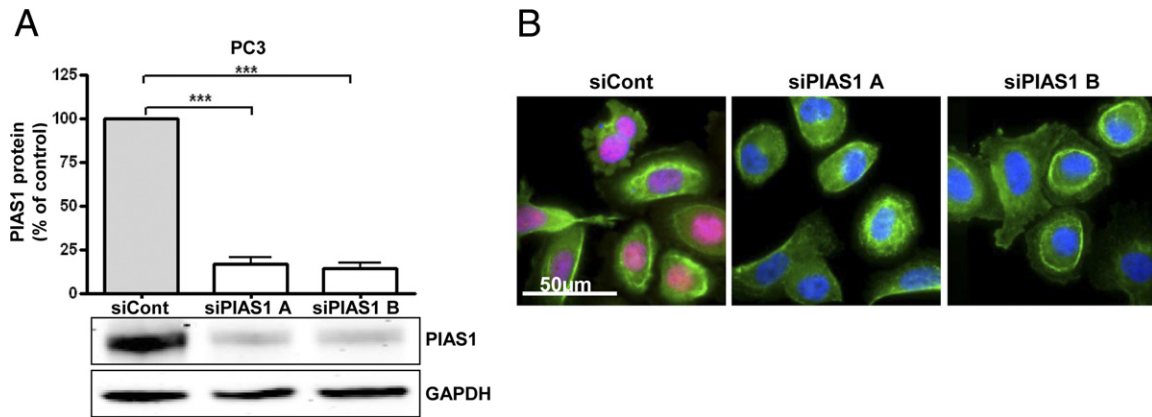


Figure 3. Inhibition of PIAS1 expression by siRNA. **A:** Western blot analysis after transfection of PC3 cells with 25 nmol/L of siPIAS1 A, siPIAS1 B, or control siRNA for 48 hours. The percentage of cells treated with control siRNA was set as 100%. Data represent mean \pm SEM from three independent experiments ($***P < 0.001$, *t*-test). **B:** PIAS1 (red) down-regulation after PIAS1 siRNA treatment was confirmed by immunofluorescence in PC3 cells. Counterstainings of nuclei (blue) and cytokeratin 8/18 (KRT 8/18; green) were performed. Original magnification, $\times 630$.

this issue, we established a siRNA approach using two specific siRNAs. Both siRNAs decreased PIAS1 mRNA (see Supplemental Figure S2 at <http://ajp.amjpathol.org>) and protein expression in PC3 cells by about 85% (Figure 3A). The efficiency of the siRNA approach was confirmed by immunofluorescence (Figure 3B).

To investigate effects of PIAS1 down-regulation on cellular proliferation, we performed [3 H]thymidine incorporation assays after down-regulation of PIAS1 in PC3, DU-145, CWR22RV1, VCaP, and RWPE-1 cells. Statistical analysis confirmed a significant decrease in cell proliferation after PIAS1 knockdown. Thymidine incorporation was reduced by approximately 50% to 75% in the investigated cell lines (Figure 4A). CWR22RV1 cells, which express very low levels of endogenous PIAS1 protein, showed no change in thymidine incorporation (Figure 4A). Similar results were obtained when siRNA B was used in PC3 cells (see Supplemental Figure S3A at <http://ajp.amjpathol.org>).

Down-regulation of PIAS1 in PC3 and DU-145 cells also resulted in a significantly reduced number of colonies formed after 12 days in a colony-forming assay. Furthermore, PIAS1 knockdown additionally reduced colony size (Figure 4B). These findings support the hypothesis that PIAS1 down-regulation negatively affects cell proliferation. In addition, PIAS knock-down with siRNA B also resulted in a reduced number of colonies (see Supplemental Figure S3B at <http://ajp.amjpathol.org>).

PIAS1 Overexpression Increases Cell Proliferation

To confirm the findings obtained by PIAS1 knockdown, we transfected PC3, DU-145, and CWR22RV1 cells with the pEGFP-C1-PIAS1 expression vector for 72 hours and subsequently measured proliferation by [3 H]thymidine incorporation. Overexpression of PIAS1 significantly increased proliferation (1.7- to 2.0-fold) in all cell lines (Figure 4C).

