Cyclin-dependent kinase 5 modulates STAT3 and androgen receptor activation through phosphorylation of Ser\textsuperscript{727} on STAT3 in prostate cancer cells

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Prostate cancer is the most common cancer in men (20). Androgen ablation therapy is the primary strategy for suppressing prostate cancer growth; however, some castration-resistant prostate cancers eventually relapse within two years. Therefore, it is imperative to further understand the molecular mechanisms of prostate cancer progression and to develop effective treatment strategies.

Signal transducer and activator of transcription 3 (STAT3), a transcription factor, has been reported to regulate prostate cancer development (4, 16). It has been suggested that the transcriptional activity of STAT3 is initiated by phosphorylation at tyrosine 705 (Tyr\textsuperscript{705}), followed by STAT3 dimerization, nuclear translocation, and DNA binding (29, 33). Phosphorylation of STAT3 at serine 727 (Ser\textsuperscript{727}) is important in activating STAT3 signaling in response to a variety of extracellular stimuli, such as growth factors, cytokines, or environmental stress (8, 44). Although Tyr\textsuperscript{705} phosphorylation is critical to STAT3 activation, Ser\textsuperscript{727} phosphorylation of STAT3 still shows the contribution of both residues for full activation of STAT3 (2).

Cyclin-dependent kinase 5 (Cdk5) is a proline-directed serine/threonine kinase. Without participating in cell cycle progression, Cdk5 has been implicated in various aspects of neural functions (11). Distinct from the other Cdk family members, Cdk5 is activated not by binding to cyclins but rather through association with its regulatory subunits p35 and p39 (37, 38). p35 (Cdk5R1), a 35-kDa protein, has been widely investigated and was originally defined as a neuron-specific activator of Cdk5 (38). Recently, p35 was indicated to play important roles in human cancers by regulating Cdk5 activity (15, 26, 27, 36), p35 overexpression was also reported in metastatic prostate cancers (36). Multiple functions of Cdk5 in addition to those in the central nervous system were recently discovered, such as its roles in cancer biology (6, 22, 26, 27, 36). In our previous research, we first identified the kinase activity and apoptotic role of Cdk5 in prostate cancer cells (27). Our recent work demonstrates the modulation of prostate cancer growth by Cdk5 through activation of androgen receptor (AR) by phosphorylation (15). Several lines of evidence have revealed that STAT3 can be modulated by Cdk5-dependent phosphorylation at the Ser\textsuperscript{727} site in mouse neurons (12), muscle cells (12), and liver cancer (34). A recent study indicates that Cdk5 prevents DNA damage through Ser\textsuperscript{727} phosphorylation of STAT3 (9). Our previous results also show that Cdk5 modulates STAT3 activation and cell proliferation of thyroid cancer (26).

STAT3 has been shown to modulate signaling cross-talk between steroid receptors such as AR (39) or glucocorticoid receptor (47) and other signaling pathways in response to
interleukin 6 (IL-6). Gene expression regulated by AR and activation of the AR NH2-terminal domain by IL-6 are accomplished through the STAT3 pathway in prostate cancer cells (7, 39, 40). Since both STAT3 and AR are substrates regulated by Cdk5, it is of interest to investigate the detailed mechanism of this regulation in prostate cancer cells. According to our in vitro data, Cdk5 interacts with STAT3 and positively regulates STAT3 activation as well as prostate cancer cell proliferation through Ser727 phosphorylation of STAT3. On the other hand, we found that Cdk5 activation might indirectly increase AR activation through an STAT3-AR protein interaction. Furthermore, we provide clinical evidence showing that p-Ser727-STAT3 level positively correlates with Gleason score, Cdk5, p35, and AR protein levels in prostate cancer patients’ specimens. We propose that Cdk5-dependent Ser727 phosphorylation of STAT3 might play important roles in prostate cancer progression.

