Cell Cycle and Senescence

Androgen Receptor Promotes the Oncogenic Function of Overexpressed Jagged1 in Prostate Cancer by Enhancing Cyclin B1 Expression via Akt Phosphorylation

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Abstract

The Jagged1, a Notch signaling pathway ligand, had been shown to have a positive correlation with prostate cancer development. Our study for Jagged1 expression in 218 prostate cancer tissue samples also supports this conclusion. However, the detailed molecular mechanism of Jagged1 in promoting the progression of prostate cancer is still unclear. Through cell proliferation examination, androgen receptor (AR) was found to promote the oncogenic function of Jagged1 to enhance the cell proliferation rate by comparing four prostate cancer cell lines, LNCaP, LAPC4, DU145, and PC3, which was further validated through analyzing the survival of 118 patients treated with androgen-deprivation therapy (ADT) with different expression levels of Jagged1 and AR. More importantly, our data showed that Jagged1 combined with AR could increase the phosphorylation level of Akt and, in turn, phosphorylated Akt plays an important role in regulating the expression level of cyclin B1 by interacting with AR and increasing the transcriptional activity of AR. These data indicate that prostate cancer progression regulated by Jagged1 can be dramatically enhanced by combining with AR through promoting Akt activity.

Implications: This study could benefit our clinical treatments for patients with prostate cancer with overexpressed Jagged1 by targeting AR and Akt. Mol Cancer Res; 12(6); 830–42. ©2014 AACR

Introduction

Prostate cancer is one of the most commonly diagnosed noncutaneous cancers and a leading cause of cancer-related deaths in American men (1). Although several new therapeutics have been approved, efficient treatments still lack for the advanced prostate cancer, such as castration-resistant prostate cancer (CRPC) and prostate cancer with metastasis (2). To obtain better outcomes for patients with prostate cancer, there is an urgent need for better understanding the detailed molecular pathways in the progression of prostate cancer to develop some new treatments. Prostate cancer is an androgen-dependent disease, and these actions are dependent on the androgen receptor (AR). AR is expressed in many prostate cancer cells and has been proved to play a significant role in the tumorigenesis and metastasis of prostate cancer (3, 4). Inhibiting the activities of AR is an important direction in the treatment of primary prostate cancer to prevent tumor progression (5, 6). A large body of evidence implicates that androgen signaling is modified after androgen-deprivation therapy (ADT), and AR overexpression is the major cause of CRPC (7–10). That is, AR still plays a critical role in CRPC development.

Except for AR, proteins that participate in a variety of signaling pathways can also affect prostate cell death or survival (11). Many cell signaling pathways play different roles in the development and progression of prostate cancer, such as NF-κB, JAK/STAT, MAPK, Wnt, PI3K/Akt, and Notch signaling. Among these, the Notch signaling pathway, which is present in most multicellular organisms, plays a role in cancer metastasis by governing embryonic development, maintaining tumor stemness, modulating the epithelial-to-mesenchymal transition (EMT), and affecting tumor angiogenesis processes (12–14). Several studies have demonstrated that the Notch signaling pathway is required for prostatic growth and plays a key role in the progression and metastasis of prostate cancer (15–17). It had been proved that the expression of both Notch receptors and their ligands would be upregulated in many prostate cancer cells compared with normal prostate cells (18–21).

In mammals, except for the four Notch receptor members (Notch1-4), five Notch ligands have been identified so far, including Delta-like 1/3/4 (Dll 1/3/4) and Jagged1/2 (17, 22). Jagged1 was found to be highly expressed in most prostate cancer cells and increased in localized prostate cancer compared with benign prostate hyperplasia in the

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Jagged1 and AR in Prostate Cancer

human prostate tumor microarray study (23–27). Moreover, a positive correlation between Jagged1 and prostate-specific antigen (PSA) recurrence after radical prostatectomy has been found (17), which suggests that high jagged1 protein expression is a strong predictor of prostate cancer recurrence. In addition, synthetic androgen R1881 can enhance Jagged1 expression in LNCaP cells, and knockdown of Jagged1 expression would inhibit LNCaP cell proliferation (25, 28), suggesting that Jagged1-mediated Notch activation of LNCaP cells plays an oncogenic role in promoting cell proliferation. Downregulation of Jagged1 has been shown to have the same property in the other prostate cancer cell lines, DU145 and PC3, through a mechanism that regulates CDK2 and p27 activities (19, 28). Therefore, growth inhibition of prostate cancer cells by downregulated Jagged1 indicates that Jagged1 could be an important target in the Notch signaling pathway for the treatment of prostate cancer.

In this study, we focused on four prostate cancer cell lines, LNCaP, LAPC4, DU145, and PC3, to further disclose the activity of Jagged1 in these cells and examined how the Notch signaling pathway can stimulate the progression of prostate cancer through Jagged1. We provide, for the first time, that tumor progression functions of the Notch signaling pathway in prostate cancer can be elevated by AR through Jagged1 by enhancing cyclin B1 expression via Akt modification.