PIAS1 Down-Regulation Has Minor Effects on Apoptosis

To test the hypothesis that decreased proliferation caused by PIAS1 down-regulation is a consequence of an altered apoptosis rate, we transfected cells twice with 25 nmol/L of siRNA against PIAS1 and measured DNA content after PI staining by flow cytometry. Analysis of the sub-G1 peak revealed a slight change in apoptosis in PC3 and RWPE-1 cells, which was not statistically significant (Figure 5A). To investigate a possible regulation of apoptosis by an alternative method, we measured cleavage of PARP after treatment with either control or PIAS1 siRNA (Figure 5B). A slight increase in cPARP was observed in PC3 and RWPE-1 cells, which confirmed the findings obtained by flow cytometry.

PIAS1 Down-Regulation Increases the Number of Cells in the G0/G1 Phase and Reduces the Number of Cells in the S-Phase

Since previous results showed that PIAS1 down-regulation leads to a reduction of proliferation, we compared cell cycle distribution in PIAS1- and control siRNA-treated PC3, DU-145, VCaP, RWPE-1, and CWR22RV1 cells. Flow cytometric analysis proved that PIAS1 down-regulation leads to a significant increase in the percentage of cells in the G0/G1 phase as well as a significant decrease of cells in the S-phase (Figure 6, A and B). This effect was confirmed when siRNA B was used for PIAS1 silencing before the cell cycle analysis (see Supplemental Figure S3C at <http://ajp.amjpathol.org>). Furthermore, we observed a reduction of the percentage of cells in the G2/M phase (Figure 6, A and B). As expected, no alterations in cell cycle distribution was observed in PIAS1-negative CWR22RV1 cells. Our findings indicate that PIAS1 depletion may prevent or delay the transition into the S

