Overexpression of Indoleamine 2,3-Dioxygenase 1 Promotes Epithelial-Mesenchymal Transition by Activation of the IL-6/STAT3/PD-L1 Pathway in Bladder Cancer

Abstract
Indoleamine 2,3-dioxygenase 1 (IDO1) is a key enzyme in tryptophan metabolism and plays an important role in tumor cell immunosuppression and angiogenesis. The molecular mechanisms of IDO1 and epithelial-mesenchymal transition (EMT) have not been elucidated or studied in bladder cancer. Therefore, the aims of this study were to detect IDO1 expression in bladder cancer tissues and then to investigate the role of IDO1 in bladder cancer cell EMT and malignant phenotypes as well as the underlying molecular mechanisms. By immunohistochemistry, Western blot, and quantitative reverse transcription–polymerase chain reaction experiments, IDO1 was found to be overexpressed in bladder cancer tissues and cell lines compared to the noncancerous ones. In addition, knockdown of IDO1 expression was shown to inhibit bladder cancer cell growth, migration, invasion, and EMT. Furthermore, we demonstrated that IDO1 may promote EMT by activation of the interleukin 6/signal transducer and activator of transcription 3/programmed cell death ligand 1 signaling pathway. Collectively, these data suggest that IDO1 may play an important role in bladder cancer and may be a novel therapeutic target for patients with bladder cancer.

Introduction
Bladder cancer is the fourth most common cancer in males in the United States, and 76,960 new cases were diagnosed in 2016 [1]. Approximately 75% of the diagnosed patients have non–muscle-invasive bladder cancer (NMIBC), while 25% present with muscle-invasive bladder cancer (MIBC). NMIBC often shows good survival after transurethral resection, but 30%-50% of the patients experience recurrence after transurethral resection, and 25% of the patients progress to MIBC. Compared to NMIBC, patients diagnosed with MIBC often have a poor prognosis. Radical cystectomy is considered the standard treatment for nonmetastatic MIBC [2]. At present, the molecular mechanisms of bladder cancer development and progression have not been fully elucidated; therefore, it is imperative to study these mechanisms so that better treatments can be discovered.

Currently, researchers have become increasingly interested in studying the metabolism of tumors as well as metabolic genes. Boyland et al. [3] have found that the urine of patients with bladder cancer contains significantly increased amounts of tryptophan metabolites. Indoleamine 2,3-dioxygenase 1 (IDO1) is a key enzyme that catalyzes the breakdown of tryptophan into kynurenine. High expression levels of IDO1 have been found in various human tumor tissues, including prostate cancer [4], lung cancer [5], and chronic lymphocytic leukemia [6]; therefore, it is believed to play an important role in the development of cancer. In addition, a high IDO expression level in tumor tissues is usually associated with a poor patient prognosis [7]. To date, studies of
IDO1 have focused on immune escape and angiogenesis. IDO1 can inhibit effector T cells by consuming tryptophan and producing kynurenine, and IDO1 can affect immune tolerance by activating circulating regulatory T cells. Moreover, in a tumor cell line with a high expression of IDO1, the number of new blood vessels was significantly increased [8]. Furthermore, Wei et al. have demonstrated that IDO1 expression is associated with breast cancer microvessel density and that IDO1 can promote angiogenesis in breast cancer; therefore, IDO1 may be a potential therapeutic target for the inhibition of angiogenesis [9]. These data suggest that, in addition to its impact on immune tolerance, IDO1 can also promote tumor progression through angiogenesis.

However, the molecular mechanisms of IDO1 and epithelial-mesenchymal transition (EMT) have not been elucidated or studied in bladder cancer. Therefore, the aims of this study were to detect IDO1 expression in bladder cancer tissues and then to investigate the role of IDO1 in bladder cancer cell EMT and malignant phenotypes as well as the underlying molecular mechanisms.

Materials and Methods

Patients and Tissue Specimens

Sixty-eight pairs of bladder cancer tissues and their adjacent normal tissues were collected from January 2008 to December 2018 at the Shanghai Tenth People’s Hospital of Tongji University (Shanghai, China). These patients did not receive any treatment before surgery. The fresh tissue specimens were immediately snap-frozen in liquid nitrogen and stored at −80°C until further use. The study was approved by the Ethics Committee of the Tenth People’s Hospital of Tongji University, and informed consent was obtained from all patients or their relatives. Our research was also performed in accordance with the ethics guidelines of the World Medical Association (Declaration of Helsinki).