Materials and Methods
RNA extraction from tissue samples of patients and survival analysis
A total of 218 samples were obtained from patients treated by radical prostatectomy at the Xinhua Hospital, Shanghai, China. All patient-provided samples were procured, and the study was conducted according to the Xinhua Hospital Review Board approval. All tissue samples were collected during surgery and stored at −80°C. Hematoxylin and eosin–stained cryostat section from each tumor sample was assessed to identify the regions of tumor free from contaminating stroma. Neoplastic and nonneoplastic epithelia were manually dissected from the frozen block. Tumor samples with greater than 70% cancer cells on histologic assessment were used for RNA extraction. Total RNAs were isolated from frozen tissue using TRIzol Reagent (Invitrogen). RNA with an A260/A280 ratio > 1.8 and concentration > 100 ng/µL was accepted.

Survival information for patients treated with ADT (either surgical or chemical castration) was tracked for a maximum of 5 years, starting from the time of ADT treatment. Since 2005, we have collected data of 118 patients for this survival study. After obtaining Jagged1 and AR mRNAs expression levels by Real-time-PCR (RT-PCR), we divided the patients into four different groups according to these two genes, with upper 50% (H) or lower 50% (L) of expression levels: Jagged1 (L)AR (L), Jagged1 (L)AR (H), Jagged1 (H)AR (L), and Jagged1 (H)AR (H). The survival percentage was estimated using the Kaplan–Meier method, with 95% confidence intervals (CI). To test the association between different patient groups, the log-rank test was used. P value for trend was also analyzed.

Cell lines and culture conditions
The human prostate cancer cell lines LNCaP, LAPC4, DU145, and PC3 were purchased from the American Type Culture Collection (ATCC) and maintained according to the ATCC protocol. Briefly, LNCaP, LAPC4, and DU145 cells were maintained in RPMI-1640 supplemented with 10% FBS. PC3 was maintained in Dulbecco’s Modified Eagle Medium (DMEM) plus 10% FBS. Penicillin and streptomycin (100 µg/mL) were added into all culture media, and all cells were cultured at 37°C in 5% CO2.

Plasmids and transfection
The Jagged1 and AR cDNA plasmids were purchased from OriGene, and the Jagged1 siRNA and siRNA control were purchased from Dharmacon. The luciferase reporter pG5-3 was purchased from Promega. All cells were transfected with plasmids or siRNA by using Lipofectamine 2000 according to the manufacturer’s instructions with some modifications. Briefly, cells were trypsinized completely to avoid the cell clump before transfection. For LNCaP and LAPC4 cells, half amount of Lipofectamine 2000 and DNA was used to reduce the toxicity totally. Half of the transfection material was used for first transfection for 4 hours. Then, cells were transferred to fresh medium and incubated for 2 hours. Finally, second transfection was performed overnight, and cells were transferred to fresh medium the next day.

Cell proliferation studies by WST-1 assay
The transfected prostate cancer cells, LNCaP (4 × 103), LAPC4 (4 × 103), DU145 (1 × 103), and PC3 (1 × 103), were seeded in a 96-well culture plate. After incubation for 2, 4, and 6 days, the cells were incubated with 10% cell proliferation reagent WST-1 (Roche Applied Science) in FBS for 1 hour at 37°C. The absorbance at day 0 was considered as baseline and labeled as 100%. Akt inhibitor MK-2206 was purchased from Selleckchem and was added into the cell culture medium at day 0 for the WST-1 assay. The results were presented as the mean ± SD of at least three independent experiments.

RT-PCR analysis for gene expression
The total RNA from transfected cells was isolated by TRIzol (Invitrogen) according to the manufacturer’s protocol. Of note, 200 ng of total RNA from each sample was used to synthesize the first-strand cDNA by the TaqMan RT Reagents Kit (Applied Biosystems) with random hexamers in a total volume of 40 µL. All primers were synthesized by ShineGene Molecular Biotech, Shanghai, for the PCR amplifications and are listed in Supplementary Table S1. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was included for every sample as the control reaction. The results were presented as the mean ± SD of at least three independent experiments.
Western blot analysis, immunoprecipitation, and tissue immunohistochemistry

After transfection for 3 days (for MK-2206 experiment), the inhibitor was added to the cultured medium 1 day after transfection, cells were washed twice with cold PBS, and then lysed for 30 minutes with RIPA cell lysis buffer (Gibco-BRL) containing the protease inhibitor cocktail (AMRESCO). After centrifugation, protein concentration in the clear cell lysis was measured by the Bio-Rad BCA assay. Eighty micrograms of proteins was resolved by 8% to 12% polyacrylamide gels and transferred onto 0.45-μm nitrocellulose membranes (Millipore). The membrane was blocked with 5% milk in PBS for 1 hour and then incubated with the primary antibodies (Supplementary Table S2) at 4°C overnight. After washing three times with PBS, the blots were incubated with an secondary antibodies. The blots were developed with an enhanced chemiluminescence reagent (Amersham) and image analyzer system.