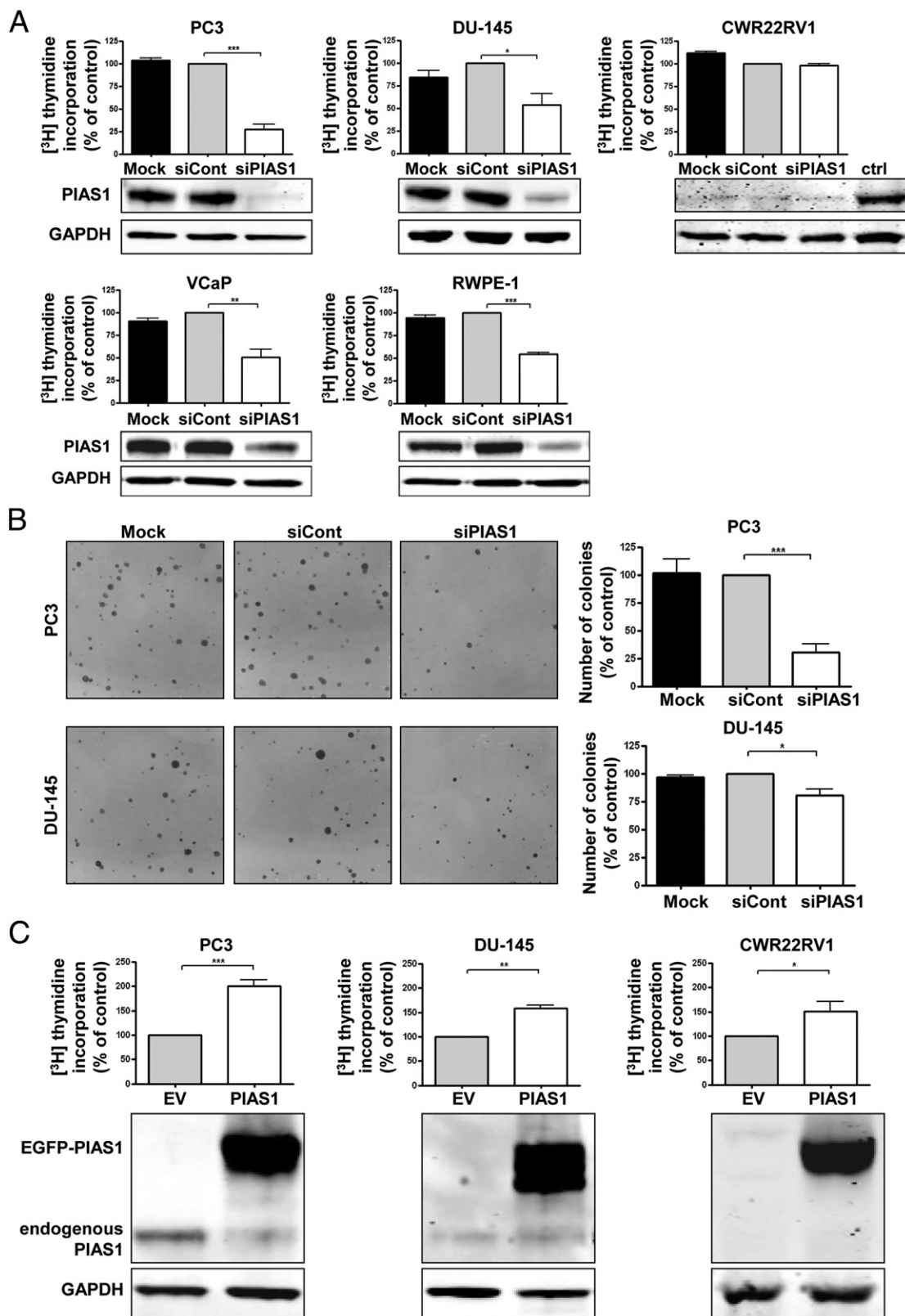


Figure 4. Expression of PIAS1 significantly influences proliferation and colony formation ability. **A:** Proliferation after PIAS1 down-regulation. Proliferation was assessed by measurement of [³H]thymidine incorporation. Efficiency of the transfections was controlled by Western blot. The percentages of cells treated with control siRNA were set as 100%. Data represent mean ± SEM from three independent experiments (**P* < 0.05, ***P* < 0.01, and ****P* < 0.001, *t*-test). **B:** Effect of PIAS1 down-regulation on colony formation ability of PC3 and DU-145 cells was assessed by counting the number of colonies after 12 days. The percentage of cells treated with control siRNA was set as 100%. Data represent mean ± SEM from three independent experiments (**P* < 0.05, ****P* < 0.001, *t*-Test). **C:** Proliferation after PIAS1 overexpression, assessed as in A. The percentages of cells transfected with empty vector were set as 100%. Data represent mean ± SEM from three independent experiments (**P* < 0.05; ***P* < 0.01; ****P* < 0.001, *t*-test).

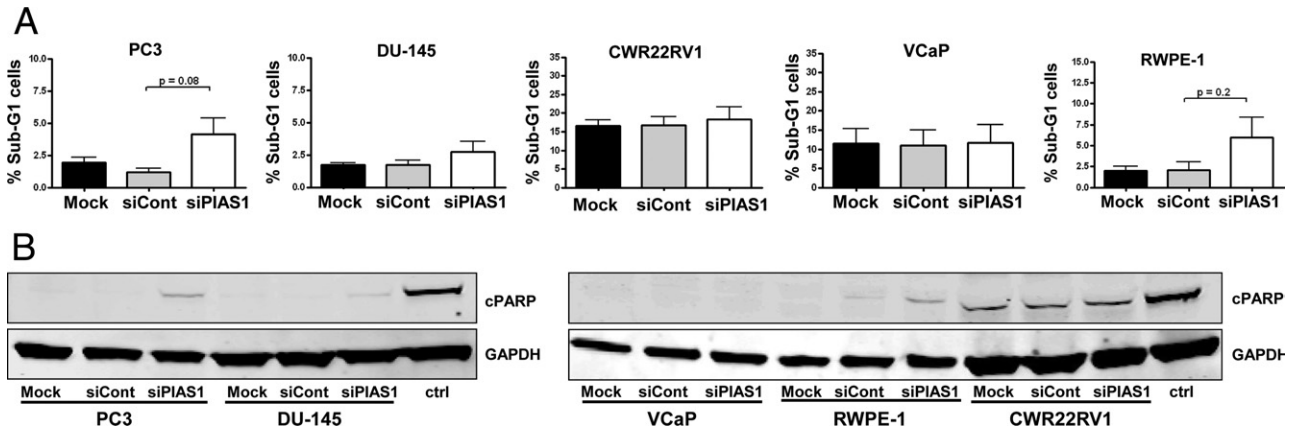


Figure 5. PIAS1 down-regulation does not significantly increase apoptosis. **A:** Cells were transfected with PIAS1 siRNA or control siRNA twice and analyzed for DNA content by flow cytometry. Data represent mean \pm SEM from three independent experiments. **B:** cPARP levels were assessed by Western blot analysis. A representative Western blot from three independent experiments is shown. Ctrl represents cells treated with 200 μ g/mL cycloheximide to induce apoptosis. GAPDH served as loading control.

phase of the cell cycle, thus being responsible for decreased cell proliferation. To prove that PIAS1 supports cell cycle progression, cell cycle analysis was also performed after overexpression of PIAS1. In con-

cordance with the results obtained by PIAS1 down-regulation, overexpression of PIAS1 resulted in a decrease of cells in the G₀/G₁ and an increase of cells in the S phase as well as the G₂/M phase (Figure 6C).