**MATERIALS AND METHODS**

**Materials.** Roscovitine (ROSC; a Cdk5 inhibitor; R7772) and cycloheximide (CHX; a protein synthesis inhibitor; C9188) were purchased from Sigma, MG132 (a proteasome inhibitor; 474791) was purchased from Calbiochem, and R881 (methylthiolute, a synthetic androgen; NLF-005) was purchased from Perkin-Elmer Life Sciences. Antibodies used for immunoblotting are as follows: β-actin (MAB1501; Millipore), AR (sc-13062 and sc-7305, Santa Cruz Biotechnology; and 554224, BD Biosciences), p-Ser81-AR (07-541, Upstate Biotechnology; AND 07-1375, Millipore), Cdk5 (sc-750 and sc-249, Santa Cruz Biotechnology; and 05-364, Upstate Biotechnology), FLAG (sc-807, Santa Cruz Biotechnology; and F3165, Sigma), c-fos (sc-52; Santa Cruz Biotechnology), junB (sc-73; Santa Cruz Biotechnology), poly(ADP)-ribose polymerase (06-557; Upstate Biotechnology), PCA (sc-7316; Santa Cruz Biotechnology), p35 (sc-820 and sc-5614; Santa Cruz Biotechnology), STAT3 (610190; BD Biosciences), p-Ser727-STAT3 (9134, Cell Signaling Technology; and 07-1375, Millipore), and sc-249, Santa Cruz Biotechnology; and 05-364, Upstate Biotechnology; AND 07-1375. Antibodies used for immunohistochemistry were Cdk5 (MAB1501; Millipore), p-Ser727-STAT3 (9134, Cell Signaling Technology; and 07-1375, Millipore), and sc-249, Santa Cruz Biotechnology.

**Cell culture.** LNCaP (BCRC-60088), 22Rv1 (BCRC-60545), DU145 (BCRC-60348), and PC3 (BCRC-60122) cell lines were purchased from the Bioresource Collection and Research Center, Food Industry Research and Development Institute in Taiwan. LNCaP and 22Rv1 cells were cultured in RPMI-1640 culture medium (Sigma) supplemented with 1.5 g/l sodium bicarbonate (NaHCO3; Sigma), 10% fetal bovine serum (FBS) (Gibco), 2 mM l-glutamine (Gibco), 4.5 g/l glucose (Sigma), 10 mM HEPES (Sigma), 1 mM sodium pyruvate (Gibco), and 100 IU/ml penicillin-100 µg/ml streptomycin (P/S; Sigma). Chinese hamster ovary (CHO) cells were kindly provided by Prof. Hong-Chen Chen, Department of Life Sciences, National Chung Hsing University, Taiwan. CHO and PC3 cells were cultured in Ham’s F-12 medium plus 10% FBS, 1.5 g/l NaHCO3, and P/S. DU145 cells were cultured in MEM plus 10% FBS, 1.5 g/l NaHCO3, 0.1 mM NEAA, 0.1 mM sodium pyruvate, and P/S. All cell lines were cultured at 37°C in a humidified atmosphere with 5% CO2. The passage numbers of LNCaP cells in all experiments are between passages 10 and 25.

**Stat3 phosphorylation, immunoprecipitation, and immunoblotting analyses.** Cell were lysed in lysis buffer [50 mM Tris-HCl (pH 8.0), 0.5% NP-40, 150 mM NaCl, 5 mM EDTA, 1 mM PMSF, 2 mM Na3VO4, and protease inhibitor cocktail (Roche Applied Science)]. Lysates were analyzed by direct immunoblots (20–35 µg/lane) or blotting after immunoprecipitation (0.5–1 mg/immunoprecipitation) using methods modified from those described previously (3, 27, 28). Immunoprecipitates were collected by binding to 25–40 µl of the FXP-Flag beads (sc-5042 and scv-5043; Santa Cruz Biotechnology) or 10–25 µl of the Mag Sepharose Xira Protein G beads (28-9670-70; GE Healthcare). To isolate subcellular proteins, cells were collected and washed in PBS-Na3VO4. Pelleted cells were resuspended in hypotonic buffer [10 mM HEPES (pH 7.9), 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 0.5% NP-40, 1 mM PMSF, 2 mM Na3VO4, and protease inhibitor cocktail]. Nuclear proteins were in the pellet, whereas the supernatant contained the cytosolic fraction. The nuclear pellet was washed three times with hypotonic buffer before lysing in nuclear extraction buffer [20 mM HEPES (pH 7.9), 0.4 M NaCl, 1 mM EDTA, 1 M EGTA, 20% glycerol, 1 mM PMSF, 2 mM Na3VO4, and protease inhibitor cocktail]. The lysates were mixed with a 1:3 volume of 5× SDS sample buffer and resolved by SDS-polyacrylamide gel electrophoresis. ECL detection reagent (PerkinElmer Life Science) was used to visualize the immunoreactive proteins on PVDF membranes (Perkin-Elmer Life Science) after transfer using a Trans-Blot SD (Bio-Rad). The quantification software was MCID Image Analysis Evaluation.

**Cell proliferation assay.** The modified colorimetric 3-(4,5-dimethylthiazol-2-y)-2,5-diphenyltetrazolium bromide (MTT) assay was performed to quantify the proliferation of LNCaP cells. The yellow MTT compound (Sigma) is converted by living cells into blue formazan, which is soluble in isopropanol. The blue staining was measured by using an optical density reader (Athos-2001) at 570 nm (background of isopropanol, 620 nm) (26, 27).