For immunoprecipitation experiments, 800 μg of total proteins was incubated with protein A/G agarose and 2 μg of AR antibody for each immunoprecipitation. After overnight immunoprecipitation at 4°C, protein A/G agarose was washed four times with lysis buffer and, finally, one time with PBS buffer. Loading buffer (40 μL, 1×) was added to the protein A/G agarose, boiled, and subjected to SDS-PAGE for Western blot analysis.

Tissue immunohistochemical analysis was performed as described in ref. (26).

Cell-cycle analysis

After transfection for 3 and 6 days, cells were trypsinized and washed twice with cold PBS. Cells were fixed with ice-cold 80% ethanol for 2 hours and then washed twice with cold PBS. Fixed cells were completely resuspended in PBS and stained with a 2-mg/mL propidium iodide solution for half an hour at room temperature in the dark. Fluorescence sorting was measured by flow cytometry in the Shanghai Jiao Tong University School of Medicine.

Luciferase reporter assays

Primers with restriction enzyme sites KpnI/BglII were designed to amplify the promoter fragment of cyclin B1 from genomic DNA with a length of 197 bp, and the positive control was 2xARE sequence (Supplementary Table S1; refs. 29, 30). LNCaP cells were transfected with the luciferase reporter constructs, and the Renilla luciferase expression construct was used as a control. Cells were harvested 48 hours after transfection and were assayed with Dual-Luciferase assays according to the manufacturer’s instructions.

Chromatin immunoprecipitation

Chromatin immunoprecipitation (ChIP) was performed as described previously (31). Briefly, cells were cross-linked with 1.5% formaldehyde solution at room temperature for 10 minutes and then stopped by the addition of glycine to a final concentration of 125 mmol/L. The cells were then sonicated for 10 30-second cycles on ice. After sonication, the solution was checked by agarose DNA gel to ensure that the average size of DNA fragments was approximately 500 bp. Five micrograms of anti-mouse AR antibody or normal anti-mouse IgG (immunoglobulin G) was added to 1 mL of clear supernatant (save 100 μL of clear supernatant as 10% total input control) and incubated at 4°C overnight on a rotating device. Then, we added 50 μL of protein A/G beads to the samples and rotated for 30 minutes at 4°C. The beads were washed four times with 1 mL of wash buffer (50 mmol/L HEPES, pH 7.9, 500 mmol/L NaCl, 1 mmol/L EDTA, 0.1% SDS, 1% Triton X-100, and 0.1% deoxycholate) and two times with 1 mL of TE buffer. Finally, we added proteinase K (20 μg/μL) and incubated at 55°C for 2 hours and then incubated at 65°C overnight to reverse cross-links. DNA in the supernatant was purified by spin columns and analyzed by RT-PCR with the primer sets targeting the cyclin B1, PSA, and TMPRSS2 promoters (Supplementary Table S1).

Statistical analysis and data presentation

The Student t test was used to evaluate the difference between different data, and P value less than 0.05 was considered significant. In this article, the control samples among nontreatment, siRNA control, empty-vector control, and DMSO (dimethyl sulfoxide) vehicle treatment showed similar results, and we only presented one of them to simplify the data.

Results

Expression level of Jag1 has a positive correlation with the progression of prostate cancer

Jagged1 and its coding gene, Jag1, were shown, in many studies, to have a closed correlation with prostate cancer (23–28), which suggests that Jagged1 functions to promote prostate cancer development. Therefore, we further analyzed Jag1 expressions in prostate cancer tissue samples and normal prostate tissue samples to examine whether Jag1 has a significant higher expression level in advanced prostate cancer. Jag1 expression levels were examined in a total of 218 tissue samples from the Xinhua Hospital, Shanghai, China, which included 130 primary prostate cancer samples, 51 prostate cancer metastasis samples, and 37 matched normal prostate samples (34 normal prostate samples matched to primary prostate cancer samples and three normal prostate samples matched to the metastasis samples; Fig. 1). Our data further proved that the mRNA expression level of Jag1 correlates with the progression of prostate cancer in patients, with the highest expression level in metastasis samples. Significant differences exist between primary tumor or metastasis samples and normal samples, with P values of 0.0012 and <0.0001, respectively. mRNA expression levels of Jag1 even have a significant difference between primary and metastasis tumor samples with a P value of 0.0086 in the study. Considering these data, we plan to continue our study to figure out which genes or pathways are involved in Jagged1 oncogenic function during prostate cancer progression.
AR promotes the oncogenic function of overexpressed Jagged1