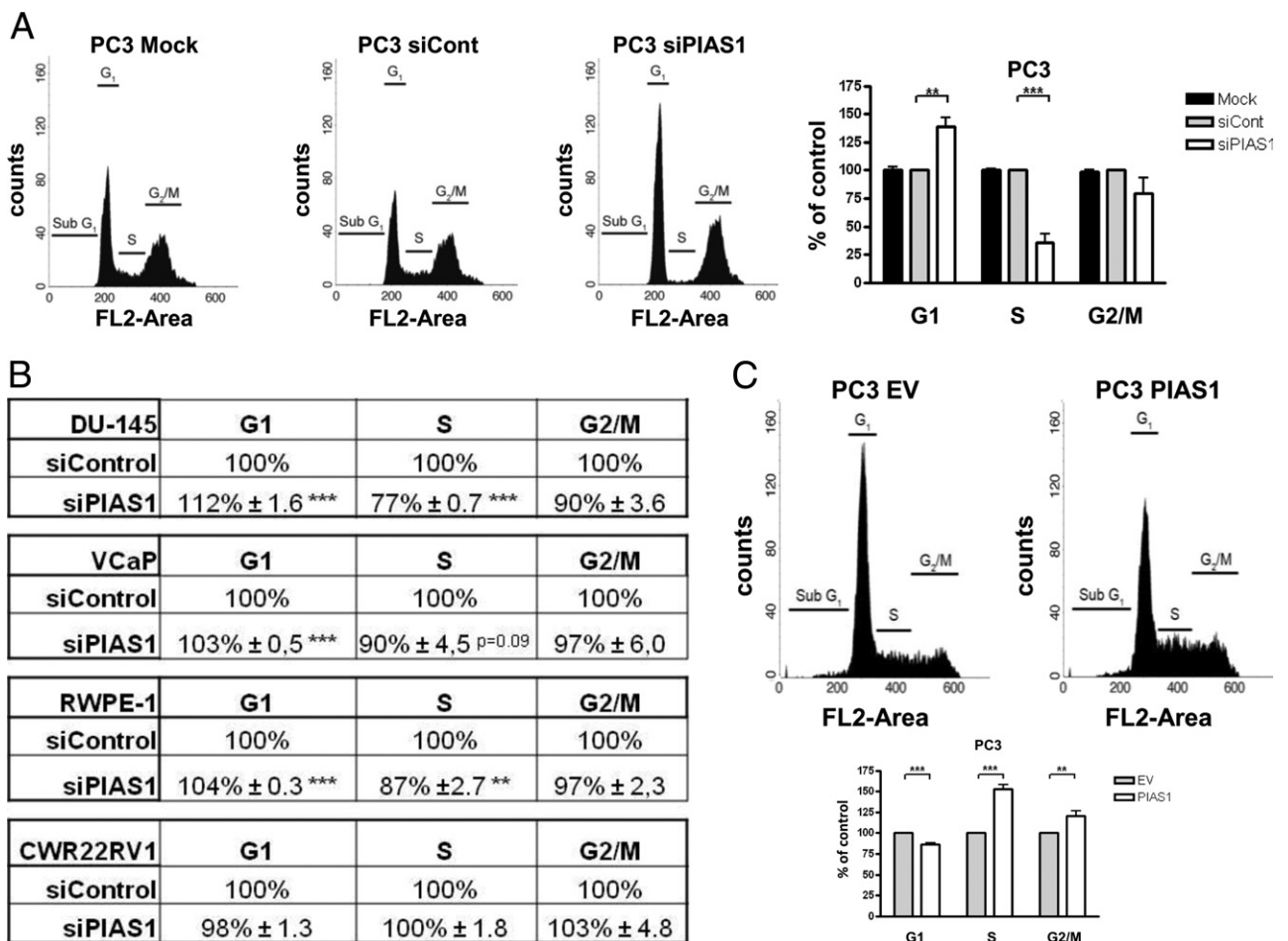


Figure 6. PIAS1 regulation influences cell cycle progression. Cells treated with siPIAS1 or control siRNA were stained with PI buffer and analyzed by flow cytometry. **A:** Representative histograms of cell cycle distribution after PIAS1 down-regulation in PC3 cells as well as statistical analysis of findings. **B:** Statistical analysis of cell cycle distribution in DU-145, VCaP, RWPE-1, and CWR22RV1 cells. The percentage of cells treated with control siRNA was set as 100%. Data represent mean \pm SEM from three independent experiments (** P < 0.01, *** P < 0.001, *t*-test). **C:** Representative histograms of cell cycle distribution after PIAS1 over-expression in PC3 cells as well as statistical analysis of findings. The percentage of cells transfected with empty vector (EV) was set as 100%. Data represent mean \pm SEM from three independent experiments (** P < 0.01, *** P < 0.001, *t*-test).