**Transfection.** siRNA-cdk5, siRNA-p35, and nonspecific control siRNA were purchased from Dharmacon (SMARTpool). shRNA plasmids of plKO.1-gfp and -cdk5 were obtained from the National
RNAi Core Facility located at the Institute of Molecular Biology/Genome Research Center, Academia Sinica, Taiwan. pcDNA3-FLAG-wild type (WT)-AR, pcDNA3-FLAG-S81A-AR (13), and pGL3-3×ARE (androgen response element) expression plasmids were kindly provided by Prof. Daniel Gioeli, Department of Microbiology, University of Virginia. Mouse mammary tumor virus (MMTV)-luciferase expression plasmid was a gift from Prof. Chawn-Shang Chang, Department of Pathology and Urology, University of Rochester Medical Center, Rochester, NY (42). pSV-β-galactosidase expression plasmid was a gift from Prof. Jeremy J. W. Chen, Institute of Biomedical Sciences, National Chung Hsing University, Taiwan. Human p35, Cdk5, and STAT3 expression plasmids were constructed by RT-PCR amplification of the human p35 and cdk5 stat3-coding sequences and inserted into the pcDNA3 and pcDNA4A vectors (Invitrogen) by TA cloning. The STAT3-S727A mutant construct was generated by PCR-based mutagenesis using the QuikChange II Site-
Directed Mutagenesis Kit (200524; Stratagene). Sequences of all constructs were verified by DNA sequencing. Immunoblotting was conducted to detect the corresponding expressed proteins after transfection. Transfections of siRNAs or plasmids into cell lines were performed using Lipofectamine 2000 (11668-019; Invitrogen) with 5 pmol of siRNA/10^5 cells and 0.8 μg DNA/10^5 cells.

**Reporter assay.** Luciferase reporter gene activity was carried out according to the dual-light system (Applied Biosystems). Cells were transfected with luciferase expression plasmids with β-galactosidase plasmids, following the manufacturer’s instructions. Cells were washed twice with PBS and lysed in lysis solution for 15–20 min. Cell lysates were centrifuged for 20 min at 4°C. Supernatants were mixed with luciferase substrate. Reporter gene activity was measured by 1420 Multilabel Counter Victor3 (Perkin-Elmer). The transfection efficiency was normalized by β-galactosidase activity.

**Analysis of clinical specimens.** The analysis of clinical specimens was performed using the tissue array of prostate cancer patients’ specimens purchased from US Biomax, and immunohistochemistry was performed to determine the expression levels of examined proteins. The specificity and selectivity of immunohistochemistry are shown in Fig. 2. The experimental procedures were modified from the paraffin immunohistochemistry protocol from Cell Signaling Technology. The slides were deparaffinized in xylene and rehydrated in graded alcohol and H2O. An antigen retrieval step with 10 mM sodium citrate (pH 6.0) at subboiling temperature was used for each primary antibody. Endogenous peroxidase activity was blocked by 3% hydrogen peroxide for 10 min, followed by a 1-h incubation with blocking serum (Vectastain ABC Kit; Vector Laboratories). The slides were then incubated for 4 h at room temperature, following incubation with biotinylated antibody (Vectastain ABC Kit) for 30 min. Finally, the slides were incubated in ABC reagent (Vectastain ABC Kit) for 30 min and in 3,3’-diaminobenzidine (DAB; Thermo Fisher Scientific) for 2 min. The slides were counterstained with diluted hematoxylin solution (1:10; Merck) and dehydrated with graded alcohol and xylene. Finally, the slides were mounted and imaged by light microscope (Bx-51; Olympus). Blue color indicated nuclei stained by hematoxylin. Brown color indicated the target proteins stained by the DAB kit. The images were blinded and evaluated by two experts in accord with a scoring system that was based on the intensity and distribution of staining signals. The scores were divided into four grades: negative (grade 0; 0%), low (grade 1; 1–17%), moderate (grade 2; 18–35%), and high (grade 3; >35%) (for details, please see Fig. 2) (15).

**Quantitative real-time RT-PCR.** Total RNA was isolated from LNCaP cells with the Total RNA Miniprep Purification Kit (GeneMark). RT-PCR was performed with the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems). The following primer pairs were used: junB, 5’-ATCACGAGCAGCGCTACAC (forward) and 5’-CTCTGTCTCTCTGGTGAC (reverse); c-fos, 5’-GGAGGAGGAGGACTGAGT (forward) and 5’-GCTGCGAGAGATGAATTCTAGTT (reverse); c-myc, 5’-TGAAGGAGACACC GGCCA (forward) and 5’-AACATCGATTTCTTCTCA (reverse); survivin, 5’-TCC ACTGCCCCACTGAGAAC (forward) and 5’-TGCTCCACGCTTCCA (reverse); β-actin, 5’-TTGCGCAAGGATGTGCAAA (forward) and 5’-GCGATCTACACCGAGATTACT (reverse). The quantitative PCR reaction was conducted by the 7300 Real Time PCR System (Applied Biosystems).