To investigate the potential tumor promotion function of overexpressed Jagged1 in prostate cancer, we first measured prostate cancer cell proliferation with different Jagged1 expression levels in four prostate cancer cell lines: LNCaP, LAPC4, DU145, and PC3. The LNCaP cell has the highest Jagged1 expression level and DU145 cell has the lowest expression level (Fig. 2A, lanes 1, 4, 7, and 13). Jagged1 can be successfully knocked down by siRNA transfection in all these cells (Fig. 2A, siJag1). Western blot analysis showed that AR was successfully overexpressed in cells, and overexpressed AR had almost no effect on the expression level of Jagged1 under each condition. We found that overexpressed AR alone increased DU145 and PC3 cell proliferation rates only to a small degree, and overexpressed AR combined with knocked down Jagged1 decreased their cell proliferation rates still to about 50% of the control (Fig. 2C). However, overexpressed AR combined with overexpressed Jagged1 significantly increased cell proliferation rates in both DU145 and PC3 cells to a level similar to that of the LNCaP cell with overexpressed Jagged1 (Fig. 2C). Because overexpressed AR does not affect the expression level of Jagged1, it suggests that AR together with Jagged1 possibly further activates the downstream targets to promote prostate cancer cell growth. Taken together, our results indicate that AR is required for prostate cancer cell proliferation enhanced by overexpressed Jagged1.

We next examined whether AR promotes the oncogenic function of Jagged1 in patients with prostate cancer to affect the survival. Of the 130 patients with primary prostate cancer, 118 patients who have the follow-up records treated with ADT were divided into four groups, as mentioned in Materials and Methods, according to Jagged1 and AR expression levels (Fig. 2D). More importantly, we showed examples that upregulated Jagged1 and AR mRNA in patients were translated to elevated protein levels by immunohistochemical analysis (Fig. 2E). High expression levels of AR in patients with low Jagged1 expression levels only decrease the median survival time from 36.30 to 32.30 months compared with that of low expression levels of AR (Fig. 2F and Table 1). However, patients with high Jagged1 expression levels show significant decrease in median survival time from 30.03 to 17.93 months between two groups of low and high AR expression levels, respectively (Fig. 2F; Table 1; P < 0.05). The percentage of survival decreases rapidly around the time of 1 year for the group of patients with both Jagged1 and AR overexpression. Therefore, AR promotes the oncogenic function of overexpressed Jagged1 in both tissue culture cells and patients with prostate cancer.

Jagged1 together with AR enhances the expression of cyclin B1

Prostate cancer cell proliferation rates affected by Jagged1 could be due to cell-cycle modification. We then analyzed the cell-cycle distribution by fluorescence-activated cell sorting (FACS) analysis among LNCaP, LAPC4, DU145, and PC3 cell lines. As shown in Fig. 3A, more cells are arrested in the S phase after knocking down Jagged1 in all of the four cells compared with control after transfection for 3 days, which explains the reason that cell proliferation rates decrease as mentioned in Fig. 2B. Although overexpression of Jagged1 significantly promotes LNCaP and LAPC4 cells entering the G2–M phase (Fig. 3A), DU145 and PC3 cells do not show this effect by overexpressed Jagged1 compared with control (Fig. 3A). We have shown that AR can cooperate with Jagged1 to promote DU145 and PC3 cell proliferation.
Figure 2. Cell proliferation rates of LNCaP, LAPC4, DU145, and PC3 cells and survival analysis for patients with different expression levels of Jagged1 and AR. A, Western blot analysis of Jagged1 and AR in LNCaP, LAPC4, DU145, and PC3 cells. All cell lysis was analyzed after 3 days of transfection. Control samples (lanes 1, 4, 7, and 13) only showing siRNA control and empty-vector transfections with almost the same results were not shown. β-Actin was used as a control for equal loading. siJag1, knocking down Jagged1 by siRNA; oeJag1, overexpression of Jagged1 by Jagged1 cDNA transient transfection; oeAR, overexpression of AR by AR cDNA transient transfection. B, cell proliferation curves of LNCaP, LAPC4, DU145, and PC3 cells with knocked down Jagged1 or overexpressed Jagged1 were measured by WST-1 assay over a 6-day time course. Data, mean ± SD. * P < 0.05. C, cell proliferation curves of DU145 and PC3 cells with AR overexpression combined with Jagged1 knockdown or overexpression were measured as in B. D, relative mRNA expression levels of Jagged1 and AR for a total of 118 patients with prostate cancer treated with ADT were measured by RT-PCR and separated for each gene to two parts: the upper 50% (H) and the lower 50% (L) of expression level. Patients were divided into four groups. E, Jagged1 and AR immunostaining in prostate cancer tissue samples from two patients: 1 patient with low expressions (left) and the other patient with high expressions of Jagged1 and AR (right). Magnification, ×400. F, Kaplan–Meier curves of percentage survival after ADT treatment for four groups of patients with prostate cancer with different Jagged1 and AR expression levels.
proliferation rates (Fig. 2C); therefore, we determined whether AR can increase the cell numbers in the G2–M phase when combined with Jagged1. Interestingly, overexpressed Jagged1 and AR together enhance the G2–M phase cell numbers significantly to a level similar to that of LNCaP/LAPC4 in both DU145 and PC3 cells (Fig. 3B). Cell-cycle analysis at day 6 after transfection also showed results similar to those at day 3, although cells entering the G2–M phase promoted by overexpressed Jagged1 decreased, which may be due to decreased Jagged1 expression level after long transfection time (Supplementary Fig. S1A and S1B). These studies suggest that AR can promote overexpressed Jagged1 to stimulate prostate cancer cells to enter the G2–M phase during the cell cycle and increase the cell proliferation rate.