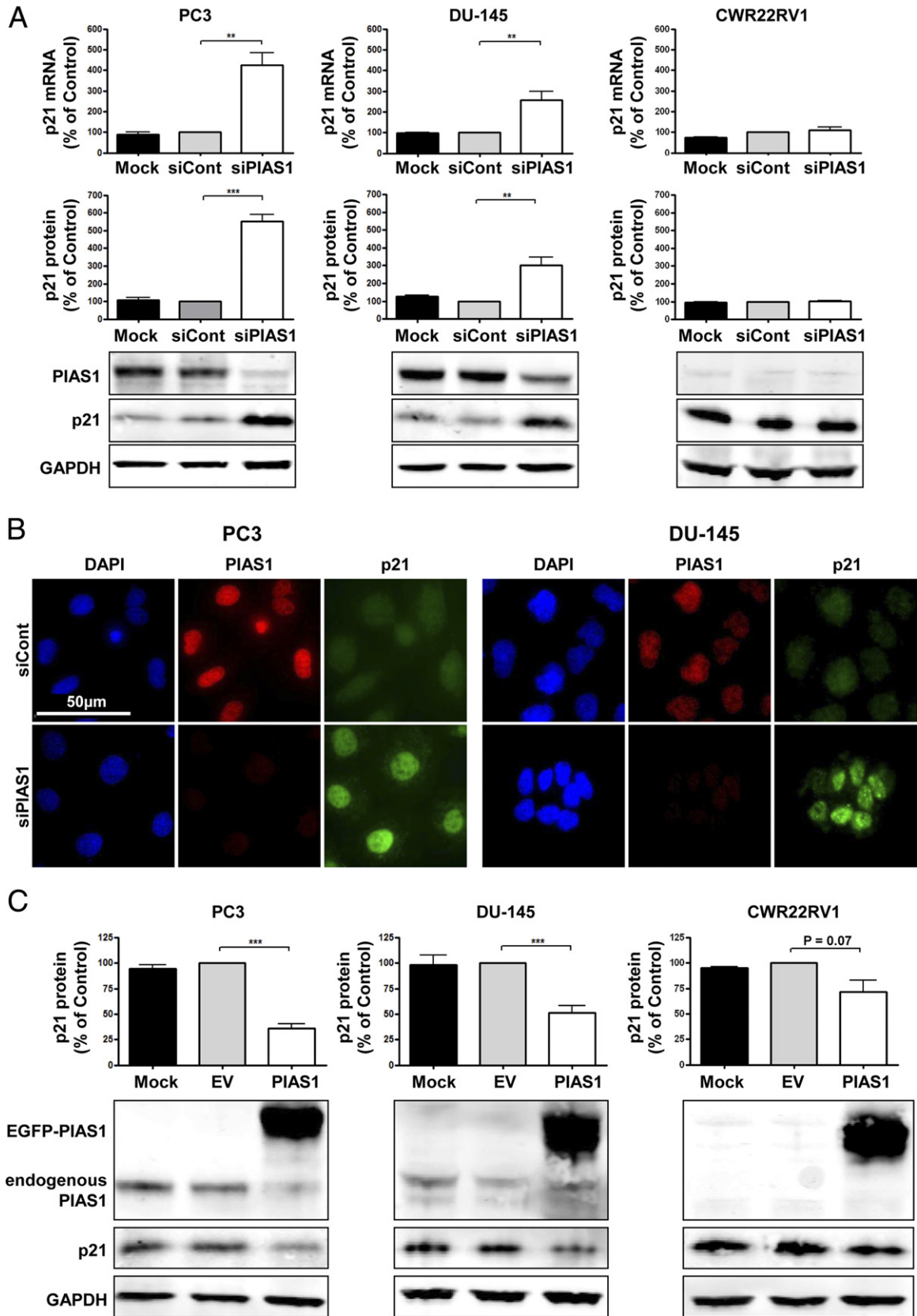


Figure 7. PIAS1 expression influences p21 levels. **A:** p21 mRNA (upper panel) and protein (lower panel) expression after down-regulation of PIAS1 were determined by qPCR and Western blot analysis, respectively. The percentages of cells treated with control siRNA were set as 100%. Data represent mean \pm SEM from three independent experiments (** $P < 0.01$, *** $P < 0.001$, *t*-test). **B:** Increased p21 (green) expression after down-regulation of PIAS1 (red) was confirmed by immunofluorescence. Original magnification, $\times 630$. **C:** Western blot analysis of p21 protein expression after overexpression of PIAS1. The percentage of cells transfected with empty