Immunohistochemistry (IHC)

Human bladder cancer and adjacent normal tissues were fixed in cold 4% paraformaldehyde. The fixed tissue samples were embedded in paraffin and sliced into 4-μm-thick sections. Following routine rehydration, antigen retrieval, and blocking procedures, the sections were incubated overnight with IDO1 antibody (1:1000 dilution, 1:1000, ab211017, Abcam, USA) at 4°C. Next, the sections were incubated with biotinylated goat anti-rabbit antibody IgG for 20 minutes at room temperature and then with streptavidin–horseradish peroxidase for 30 minutes. Subsequently, diaminobenzidine-H2O2 was used as a substrate for the peroxidase. Two pathologists read the pathological sections and determined positive results when the cytoplasm of the cancer cells was stained yellow. The proportion of positive cells was evaluated by five fields of view, regardless of the intensity of staining. We divided the expression of IDO1 into two grades: a high-expression group with at least 50% of cancer cells being positive and a low-expression group with a percentage of positive cancer cells <50%.

Reagents and Enzyme-Linked Immunosorbent Assay (ELISA) Assay

Human recombinant interleukin 6 (IL-6; PeproTech, Rocky Hill, NJ) was dissolved in trehalose at a concentration of 100 μg/ml and stored at −20°C. At the time of use, the final concentration of IL-6 was adjusted to 100 ng/ml in the appropriate medium. AG490 (Med-ChemExpress, Monmouth Junction, NJ), a tyrosine kinase inhibitor that can inhibit the signal transducer and activator of transcription 3 (STAT3) signaling pathway, was dissolved in dimethyl sulfoxide at a concentration of 5 mg/ml, stored at −20°C, and protected from light. AG490 was diluted to a concentration of 10 nmol in the appropriate medium before use [10]. Human IL6 ELISA kit was purchased from Anogen-Yes Biotech Laboratories Ltd. (EL10023, Ontario, Canada), and the concentration of IL6 in the cell supernatant was determined according to the manufacturer’s instructions.

Cell Lines

Four human bladder cancer cell lines (T24, UMUC3, 5637, and J82) and one immortalized human normal bladder epithelial cell line (SV-HUC-1) were obtained from the American Type Culture Collection (ATCC, Rockville, MD). T24, UMUC3, and 5637 cells were maintained in RPMI-1640 (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA); J82 cells were cultured in Dulbecco’s modified Eagle’s medium (Gibco); and SV-HUC-1 cells were maintained in F12K medium (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany). All cell lines were cultured at 37°C in a humidified incubator with 5% CO2. All cell culture media were supplemented with 10% fetal bovine serum (FBS; Gibco) and 1% penicillin/streptomycin (HyClone; GE Healthcare Life Sciences, Logan, UT).

RNA Extraction and Quantitative Reverse Transcription–Polymerase Chain Reaction (qRT-PCR)

Total RNA was extracted from human tissues or cultured cells using Trizol reagent (Invitrogen, Carlsbad, CA), and 5 μg of RNA was used to generate the first strand of cDNA by using a cDNA synthesis kit (Takara, Kyoto, Japan). qRT-PCR was performed using a SYBR Green PCR Kit (Takara Biotechnology, Dalian, China) with an ABI Prism 7500 Sequence Detection System (Applied Biosystems, Foster City, CA). The primers used were as follows: IDO1 forward, 5′-GCTTGCAAGAATCAGATG-3′ and reverse, 5′-GGC AAAGTCATGAGATGT-3′; GAPDH forward, 5′-ATGTCG TGGAGTCTACT GGC-3′ and reverse, 5′-TGACCTTGCCA CAGCCTTG-3′. The PCR parameters for relative quantification were as follows: 2 minutes at 95°C followed by 40 cycles of 45 seconds at 57°C and 45 seconds at 72°C. The IDO1 mRNA level was normalized to that of GAPDH. The relative expression levels of IDO1 were analyzed by the 2−ΔΔCt method [11]. Assays were repeated three times.