To detect which cell-cycle genes could be affected by Jagged1 and AR, we analyzed the G1, S, and G2–M cell-cycle key genes, CDK4 and cyclin D1, CDK2 and cyclin A2, and CDK1 and cyclin B1, respectively, in these four cells. For the DU145 cell, knocking down Jagged1 decreases the S phase cell-cycle genes, especially for CDK2, whereas overexpressed Jagged1 only significantly increases CDK1 expression in the G2–M phase, not cyclin B1 (Fig. 3C). CDK and cyclin in the cell cycle need to form a complex to perform their active functions (32). That is, the cell cycle can be enhanced by increasing coupled CDK and cyclin expression levels together, while the expression level decreased by only one of them will induce the cell-cycle arrest in the correspondent phase, which explains why the cell proliferation rate can be decreased by knocking down Jagged1 (S-phase arrest) and can be increased by overexpressed Jagged1 only to a small level in DU145 cells. Although overexpressed AR together with Jagged1 knockdown has no further effect on the cell-cycle genes, it can remarkably increase the expression level of cyclin B1 when we increase Jagged1 expression. PC3 cells also showed results similar to those of DU145 cells (Supplementary Fig. S1C). We had tried to knock down AR in LNCaP cells to examine whether decreasing AR expression could downregulate the G2–M cell-cycle genes. Unfortunately, LNCaP cells are not healthy without AR expression and, therefore, we did not continue to examine the outcomes of LNCaP cells with AR knockdown. However, knockdown or overexpression of Jagged1 in LNCaP/LAPC4 cells affects the expression of cell-cycle genes CDK2 and CDK1/cyclin B1, respectively, which are similar to that of DU145/PC3 cells with AR overexpression (Fig. 3C and Supplementary Fig. S1C). Taken together, it indicates that AR promotes the function of overexpressed Jagged1 in increasing the cell proliferation through activating cyclin B1 and enhancing cell numbers in the G2–M phase.

Except for cell line studies, we also examined whether the expression level of cyclin B1 correlates with Jagged1 and AR in our clinical samples. Expression levels of cyclin B1 from the 118 patients mentioned above were measured by RT-PCR, and we found that patients with high expression levels of Jagged1/AR have significantly higher cyclin B1 expressions, whereas lower cyclin B1 expressions are associated with patients of low expression levels of Jagged1/AR (Fig. 3D). Therefore, Jagged1 together with AR enhances the expression of cyclin B1 in patients with prostate cancer.

### Jagged1 together with AR enhances the expression level of cyclin B1 by activating Akt

The PI3K/Akt pathway plays an important role in regulating prostate cancer cell proliferation (33–36), and the Notch signaling pathway has been shown to activate Akt (37, 38). It is, therefore, reasonable to examine whether the activity of Akt can be enhanced by Jagged1 together with AR, and whether some relationships exist among them to enhance the cyclin B1 expression level as determined in Fig. 3. First, we examined the level of active form of Akt (phosphorylated Akt, p-Akt) by analyzing DU145, LNCaP, and LAPC4 cells with different expression levels of Jagged1 or AR. We found that p-Akt has a positive relationship with the expression level of Jagged1 and AR, but the total amount of Akt cannot be affected by Jagged1 and AR (Fig. 4A). More importantly, the level of p-Akt is obviously increased in DU145 cells with co-overexpression of Jagged1 and AR (Fig. 4A). This result indicates that Jagged1 and AR together can greatly enhance the phosphorylation level of Akt.

To study the effect of p-Akt in prostate cancer cells, we applied MK-2206, an Akt inhibitor, by suppressing Akt phosphorylation (39), to the DU145, LNCaP, and LAPC4 cells with overexpressed Jagged1 or AR (Fig. 4B). MK-2206

### Table 1. Comparison of survival for patients with prostate cancer (n = 118) with different Jagged1 and AR expression levels

<table>
<thead>
<tr>
<th>Group</th>
<th>No.</th>
<th>Median survival (months)</th>
<th>HR (95% CI)</th>
<th>P log-rank (Mantel-Cox)</th>
<th>P log-rank for trend</th>
</tr>
</thead>
<tbody>
<tr>
<td>Jagged1(L)AR(L)</td>
<td>32</td>
<td>36.30</td>
<td>0.8599 (0.4714–1.5690)</td>
<td>0.6226</td>
<td></td>
</tr>
<tr>
<td>Jagged1(L)AR(H)</td>
<td>27</td>
<td>32.10</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Jagged1(H)AR(L)</td>
<td>27</td>
<td>30.03</td>
<td>0.5428 (0.3049–0.9661)</td>
<td>0.0378ab</td>
<td>0.0105a</td>
</tr>
<tr>
<td>Jagged1(H)AR(H)</td>
<td>32</td>
<td>17.93</td>
<td></td>
<td></td>
<td>0.0041b</td>
</tr>
</tbody>
</table>

Abbreviations: H, high expression level; L, low expression level.