vector was set as 100%. Data represent mean \pm SEM from three independent experiments (** $P < 0.01$, *** $P < 0.001$, *t*-test).

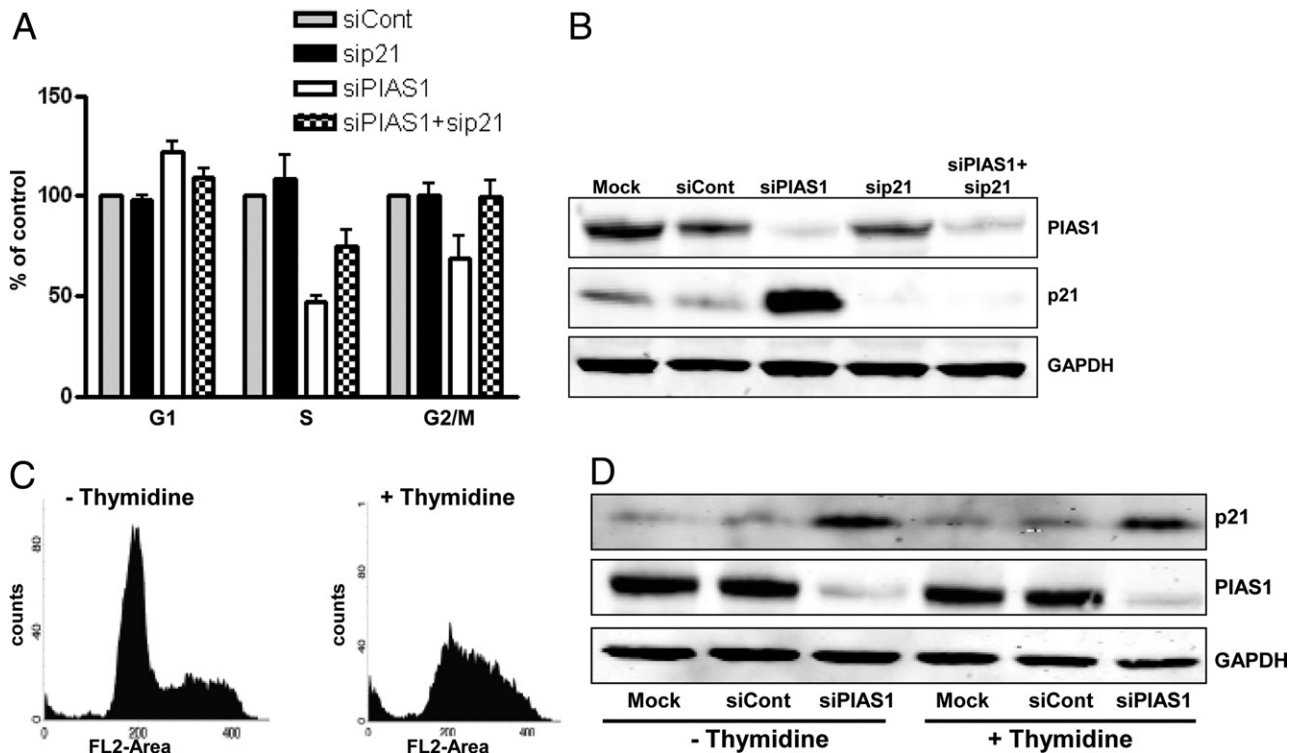


Figure 8. p21 accumulation is involved in mediating the cell cycle arrest after PIAS1 knockdown. PC3 cells were treated with siRNA against p21, PIAS1, or a combination of both. Cell cycle analysis was performed by flow cytometry (A), and expression of PIAS1 and p21 were determined by Western blot analysis (B). GAPDH was used as an internal control. PC3 cells were growth arrested by thymidine block followed by cell cycle analysis (C) and determination of p21 and PIAS1 expression levels (D). Three independent experiments were performed.