Transient Transfection

Small interfering RNAs that specifically target human IDO1 (si-IDO1) and nonspecific negative control oligonucleotides (si-NC) were purchased from GenePharma (Shanghai, China). Lipofectamine 3000 (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA) was used for cell transfection according to the manufacturer’s instructions. The si-IDO1 sequences were 5′-GAGCGGGAACA CUUUGCUAA-3′ (sense) and 5′-UUAGCAAGAGUC GCUCUCU-3′ (antisense). The si-NC sequences were 5′-UUCUCGGAACUGUCAGTTT-3′ (sense) and 5′-ACUGA CACGUUGGAGATTT-3′ (antisense). Total RNA or protein was extracted at 48 hours after transfection.

Cell Viability Assays

The cell proliferation rate was detected using a Cell Counting Kit-8 (CCK-8; Dojing Molecular Technologies, Inc., Kumamoto, Japan). Cells were seeded into 96-well plates at a density of 1 × 103 cells/well and transfected with siRNA or si-NC for 4 days. CCK-8 reagent (10 μl) was added to each well, and the cells were maintained at 37°C.
for 2 hours. The absorbance at 450 nm was measured using a microplate spectrophotometer (BioTek, Winooski, VT).

**Scratch Wound Healing Assay**

For the scratch healing assay, cells were plated and transfected, wounded using a 200-μl sterile pipette tip, and washed three times with phosphate-buffered saline (PBS), and RPMI-1640 without FBS was added. The wounds were continuously imaged at a magnification of ×5 using an optical system microscope for 24, 36, and 48 hours.

**Colony Formation Assays**

For colony formation assays, cells were transfected with si-NC or si-IDO1 for 24 hours, seeded into six-well plates at $1 \times 10^5$/well, and cultured for 14 days. Subsequent to culturing, the six-well plates were washed three times with cold PBS, fixed with 75% ethanol, and stained with 0.1% crystal violet. Images of stained tumor cell colonies were recorded with a digital camera.

**Cell Migration and Invasion Assays**

The cell migration ability was measured using a Transwell chamber (Corning, Lowell, MA). A total of $5 \times 10^4$ transfected cells were inoculated into each insert of a 24-well plate in FBS-free RPMI-1640 medium (200 μl), and 600 μl of cell culture medium containing 10% FBS was added to the bottom chamber. The cells were incubated in a 37°C incubator for 16 hours. After 16 hours, the Transwell chamber was washed three times with cold PBS. The nonmigrated cells in the upper insert were carefully wiped with a moist cotton swab, while the cells on the opposite side of the filter, the migrated cells, were fixed with 70% ethanol for 30 minutes and then stained with 0.1% crystal violet for 10 minutes. The cell migration was observed under a microscope (Olympus Corporation). For the tumor cell invasion experiments, the Transwell filters were precoated with Matrigel (BD Biosciences, Franklin Lakes, NJ), and the cells were allowed to invade for 24 hours. The rest of the experimental procedure was the same as that of the tumor cell migration assay. Each experiment was performed in triplicate.

**Western Blot Analysis**

Total protein extracts of cell lines or tissues were prepared by using radioimmunoprecipitation assay buffer (Sigma-Aldrich) containing protease inhibitors. The protein concentration of lysates was quantified using a bicinchoninic acid protein assay (Beyotime, China). A total of 5 × 10^6 transfected cells were harvested, and were washed three times with cold PBS, fixed with 75% ethanol, and stained with 0.1% crystal violet for 10 minutes. The labeled protein bands were visualized and quantified by using the Odyssey two-color infrared laser imaging system (LI-COR Biosciences, Lincoln, NE, USA).

**Statistical Analysis**

SPSS (version 22; SPSS, Inc., Chicago, IL) was used to analyze the resulting data. Chi-square test or Fisher’s exact test was used to assess the association between clinical pathological features and IDO1 expression, and Student's $t$ test was used to assess differences between groups. $P < .05$ was considered to be a statistically significant difference.

**Results**

**Overexpression of IDO1 in bladder cancer tissues and cell lines**

In this study, we examined the expression of protein and mRNA levels of IDO1 in bladder cancer tissues and cell lines. H&E staining indicated that IDO1 high expression was observed in 63.2% (43/68) of bladder cancer tissues compared with 29.4% (20/68) of the adjacent normal tissues and was statistically significant between bladder tumor tissues and adjacent normal tissues (Figure 1A, $P < .05$). At the same time, the expression of IDO1 was significantly correlated with tumor size, T stage, and N stage but not related to other clinicopathological features, including age, gender, smoking status, M stage, and histologic grade (Table 1, $P > .05$). Survival analysis showed that the prognosis of the IDO1 high-expression group was significantly worse, and the difference was statistically significant (Figure 1B, $P < .05$). In addition, we examined 20 pairs of samples showing that the protein and mRNA levels of IDO1 were higher in bladder cancer tissues than in normal bladder tissues by the Western blot and qRT-PCR analyses (Figure 1, C and D). Consistently, IDO1 expression was also significantly increased in T24, UMUC3, and 5637 bladder cancer cells compared to the normal bladder epithelial cell line SV-HUC-1 (Figure 2, A and B).