**Xenograft tumor growth in nude mice.** The BALB/c nude mice were purchased from the National Laboratory Animal Center in Taiwan. 22Rv1 cells (10^7 cells/mouse) were subcutaneously injected into the backs of BALB/c nude mice. When the tumor volumes reached 500–1,000 mm³, 10 μg of Cdk5 plasmids was mixed with in vivo jet PEI transfection reagent (Polyplus) and injected directly into the xenograft tumors every 3 days. The mice in the mock group received enhanced green fluorescent protein plasmids. The major axis (L) and the short axis (W) were measured every day. Tumor volumes were estimated using the following formula: L × W × W × 3.14/6.

The mice were euthanized 3 days after the final injection. The tumors were obtained, and the protein expression was analyzed by immunoblotting. All animal experiments were conducted in accordance with the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals and approved by the Institutional Animal Care and Use Committee of National Chung Hsing University, Taiwan.

**Statistics.** All values are given as means ± SE. Student’s t-test was used in the cell proliferation experiment, quantitative RT-PCR experiments, and reporter assay. A difference between two means was considered to be statistically significant when P < 0.05. The correlations between p-Ser^727^-STAT3 protein level and Gleason score, Cdk5, p35, and AR protein levels in clinical specimens were analyzed using χ² test by S-PLUS 6.2pProfessional software.

**RESULTS**

The correlation between STAT3 and Cdk5 in prostate cancer cells. Here, we show the biochemical interaction of Cdk5 and STAT3 in the prostate cancer LNCaP cell line by coimmunoprecipitation (Fig. 1A). Consistent with our previous observation (15), Cdk5 also interacted with AR. Interestingly, the Cdk5-STAT3 protein interaction and STAT3 Ser^727^-phosphorylation were significantly decreased by treatment with the Cdk5 inhibitor ROSC (Fig. 1B). On the contrary, p35 overexpression increased the Cdk5-STAT3 protein interaction and STAT3 Ser^727^-phosphorylation in LNCaP cells (Fig. 1C). Consistently, Cdk5 overexpression elevated p-Ser^727^-phosphorylation level, whereas Cdk5 knockdown by siRNA reduced it (Fig. 1D). The quantification of p-Ser^727^-STAT3 and Cdk5 levels normalized by actin level was performed and labeled in Fig. 1D. The numbers below the immunoblotting image represent the fold change normalized to respective control groups (Con and siCon). As the data show, Cdk5 overexpression led to a 4.9-fold increase in Cdk5 protein level and a 2.4-fold increase in p-Ser^727^-STAT3 level (Fig. 1D, left). In addition, a 30% decrease in Cdk5 level and a 60% decrease in p-Ser^727^-STAT3 level were observed after Cdk5 knockdown (Fig. 1D, right). In our published data (15), Cdk5 may positively regulate in vivo prostate tumor growth, and those tumor lysates were also collected to analyze protein expression in this study (Fig. 1E). Consistently, Cdk5 levels positively modulated Ser^727^-phosphorylation of STAT3 in 22Rv1 xenograft tumors (Fig. 1E). However, the change in Tyr^705^-phosphorylation of STAT3 by Cdk5 overexpression was not observed in two different prostate cancer cell lines (Fig. 1F). Intriguingly, the S727A (Ser/Ala) mutant of STAT3 decreased its interaction with Cdk5 (Fig. 1G). These results suggest that the Ser^727^-site of STAT3 might be phosphorylated by Cdk5 before their biochemical interaction.

In addition to the results from cell lines, we analyzed the correlations of protein levels between p-Ser^727^-STAT3 and Cdk5 or p35 in clinical samples. Prostate carcinoma specimens from a cohort of 110 patients were collected from tissue array product (Biomax). The protein levels in the specimens were observed by immunohistochemical staining. The intensity of protein staining was defined and divided into two levels (15), negative/low and moderate/high, as indicated in Tables 1–3. Representative images for each grade of p-Ser^727^-STAT3, Cdk5, p35, and AR proteins were shown in Fig. 2. The correlations between p-Ser^727^-STAT3 levels and Cdk5, p35, or AR levels were analyzed by a χ² test. As summarized in Table

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1, the level of p-Ser727-STAT3 was shown to correlate significantly with both Cdk5 and p35 levels (both \( P < 0.0001, \chi^2 = 27.08 \) and 29.69, respectively; Table 1). These results again suggest that Cdk5 regulates STAT3 Ser727 phosphorylation in prostate cancer.