*P < 0.05.

abP < 0.01.
(1.0 μmol/L) in culture medium can successfully block the phosphorylation of Akt to an undetectable level without changing the total expression level of Akt (Fig. 4B, lanes 5–8, 11, 12, 15, and 16). Consistent with previous results, the expression level of cyclin B1 could not be affected by overexpressed Jagged1, whereas overexpressed AR increases the expression level of cyclin B1. However, co-overexpression of Jagged1 and AR increases the expression of cyclin B1 to a higher level. In addition, an increase in cyclin B1 expression level was also observed in AR-positive LNCaP and LAPC4 cells with Jagged1 overexpression. However, cyclin B1 expression level decreased when MK-2206 was applied to the cells even with both Jagged1 and AR overexpression. This finding suggests that cyclin B1 expression level is enhanced by Jagged1 and AR through p-Akt. From Fig. 4B, it is clear that the phosphorylation level of cyclin B1 has a positive correlation with both the expression level of cyclin B1 and the phosphorylation level of Akt, which suggests that Akt phosphorylation may affect the phosphorylation level of cyclin B1 in some degree, which needs further studies. In addition, we also examined the expression and phosphorylation levels of cyclin B1 in LNCaP and LAPC4 cells (Fig. 4B, lanes 9–16), which further confirmed that overexpressed Jagged1 together with AR can activate the downstream

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**Figure 3.** Cell cycle and cell-cycle genes affected by Jagged1 and AR. Each experiment was performed in triplicate, and data represent the mean ± SD at day 3 after transfection. A, changes in cell-cycle distribution analyzed by flow cytometry after knocked down or overexpressed Jagged1 after 3 days in LNCaP, LAPC4, DU145, and PC3 cells. B, cell-cycle analysis for DU145 and PC3 cells in overexpressed AR combined with Jagged1 knockdown or overexpression. C, cell-cycle genes were analyzed by RT-PCR with different combinations of Jagged1 and AR expression levels in DU145 and LNCaP cells. D, relative mRNA expression levels of cyclin B1 for a total of 118 patients with prostate cancer treated with ADT were measured by RT-PCR in four groups according to Jagged1 and AR expression levels. The paired t test was performed for statistical analysis. *, P < 0.05; **, P < 0.01; ***, P < 0.001.
target, Akt; as a result, cyclin B1 expression and phosphorylation levels are increased.

To validate whether activated Akt regulated by Jagged1 and AR is required for the enhancement of cyclin B1 to promote the cell proliferation rate in prostate cancer cells, we detected the cell proliferation rate of DU145 cells with 1.0 μmol/L MK-2206 and overexpressed Jagged1 and AR (Fig. 4C). We found that the increased cell proliferation rate was blocked by MK-2206 by the overexpression of both Jagged1 and AR. As anticipated, LNCaP and LAPC4 cell proliferation rates were also inhibited by MK-2206, even though Jagged1 was overexpressed in cells (Fig. 4C). Taken together, our results show that Jagged1 together with AR can coactivate Akt, which enhances the G2–M cell-cycle gene cyclin B1 expression and promotes prostate cancer growth.

**p-Akt increases cyclin B1 expression by enhancing AR recruitment to the promoter of cyclin B1**

Our data have shown that the expression level of cyclin B1 can be upregulated by Akt phosphorylation when Jagged1
and AR are overexpressed in prostate cancer cells. Li and colleagues found that transactivation of cyclin B1 in LNCaP cell was increased by androgen and AR (40). We assumed that AR transcription activity for cyclin B1 might be enhanced by overexpressed Jagged1 through Akt phosphorylation. To examine this possibility, we studied cyclin B1 expression in LNCaP cells by dihydrotestosterone (DHT) induction with or without Jagged1 overexpression and in the presence or absence of MK-2206. LNCaP cells with different expression levels of Jagged1 and treated with MK-2206 or DMSO were cultured in an androgen-depleted culture medium for 2 days. The cells were then treated with DHT (or not) for additional 1 day, and then cyclin B1 expression level was analyzed. The cyclin B1 expression level increased about 5-fold by DHT in LNCaP cells for the control (DMSO; Fig. 5A). Consistent with previous results (Fig. 3C), cyclin B1 expression level is further increased by overexpressed Jagged1 in AR-positive LNCaP cells. As anticipated, Akt phosphorylation inhibition by MK-2206 abolished the increase of cyclin B1 expression level, even though DHT could still induce the expression. The result in this figure indicates that AR transcription regulation activity is involved in cyclin B1 expression, and this transcription activity can be enhanced by Akt phosphorylation induced by overexpressed Jagged1 and AR. We next performed luciferase reporter assays to further examine whether cyclin B1 expression is regulated by AR and p-Akt. We prepared three luciferase reporter constructs: empty pGL-3 basic vector (negative control), vector with two copies of androgen response element (ARE; positive control), and vector with the promoter region of cyclin B1. It is clear that luciferase signal is induced by DHT, and this induction is reduced by MK-2206 (Fig. 5B). Our results here suggest that p-Akt can regulate AR activity. We then assess the expression of AR-regulated genes, including PSA, KLK2, and TMPRSS2, in LNCaP and LAPC4 cells upon Jagged1 depletion and Akt inactivation. We found that knocking down Jagged1 has no effect on these genes’ expression. However, Akt inactivation by MK-2206 significantly inhibits these genes’ expression, especially for PSA and KLK2 (Supplementary Fig. S2).