PIAS1 Influences Expression of the Cell Cycle Regulator p21

To further analyze the mechanism by which PIAS1 regulates cell cycle progression, we determined p21 expression after down-regulation or overexpression of PIAS1. p21 is a member of the CIP/KIP family of cyclin-dependent kinase G1/S phase transition inhibitors.^{20–22} Furthermore, it was shown that p21 induction reduces prostate cancer cell growth.²³ We transfected PC3, DU-145, and CWR22RV1 cell lines with PIAS1 siRNA and measured p21 expression by qPCR and Western blot (Figure 7A). Both analyses confirmed a significantly increased p21 expression after PIAS1 silencing in PC3 (~5.5-fold) and DU-145 cells (approximately threefold). Taken together, our data reveal that the increase in p21 expression is a result of transcriptional regulation. The results were confirmed with siRNA B (see Supplemental Figure S3D at <http://ajp.amjpathol.org>). As a control, p21 expression was not affected in CWR22RV1 cells which express very low levels of PIAS1 protein (Figure 7A). In addition, these cells did not show any change in proliferation after siRNA treatment (Figure 4A). We confirmed those findings by immunofluorescence in PC3 and DU-145 cell lines. p21 staining highly increased in siRNA-treated cells (Figure 7B). Immunofluorescence also revealed that p21, following PIAS1 siRNA treatment, predominantly shows nuclear localization, which is known to be associated with its cell cycle inhibitory activity.^{24,25} To provide further support for the hypothesis that PIAS1 regulates p21 expression in prostate cancer, we also measured p21 levels after overexpression

of PIAS1. Therefore, we transfected PC3, DU-145, and CWR22RV1 cells with pEGFP-C1-PIAS1 or empty vector. Western blot analysis revealed that PIAS1 overexpression leads to a significant decrease in p21 levels in PC3 and DU-145 cells and to a slight decrease in its expression in the CWR22RV1 cell line (Figure 7C). Taken together, these findings clearly demonstrate that PIAS1 significantly influences expression levels of the cell cycle inhibitor p21.

Cell Cycle Arrest Following PIAS1 Silencing Is Mediated by p21

To address the question whether the observed cell cycle arrest after PIAS1 down-regulation is enforced by p21, we treated the cells with siRNAs against PIAS1 or p21 or the combination of both and measured cell cycle distribution under those conditions. As demonstrated before, down-regulation of PIAS1 leads to an increase in p21 levels and, subsequently, to an enrichment of cells in the G1 phase as well as a decrease of cells in the S phase. These effects could be reversed after a combined down-regulation of PIAS1 and p21 (Figure 8, A and B). To exclude the possibility that p21 accumulation is mediated by the G1 arrest, we measured p21 levels in S phase-arrested cells (Figure 8, C and D). Western blot results confirmed that treatment with PIAS1 siRNA leads to p21 accumulation independently of cell cycle position, thus indicating that cell cycle arrest is a consequence of p21 increase.

Discussion

The functional role of PIAS proteins in human malignancies has not been clarified yet. Brantley and colleagues²⁶ demonstrated that loss of PIAS3 in glioblastoma multiforme leads to enhanced cellular proliferation through increased STAT3 phosphorylation, while Coppola et al²⁷ show a reduced expression of PIAS1 associated with colon cancer development. Studies that systematically address PIAS1 protein expression patterns and its role in regulation of proliferation and cell death in cancer have not been performed yet.

In this work, we prove for the first time that PIAS1 protein expression is significantly increased in tumor tissue in comparison to benign areas of specimens obtained from patients with prostate cancer. These results are in line with the observations from Li and colleagues²⁸ who reported on an increase in PIAS1 mRNA in prostate tumor cases.

It was shown that androgenic hormones up-regulate PIAS1 expression.²⁹ Other steroids or peptide growth factors, whose expression is dysregulated at diverse stages of prostate carcinogenesis, may also influence PIAS1 levels, and regulation of PIAS1 by these molecules may be a subject of future investigations. In view of our results, previous findings by Gross and colleagues¹⁶ who described the role of PIAS1 as a co-activator of androgen receptor may be particularly relevant in prostate cancer. Thus, PIAS1 may in addition contribute to enhanced proliferation or decreased apoptosis of prostate cancer cells through stimulation of androgen receptor activity.