The correlation of p-Ser727-STAT3 and prostate cancer progression. Since p-Ser727-STAT3 might be an important factor for prostate cancer, the correlation of STAT3 Ser727 phosphorylation and prostate cancer progression was investigated. The correlation between p-Ser727-STAT3 level and Gleason score (specific scoring system for prostate cancer and provided by Biomax) was analyzed by \( \chi^2 \) test. Notably, the significant correlation between p-Ser727-STAT3 level and Gleason score (\( P = 0.0115, \chi^2 = 11.035; \) Table 2) was identified. These observations imply that Ser727 phosphorylation of STAT3 might play an important role in prostate cancer progression.

\[ \text{Cdk5 responds to STAT3 activation.} \] A previous study showed that the levels of nuclear p-Ser727-STAT3 are increased in breast cancer lesions and correlate to pathogenesis (46). In our data, Cdk5 inhibition reduced the level of p-Ser727-STAT3 in the nucleus of LNCaP cells, whereas the amount of total STAT3 protein was unaffected (Fig. 3A). JunB (\( \text{junB} \)), a gene targeted by STAT3 in response to cell proliferation (32), was upregulated in both levels of mRNA and protein after Cdk5 or p35 overexpression (Fig. 3B and C). Conversely, treatment with a Cdk5 inhibitor significantly decreased junB protein expression (Fig. 3D). In addition to \( \text{junB} \), we also found that the expressions of other STAT3-regulated genes, including survivin (18), \( \text{c-myc} \) (10), and \( \text{c-fos} \) (30), were all affected by Cdk5 activity (Fig. 3E-H). Taken together, our data reveal that STAT3 transactivation is modulated by Cdk5 activity in prostate cancer cells.

### Table 1. Correlations between p-Ser727-STAT3 and Cdk5 or p35 expression levels in human prostate cancer tissues

<table>
<thead>
<tr>
<th>Expression Level</th>
<th>p-Ser727-STAT3, n (%)</th>
<th>Total (n)</th>
<th>( P ) Value (( \chi^2 ))</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cdk5</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Negative, low</td>
<td>45 (41)</td>
<td>55</td>
<td>(&lt;0.0001(27.08)^*)</td>
</tr>
<tr>
<td>Moderate, high</td>
<td>10 (8.8)</td>
<td>55</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>55</td>
<td>110</td>
<td></td>
</tr>
<tr>
<td>p35</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Negative, low</td>
<td>32 (29)</td>
<td>55</td>
<td>(&lt;0.0001(29.69)^*)</td>
</tr>
<tr>
<td>Moderate, high</td>
<td>23 (21)</td>
<td>55</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>55</td>
<td>110</td>
<td></td>
</tr>
</tbody>
</table>

\( \text{Cdk5, cyclin-dependent kinase 5.} \) \( *P < 0.05, \) statistically significant.

### Table 2. Correlation between p-Ser727-STAT3 level and Gleason score in human prostate cancer tissues

<table>
<thead>
<tr>
<th>Gleason Score</th>
<th>p-Ser727-STAT3, n (%)</th>
<th>Total (n)</th>
<th>( P ) Value (( \chi^2 ))</th>
</tr>
</thead>
<tbody>
<tr>
<td>2–4</td>
<td>12 (11)</td>
<td>14</td>
<td>( 0.0115 (11.035)^*)</td>
</tr>
<tr>
<td>5–6</td>
<td>4 (3.6)</td>
<td>9</td>
<td>13</td>
</tr>
<tr>
<td>7–8</td>
<td>14 (13)</td>
<td>22 (20)</td>
<td>36</td>
</tr>
<tr>
<td>9–10</td>
<td>25 (23)</td>
<td>47</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>55</td>
<td>55</td>
<td>110</td>
</tr>
</tbody>
</table>

\( \text{\*}P < 0.05, \) statistically significant.

Fig. 2. Representative images of immunohistochemical staining. The scoring standard for the intensities of protein levels in the sections of patients’ specimens was listed and categorized as negative (0, 0%), low (1, 1–17%), moderate (2, 18–35%), and high (3, >35%) levels of 4 proteins, including p-Ser727-STAT3, AR, Cdk5, and p35. Representative fields for the intensities of the immunohistochemical staining are shown.
Furthermore, the data showed that the overexpression of S727A-mutated STAT3 significantly prevented LNCaP cell proliferation, whereas WT-STAT3 increased proliferation significantly (WT and S727A; Fig. 4). Treatment with a Cdk5 inhibitor significantly inhibited the proliferation stimulated by WT-STAT3 overexpression; however, combining expression of S727A-STAT3 mutant and Cdk5 inhibitor treatment did not cause any further decrease in proliferation (WT/ROSC and S727A/ROSC; Fig. 4). These results suggest that Cdk5 activity mediates prostate cancer cell proliferation by phosphorylating STAT3.