**Inhibition of bladder cancer cell proliferation by knockdown IDO1**

To determine the biological effects of IDO1 on bladder cancer cell growth, si-IDO1 was transfected into bladder cancer cells to inhibit the expression of IDO1, while si-NC was used as a control. qRT-PCR and Western blot analyses confirmed that the expression of IDO1 was inhibited with si-IDO1 transfection (Figure 2, C and D). CCK-8 and colony formation assays were used to study cell proliferation. The CCK-8 assay showed that knockdown of IDO1 significantly inhibited T24 and UMUC3 cell proliferation, while si-NC transfection had no significant effect on their proliferation (Figure 3, A and B). Consistently, after culturing for 2 weeks, the colony formation rate of cells with IDO1 knockdown was significantly inhibited compared with the control group (Figure 3, C and D).

**Inhibition of bladder cancer cell migration and invasion by knockdown IDO1**

We evaluated the effect of si-IDO1 on the metastatic capacity of bladder cancer cells. First, we performed cell scratch assays using T24 and UMUC3 cells. The knockdown of IDO1 reduced the ability of these bladder cancer cells to migrate compared to the control group (Figure 4A). Next, we performed a tumor cell migration assay. Consistent with the above results, IDO1 downregulation reduced the
migration ability of bladder cancer cells (Figure 4B). In addition, tumor cell invasion assays also demonstrated that downregulation of IDO1 significantly reduced T24 and UMUC3 cell invasiveness (Figure 4C).

Table 1. Correlation of IDO1 Expression with Clinicopathological Factors in 68 BCa Patients

| Parameters                  | No. of Patients | High Expression | Low Expression | P Value |
|-----------------------------|-----------------|-----------------|----------------|---------|
| Gender                      |                 |                 |                |         |
| Male                        | 63              | 39              | 24             | .744    |
| Female                      | 5               | 4               | 1              |         |
| Age (years)                 |                 |                 |                | .881    |
| ≤65                         | 28              | 18              | 10             |         |
| >65                         | 40              | 25              | 15             |         |
| BMI                         |                 |                 |                | .988    |
| ≤24                         | 40              | 24              | 14             |         |
| >24                         | 28              | 19              | 11             |         |
| Smoking status              |                 |                 |                | .718    |
| Yes                         | 40              | 26              | 14             |         |
| No                          | 28              | 17              | 11             |         |
| Tumor size                  |                 |                 |                | .044    |
| ≤3                          | 34              | 17              | 17             |         |
| >3                          | 34              | 26              | 8              |         |
| Histologic grade            |                 |                 |                | .030    |
| High                        | 64              | 43              | 21             |         |
| Low                         | 4               | 0               | 4              |         |
| Tumor stage                 |                 |                 |                | .032    |
| I-II                        | 23              | 10              | 13             |         |
| >II                         | 45              | 33              | 12             |         |
| Lymphatic invasion          |                 |                 |                | .032    |
| Positive                    | 48              | 26              | 22             |         |
| Negative                    | 20              | 17              | 3              |         |
| Distant metastasis          |                 |                 |                | .197    |
| Positive                    | 63              | 38              | 25             |         |
| Negative                    | 5               | 5               | 0              |         |
| Tumor progress              |                 |                 |                | .211    |
| Yes                         | 38              | 27              | 11             |         |
| No                          | 30              | 16              | 14             |         |

Figure 1. IDO1 is overexpressed human bladder cancer tissues and cell lines. (A) IHC staining of IDO1 in bladder cancer tissues (T) and adjacent normal tissues (ANT). The representative images are shown. Scale bar = 200 μm for 10× and 100 μm for 20×. (B) Kaplan-Meier survival analysis of patients with gastric cancer according to the IDO1 expression status. Kaplan-Meier analysis of cumulative survival in the low–IDO1 expression group (25 patients) and high–IDO1 expression group (43 patients). The P value was based on the log-rank test. Patients with a high IDO1 expression had a worse survival (P < .05). (C) and mRNA (D) levels of IDO1 in bladder cancer tissues (tumor) and adjacent normal tissues detected by Western blot and qPCR, respectively.