In our study, the staining pattern of PIAS1 protein in prostate tissues correlated with that of the proliferation markers PCNA and Ki-67, thus suggesting a growth-promoting role for PIAS1 in prostate cancer.¹⁵ This assumption was supported by our *in vitro* results. We show that PIAS1 down-regulation decreases proliferation and colony formation ability of prostate cancer cell lines. This decrease can be explained by a p21-induced cell cycle arrest. Furthermore, PIAS1 over-expression leads to an increase in proliferation and reduced p21 levels. Our results also indicate that the presence of p21 in cancer cells plays an important role in the inhibitory effect of PIAS1 on cell growth.

In prostate cancer, induction of p21 after inositol hexaphosphate or silibinin treatment was reported.^{30,31} Those results are consistent with our data, that p21 induction leads to a G1 arrest and growth inhibition of prostate cancer cells. Furthermore, Chen³² et al showed in a model of acute pancreatitis that PIAS1 down-regulation leads to an increase in p21 levels which induces cell cycle arrest and subsequent apoptosis thus supporting our results. Interestingly, a tumor suppressive effect of p21 was not observed in many cellular systems.^{25,33} It was reported that p21 has multiple functions depending on binding partners and its subcellular localization.³⁴ It was proposed that nuclear retention of p21 is associated with enhanced G1 arrest and cell death in cells in which heat-shock protein 27 or Akt were silenced, whereas cytoplasmic sequestration of p21 delays apoptosis.^{35,36} The subcellular localization of p21 may be a determinant for the regulatory functions of p21. In the present study, p21 was expressed in the nucleus following PIAS1 down-regulation. This indicates a tumor-suppressive effect of

p21 thus explaining the observed cell cycle arrest and inhibition of proliferation after siRNA treatment.

The regulation of p21 by PIAS1 in prostate cancer has not been completely clarified but several mechanisms could be considered. For example, SUMOylation of p53 or p73 by PIAS1 could account for the observed effects. Both tumor suppressors can directly influence p21 expression.^{37,38} Since p53 is absent in PC3 and mutated in DU-145 cells, a p73-dependent mechanism being responsible for the effects of PIAS1 is more probable.³⁹ This hypothesis is supported by Munarriz and colleagues,³⁸ who showed that PIAS1, which is expressed predominantly during the S phase of the cell cycle, can bind to and SUMOylate p73, thereby inhibiting the transcriptional activity of p73, which leads to a decrease in p21 in lung cancer and osteosarcoma cells. Furthermore, it has been shown that also PIAS1 can interact with p73 in HEK293 cells.⁴⁰ However, on the basis of our results, we cannot exclude p73-independent regulation of p21 by PIAS1.

The present results indicate that the molecules that inhibit cytokine signaling are involved in prostate cancer regulatory processes by multiple mechanisms. In this context, PIAS1 and SOCS-3 promote tumorigenesis in prostate cancer cell lines through different mechanisms. We demonstrated that SOCS-3 prevents apoptosis through inhibition of extrinsic and intrinsic apoptotic pathways and regulation of Bcl-2 expression in androgen-insensitive cell lines.¹³ In androgen-sensitive prostate cancer cells, androgenic up-regulation of SOCS-3 resulted in inhibition of proliferation and secretion of prostate-specific antigen induced by male sexual hormones.⁴¹ Tumor-promoting effects of PIAS1 in prostate cancer were observed in this study in cell lines irrespective of their androgen sensitivity. In contrast to PIAS1, SOCS-1 retards cell cycle progression. It inhibits expression of cyclin-dependent kinases 2 and 4 and cyclins D1 and E.⁴² The interest for the role of endogenous inhibitors of cytokine signaling could be explained by the fact that chronic inflammation is associated with prostate cancer development, at least in a subgroup of patients.⁴³ A better understanding of action of cytokines and their endogenous inhibitors in pre-malignant prostate lesions and cancer is therefore necessary to improve therapy and prevention of prostate carcinogenesis. For this reason, all elements of cytokine signaling pathways are a subject of intensive investigations. As stated above, suppressors of cytokine signaling are likely to regulate tumorigenesis also by inflammation-independent mechanisms, in particular in advanced cancer.

In summary, we show for the first time that PIAS1 protein expression is increased in human prostate cancer and identify a new functional role for PIAS1 in the regulation of proliferation and cell cycle in prostate cancer cell lines. On the basis of these results, we suggest that PIAS1 may be considered a potential target for the development of improved therapies in prostate cancer.

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