Cdk5 promotes the interaction between STAT3 and AR. STAT3 has been reported to positively activate AR (40). In Fig. 1C, we observed the biochemical interaction between p-S727-STAT3 and AR. These results suggest that Cdk5 activity regulates the interaction between STAT3 and AR.

Fig. 3. STAT3 activation is regulated by Cdk5. A: LNCaP cells were treated with ROSC (10 μM) for 24 h, and protein fractionation was performed. p-Ser727-STAT3 and STAT3 proteins were immunoblotted in both nuclear (N) and cytosolic (C) fractions. Poly(ADP)-ribose polymerase (PARP) and α-tubulin represent markers of N and C fractions, respectively. B and C: the levels of junB (STAT3-regulated gene) mRNA and junB proteins after Cdk5 or p35 overexpression in LNCaP cell lysates were evaluated by quantitative real-time PCR and immunoblotting, respectively. The control groups were transfected with pcDNA3 empty vector. The experiments (B) were repeated 3 times. Data are represented as means ± SE. D: the protein level of junB was examined after 24-h treatment with the Cdk5 inhibitor ROSC (10 μM) in LNCaP cells. The effects of Cdk5/p35 overexpression on expressions of survivin (E) and c-myc (F) and the effect of ROSC (10 μM, 24 h) treatment on c-fos expression (G) in LNCaP cells were evaluated by quantitative real-time RT-PCR, as described in MATERIALS AND METHODS. H: the protein levels of c-fos were evaluated by immunoblotting, as described in MATERIALS AND METHODS. The experiments were repeated 3 times, and data are represented as means ± SE. *P < 0.05, **P < 0.01 vs. control group.
Data are represented as means ± SEM. *P < 0.05 and **P < 0.01 vs. WT-STAT3 group.

DISCUSSION

Cdk5 has recently been shown to play numerous roles in nonneuronal human cells (11). Previously, we reported that Cdk5 stabilizes AR protein through phosphorylation and thereby promotes the growth of prostate cancer cells (15). In this study, we discovered that Cdk5 is a positive modulator to STAT3 activation, cell growth, and STAT3-dependent AR transactivation by phosphorylation of STAT3 on Ser727 on STAT3. Although Cdk5-dependent STAT3 regulation by phosphorylation has been shown in many studies (9, 12, 26, 34, 43), this study is the first one to investigate Cdk5 regulation of STAT3 Ser727 phosphorylation and activation in prostate cancer. Based on our findings, the regulation of Ser727 phosphorylation of STAT3 modulated by Cdk5 is involved in the proliferation of prostate cancer cells and will become an important target of future prostate cancer research.

STAT3 has been reported to be constitutively activated in various tumors (17) and functions as an oncogene by contributing to cell proliferation, cell cycle progression, cellular transformation, differentiation, immune responses, and prevention of apoptosis (5, 14, 21, 31, 41). Although Tyr705 phosphorylation by nonreceptor or receptor tyrosine kinases has been believed to be a prerequisite for STAT3 activation, the role of Ser727 phosphorylation of STAT3 in biological function is still controversial. The serine residue is located in a conserved PMSP (or PSP) motif within the COOH-terminal transcriptional activation domain of STAT3 (35) and has been reported to be phosphorylated by many kinases, such as Cdk5.
as Cdk5 (9, 12, 26, 34), Cdk1 (35), Erk (24, 25), p38 (45), Pin1 (29), PKC8 (19), and PKCε (2), upon exposure to extracellular stimuli. Several lines of evidence have indicated recently that Ser727 phosphorylation directly promotes STAT3 activation, anchorage-independent growth of noncancerous prostate epithelial cells (RWPE-1) and prostate cancer cells (LNCaP), prostatic tumorigenesis in NOD/SCID mice, and cell invasion in the absence of Tyr705 phosphorylation (33). According to our observations, Tyr705 phosphorylation of STAT3 in AR-positive prostate cancer cell lines was relatively weaker than Ser727 phosphorylation, and there is no obvious correlation between Cdk5 activation and STAT3 Tyr705 phosphorylation in prostate cancer cells (Fig. 1F). The role of STAT3 is thought to be that of conveying signals into the nucleus in combination with Cdk5 (12, 23). Our published results demonstrate that Cdk5-dependent Ser727 phosphorylation of STAT3 is important in promoting thyroid cancer growth (26). Moreover, a recent study indicates that Cdk5 prevents DNA damage through STAT3 Ser727 phosphorylation (9). These observations im-