To examine how p-Akt affects cyclin B1 expression by Jagged1 and AR, ChIP assays were performed with anti-AR antibody through AREs (40, 41) in the cyclin B1 promoter region in AR-positive LNCaP cells with different Jagged1 expression levels. LNCaP cells cultured in androgen-depleted medium were used as a negative control as there was no DHT-induced AR binding. In addition, standard AR-regulated genes, PSA and TMPRSS2, were also included to assess the role of Jagged1/Akt in AR regulation at additional receptor-target genes. Our data showed that AR is recruited to the promoter region of cyclin B1 in wild-type LNCaP and is significantly enhanced in the presence of overexpressed Jagged1 (Fig. 5C). This indicates that p-Akt can enhance AR recruitment to the promoter region of cyclin B1. We also found that AR in the promoter region of cyclin B1 dramatically decreases both control and Jagged1-overexpressed LNCaP cells when MK-2206 is present, which confirmed our hypothesis that p-Akt can increase the expression level of cyclin B1 by enhancing the recruitment of AR to the promoter region of cyclin B1. Moreover, Jagged1/Akt affects the AR binding to additional AR-regulated genes such as PSA and TMPRSS2.

Finally, we immunoprecipitated AR in LNCaP cells with different expression levels of Jagged1 in the presence or absence of MK-2206 to examine whether p-Akt is involved in AR complexes for AR transcriptional activity. Figure 5D clearly shows that p-Akt, not unmodified Akt, interacts with AR and participates with AR transcriptional complexes. Taken together, our data suggest that p-Akt induced by Jagged1 and AR further interacts with AR to enhance AR recruitment to the promoter region of cyclin B1 and then promotes prostate cancer cell proliferation by increasing the expression level of cyclin B1.

Discussion

Jagged1, a Notch signaling pathway ligand, is expressed in both tumor epithelial and endothelial cells, and is associated with tumor progression in patients with poor prognosis breast and prostate cancer (26, 42). The reason why Jagged1 overexpression in prostate cancer correlates with a poor prognosis is still unknown. In the present study, we try to find out molecular regulations and downstream targets of Jagged1 in prostate cancer cells. We investigated the oncogenic activities of Jagged1 with different expression levels and found that Jagged1 can increase prostate cancer cell proliferation rates and regulate the cell cycle. More importantly, we found that AR promotes the oncogenic function of overexpressed Jagged1 to active their downstream targets to promote prostate cancer development.