Fig. 5. The interaction between STAT3 and AR is regulated by Cdk5-dependent Ser727 phosphorylation. A: the interaction among Cdk5, STAT3, and AR after ROSC (10 μM, 24 h) treatment in LNCaP cells or (B) p35 overexpression in PC3 cells was evaluated by IP with anti-AR antibody. C: the protein interaction between AR and STAT3 after p35 overexpression or knockdown was analyzed by immunoprecipitating AR proteins in LNCaP cells. D: the interactions between WT-STAT3 or S727A-STAT3 mutant and AR after transient expression of the aforementioned proteins were evaluated by IP with anti-AR antibody in 22Rv1 and Chinese hamster ovary (CHO) cells. The inputs were evaluated by immunoblotting. E and F: the interactions between exogenous FLAG-tagged WT-AR or S81A-AR mutant with STAT3 were evaluated by IP with FLAG antibody in LNCaP and DU145 cells.
Fig. 6. The protein phosphorylation, stability, and transactivation of AR are mediated by STAT3 through Ser727 phosphorylation. 

A: Ser81 phosphorylation status was evaluated after overexpressing WT-STAT3 or S727A-STAT3 mutant in LNCaP cells. B: LNCaP cells were treated with cycloheximide (CHX; an inhibitor of protein synthesis, 10 μg/ml) for 0, 4, or 7 h, and AR protein degradation was monitored by immunoblotting after the overexpression of WT-STAT3 or S727A-STAT3 mutant. C: the quantitative results revealed the mean percentages of AR stability compared with respective time = 0 groups (the value is 1). The independent experiments were repeated 4 times. Data are represented as means ± SE; *P < 0.05 vs. WT-STAT3-overexpressed group. D: the ubiquitination of exogenous AR was detected after overexpressing WT-STAT3 or S727A-STAT3 mutant by IP with anti-AR antibody. MG132 (proteasome inhibitor; 5 μM, 6 h) was used to block the proteasome-dependent degradation. E and F: mouse mammary tumor virus (MMTV) luciferase (black bars) and 3XARE (androgen response element)-luciferase (open bars) reporter assays were performed in LNCaP (E) and 22Rv1 cells (F) after WT-STAT3 or S727A-STAT3 overexpressions. The expression of β-galactosidase served as the internal control. Data are represented as means ± SE; #P < 0.05 vs. WT-STAT3-overexpressed group by using MMTV-luciferase expression plasmid and **P < 0.01 vs. WT-STAT3-overexpressed group by using 3XARE-luciferase expressing plasmid. G: the levels of PSA (AR-regulated gene) protein after WT-STAT3 or S727A-STAT3 mutant overexpressions in LNCaP cells were evaluated by immunoblotting.
CDK5 REGULATES STAT3/AR IN PROSTATE CANCER CELLS

Table 3. Correlation between p-Ser727-STAT3 and AR expression levels in human prostate cancer tissues

<table>
<thead>
<tr>
<th>Expression Level (AR)</th>
<th>p-Ser727-STAT3, n (%)</th>
<th>P Value (χ²)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Negative, low</td>
<td>35 (32)</td>
<td></td>
</tr>
<tr>
<td>Moderate, high</td>
<td>20 (18)</td>
<td></td>
</tr>
<tr>
<td>Total (n)</td>
<td>55</td>
<td>&lt;0.0001 (32.70)*</td>
</tr>
</tbody>
</table>

AR, androgen receptor. *P < 0.05, statistically significant.

...ple that the regulation of Cdk5-dependent STAT3 Ser727 phosphorylation is a relevant finding in cancer biology.

In this study, we first demonstrate Cdk5 activity-dependent STAT3-Cdk5 interaction and Ser727 phosphorylation of STAT3 in prostate cancer cells and 22Rv1 xenograft tumor cells (Fig. 1). Significant correlations of p-Ser727-STAT3 levels with Cdk5 or p35 expression in clinical specimens of prostate cancer were also shown (Table 1). This evidence suggests that p-Ser727-STAT3 levels were mediated by Cdk5 in prostate cancer. In regard to the function of STAT3 transactivation, our data show that inhibition of Cdk5 by ROSC treatment decreased only the level of p-Ser727-STAT3 in the nucleus but not total STAT3 levels (Fig. 3A). We further examined the expression of several STAT3-targeted genes related to cell proliferation and confirmed that Cdk5 does promote STAT3 transactivation (Fig. 3, B–H). Therefore, we infer that Cdk5 primarily affects nuclear STAT3 by phosphorylation, in turn promoting cell proliferation (Fig. 4). The experiment in Fig. 4 was performed by LNCaP cells that contain a relatively high basal level of phospho-Ser727-STAT3 compared with other prostate cancer cell lines in our previous observation. This phenomenon suggests that LNCaP cells might rely more on p-Ser727-STAT3-related signaling than other cell lines. It is hard to obtain more active STAT3 by overexpression since the basal activation of STAT3 has already been high. This might explain why STAT3 overexpression in LNCaP cells induced only a 10% proliferation increase. On the other hand, overexpression of the S727A-STAT3 mutant dominant negatively reversed the function (cell proliferation) of endogenous STAT3 (S727A; Fig. 4), which supports the above explanation. Furthermore, combining overexpression of the S727A-STAT3 mutant and ROSC treatment did not cause further inhibition of proliferation compared with WT-STAT3 overexpression with ROSC treatment (WT/ROSC and S727A/ROSC; Fig. 4). These results again suggest that STAT3 serves as a downstream signaling protein of Cdk5 in the regulation of cell proliferation.

It has been shown that the biochemical interaction of STAT3 with AR can be enhanced by IL-6 challenge (7). In addition, the STAT3 S727A mutant significantly blocks IL-6-induced AR transactivation and STAT3-AR complex formation (1). Therefore, Cdk5 regulation on the interaction between AR and STAT3 becomes interesting to investigate. The results show that the coimmunoprecipitation of Cdk5, AR, and STAT3 molecules was observed (Figs. 1, A and C, and S, A, B, and E). Besides, our data indicate that Cdk5 activation is important to the interaction between STAT3 and AR (Fig. 5, A–C). The STAT3 S727A mutant diminished its binding to AR (Fig. 5D). These findings suggest that S727 phosphorylation of STAT3 plays an essential role in the interaction between STAT3 and AR in prostate cancer cells, and this phenomenon is controlled by Cdk5. Additionally, the data in Fig. 3A reveal that Cdk5 phosphorylates STAT3 in the nucleus. Thus, we assume that p-Ser727, STAT3 proteins would also interact with AR proteins in the nucleus. Interestingly, a slight increase in Tyr705 phosphorylation was observed in the S727A-STAT3 mutant (Fig. 5D), implying that there might be a compensatory effect between Tyr705 and Ser727 phosphorylation in prostate cancer cells. On the other hand, our previous report indicates that Ser61 of AR is a Cdk5-phosphorylated site and important to AR function (15). Interestingly, distinct from the results in Fig. 5D, the interaction between STAT3 and the S81A-AR mutant was significantly increased compared with that of STAT3 and WT-AR (Fig. 5, E and F). These results suggest that STAT3 might activate mutant AR by increasing their interaction in a negative feedback manner, whereas Cdk5-dependent AR activation (15) is blocked by S81A-AR mutant. Finally, since Ser61 phosphorylation contributes to AR stability and activation (15), we found that AR protein stability, AR transactivation, and the expression of the AR-regulated gene (PSA) were all regulated by Ser727 phosphorylation of STAT3 (Fig. 6). Qin et al. (33) demonstrated that high levels of STAT3 Ser727 phosphorylation are observed in malignant prostate specimens. Table 2 and our unpublished data also indicate that p-Ser727-STAT3, p35 (Cdk5 activator), and AR proteins all correlated with the Gleason score in prostate cancer patients. Consistent with the clinical observations, we discovered that protein levels of Cdk5, p35, AR, and p-Ser727, STAT3 were all higher in androgen-independent LNCaPVec cells [screened from LNCaP cells in androgen-deprived conditions (15)] than in parental LNCaP cells (unpublished data). Altogether, our clinical evidence and our in vitro data show that Cdk5-dependent STAT3 Ser727 phosphorylation plays an important role in the transition of androgen requirement of prostate cancer cells and prostate cancer progression.

In conclusion, our results illustrate that Cdk5 activity contributes to the proliferation of prostate cancer cells by directly regulating STAT3 function through Ser727 phosphorylation and indirectly mediating AR activation through p-Ser727, STAT3 interaction. These findings lead us to hypothesize that a Cdk5-STAT3-AR axis plays a decisive role in the development and progression of prostate cancer and will become a research target or a diagnostic and therapeutic target in the near future.

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DISCLOSURES

The authors have no conflicts of interest, financial or otherwise, to declare.
AUTHOR CONTRIBUTIONS
F.-N.H., M.-C. Chen, Y.-T.P., P.-C.L., and M.-C. Chiang prepared the experiments; F.-N.H., M.-C. Chen, K.-C.L., Y.-T.P., E.L., and H.L. analyzed the data; F.-N.H., E.L., and H.L. interpreted the results of the experiments; F.-N.H. prepared the figures; F.-N.H. drafted the manuscript; M.-C. Chen, E.L., and H.L. contributed to the conception and design of the research; M.-C. Chen, J.-T.H., and H.L. edited and the revised manuscript; H.L. approved the final version of the manuscript.

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