The study of expression level of Jagged1 in human prostate cancer was first reported by Santagata and colleagues (26). They showed that Jagged1 was significantly highly expressed in metastatic prostate cancer as compared with localized prostate cancer or benign prostatic tissues by immunohistochemical analysis from 154 men. In addition, they also demonstrated that overexpression of Jagged1 in a subset of clinically localized tumors was significantly associated with recurrence, which suggests that Jagged1 has a close association with prostate cancer progression and metastasis. Another recent immunohistochemical study from Zhu and colleagues also showed similar results that Jagged1 had a high expression level in both metastatic prostate cancer and high-grade prostate cancer (27). Our data further confirmed the previous conclusion by measuring the mRNA level of Jag1 in normal prostate, primary prostate cancer, and metastasis tissue samples. To examine the potential oncogenic activity of Jagged1 and to uncover its coregulators or downstream targets at the molecular level, we measured prostate cancer cell proliferation and the cell cycle affected by knockdown or overexpression of Jagged1. Consistent with previous results (19, 28), we found that downregulation of Jagged1 significantly inhibited cell proliferation with a similar activity in all tested prostate cancer cell lines, including LNCaP, LAPC4, DU145, and PC3, and inhibited the cell cycle at S phase, which is direct evidence that...
Figure 5. p-Akt interacts with AR and enhances AR transcriptional activity by binding to the promoter region of cyclin B1. A, DHT induction for cyclin B1 expression in LNCaP cells with different expression levels of Jagged1 and in the presence or absence of MK-2206 was analyzed by RT-PCR. Each experiment was performed in triplicate, and data represent the mean ± SD. B, luciferase reporter assays were performed in LNCaP cells with three constructs [empty pGL-3 basic vector (negative control), vector with two copies of ARE elements (positive control), and vector with the promoter region of cyclin B1] with or without MK-2206. Luciferase signal was assayed with or without DHT induction. Each experiment was performed in triplicate, and data represent the mean ± SD. C, anti-AR ChIP assay was performed in LNCaP cells with or without Jagged1 overexpression in the presence or absence of MK-2206 or androgen. Anti-IgG used in the assay was treated as control, and all PCR signals for cyclin B1, PSA, and TMPRSS2 promoters were calculated to the percentage of the input (10%). Each experiment was performed in triplicate, and data represent the mean ± SD. c, anti-AR ChIP assay was performed in LNCaP cells with or without Jagged1 overexpression in the presence or absence of MK-2206 or androgen. Anti-IgG used in the assay was treated as control, and all PCR signals for cyclin B1, PSA, and TMPRSS2 promoters were calculated to the percentage of the input (10%). Each experiment was performed in triplicate, and data represent the mean ± SD. D, anti-AR immunoprecipitation for LNCaP cells with or without Jagged1 overexpression in the presence or absence of MK-2206 was analyzed by Western blotting. The proteins of AR, Jagged1, p-Akt, and Akt were detected in both input and immunoprecipitation samples.
Jagged1 has an oncogenic function in prostate cancer. However, AR-positive LNCaP/LAPC4 cells and AR-negative DU145/PC3 cells showed different phenomena for cell proliferation and cell cycle when we overexpressed Jagged1 in these cells. This suggests that AR may play an important role for overexpressed Jagged1 through the Notch signaling pathway during prostate cancer progression. It also suggests that the inhibition of cell proliferation by downregulation of Jagged1 may, through some mechanism, be independent of AR activity in prostate cancer, which is now being studied in our laboratory.

Interestingly, our results showed for the first time that AR promotes the oncogenic functions of overexpressed Jagged1 in prostate cancer cells. Except for prostate cancer cell lines, more importantly, we showed that patients with prostate cancer treated with ADT have more than 1 year shorter median survival time if they have both Jagged1 and AR high expression levels. In addition, cyclin B1 was shown to be highly expressed by co-overexpression of Jagged1 and AR in both prostate cancer cells and patient tissue samples, which can explain why prostate cancer cell growth is stimulated by both Jagged1 and AR overexpression.

The AR signaling pathway has been shown to cross-talk with many other cell signaling pathways in prostate cancer, including an array of growth factor signal transduction events, MAPK, and PI3K/Akt pathways (43–45). In addition, Akt was proved as a downstream target of the Notch signaling pathway in affecting cell growth and apoptosis in prostate cancer cells (46). Therefore, we hypothesized that AR promoting the oncogenic functions of overexpressed Jagged1 may be through activating Akt. Our data supported our hypothesis and showed that the phosphorylation level of Akt was highest when we co-overexpressed Jagged1 and AR in prostate cancer cells, and inhibiting Akt phosphorylation by inhibitor MK-2206 abolished the enhancement of cell proliferation induced by co-overexpression of Jagged1 and AR. More importantly, cyclin B1 expression level is affected dramatically by the phosphorylation level of Akt, which indicates that p-Akt might be involved in the transcription regulation of cyclin B1. AR has been shown in prostate cancer epithelial cells to influence the expression levels and activities of several cell-cycle genes essential for the G1–S and G2–M cell-cycle transition (40, 47). In addition, Akt regulates the expression and stability of AR at multiple levels, ranging from transcriptional to posttranslational modulation (48). We, therefore, hypothesized that AR and p-Akt may regulate the expression of cyclin B1 together. Our DHT induction, luciferase reporter assay, and anti-AR ChIP experiments confirmed that AR binds to the promoter region of cyclin B1 and regulates cyclin B1 expression. p-Akt functions as a coactivator to enhance AR binding to the promoter region of cyclin B1, and AR immunoprecipitation experiments showed that p-Akt interacts with the AR transcriptional complexes. Our data indicate a molecular regulation pathway among Jagged1, AR, cyclin B1, and p-Akt as
shown in our model (Fig. 6), which showed the different activities of the Jagged1-related Notch signaling pathway in AR-negative and AR-positive prostate cancer cells. Some questions still remain unanswered, such as how p-Akt is regulated by Jagged1 combined with AR and why p-Akt can enhance AR recruitment to the cyclin B1 promoter region, which need further studies in prostate cancer.

In summary, our study demonstrates that patients with prostate cancer with overexpressed Jagged1 can activate Akt, the downstream target of the Notch signaling pathway, to stimulate the tumor progression via promoting the cell cycle through enhancing the expression level of cyclin B1, and this activity can be significantly promoted by AR. Our results suggest that targeting AR or inhibiting Akt activity may be a clinically useful strategy for patients with prostate cancer with overexpressed Jagged1.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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