Inhibition of bladder cancer EMT by knockdown IDO1

The expression of several EMT-related markers also changed due to the effects of IDO1 knockdown. During EMT, the N-cadherin and vimentin levels are upregulated, while the E-cadherin levels are downregulated [12]. In this study, si-IDO1 transfection significantly reduced the protein expression of N-cadherin and vimentin compared to the si-NC group. However, the expression level of E-cadherin was significantly upregulated (Figure 4D). Therefore, our results suggest that knockdown of IDO1 can inhibit EMT.

Inhibition of EMT through the IL-6/STAT3/PD-L1 signaling pathway by knockdown IDO1

As an important inflammatory factor, IL-6 has been shown to affect the EMT [13]. ELISA experiments confirmed that IL-6 secretion was significantly reduced in the supernatant of si-IDO1–treated cells compared to the control group (Figure 5A). The Western blot results also showed that knockdown of IDO1 caused a decrease in STAT3 phosphorylation (Figure 5B). Therefore, we hypothesized that IL-6/STAT3/PD-L1 signaling is required for IDO1-mediated EMT in bladder cancer. To test this hypothesis, we first added exogenous IL-6 and found that it reversed the expression level of phosphorylated STAT3 as well as upregulated PD-L1 expression (Figure 5C). We further examined the role of exogenous IL-6 in EMT in cells by analyzing the expression of EMT-related markers. The addition of exogenous IL-6 significantly downregulated E-cadherin expression, while it upregulated N-cadherin and vimentin expression (Figure 5D). Second, we used the STAT3–specific inhibitor AG490 to inhibit STAT3 activation. We observed that AG490 reduced STAT3 phosphorylation and PD-L1 expression (Figure 6A). At the same time, we found that the expression of EMT-related markers of the si-IDO1 groups of T24 and UMUC3 cells also changed significantly upon AG490 treatment compared with the control group (Figure 6B).
Discussion
In this study, our aim was to elucidate the mechanisms related to tumor EMT in bladder cancer. We focused on IDO1, a key enzyme in tryptophan metabolism. We found that knocking down the expression of IDO1 could reduce the expression level of EMT-related genes as well as inhibit the invasion and migration of bladder cancer cells in vitro. Furthermore, we demonstrated that IDO1 may promote EMT by activation of the IL-6/STAT3/PD-L1 pathway. Uyttenhove et al. detected the expression of IDO1 in various human tumor tissues by IHC [14]. Their research reveals that IDO1 is highly expressed in a variety of human tumors and is closely related to the tumor immune microenvironment and clinical outcome. In addition, Suzuki et al. studied the serum levels of tryptophan and kynurenine in 123 patients with lung cancer, and they expressed the activity of IDO1 with the kynurenine/tryptophan ratio. Their results suggest that IDO1 may promote tumor progression in vivo by depleting tryptophan and accumulating the toxic metabolite kynurenine [15]. Moreover, Folgiero et al. demonstrated that children with acute myeloid leukemia and a high level of IDO1 expression had a significantly reduced 8-year event-free survival compared with those who did not express IDO1 [16]. Together with our data, these data indicate that IDO1 expression is closely related to the development of bladder cancer.

EMT is an important biological process for tumor migration and invasion. EMT is characterized by the conversion of polar, immobile epithelial cells into mesenchymal cells, which can lead to increased cell migration, resistance to apoptosis, and invasiveness. Simultaneously, the expression of EMT-related molecular markers also changes: the levels of E-cadherin, an epithelial cell marker, decrease, while the levels of N-cadherin and vimentin, mesenchymal cell markers, increase [17, 18]. Similar to our results, Kolijn et al. [4] have demonstrated that increased IDO1 expression leads to the

Figure 2. IDO1 is overexpressed human bladder cancer cell lines. Relative protein (A) and mRNA (B) levels of IDO in bladder cancer cell lines (T24, UMUC3, and 5637) and the immortalized human normal bladder epithelial cell line SV-HUC-1 detected by Western blot and qRT-PCR, respectively. (C) mRNA and (D) protein levels of IDO1 in transfected T24 and UMUC3 cells were analyzed using RT-qPCR and Western blot analysis, respectively. GAPDH and β-actin served as the internal controls, respectively. *P < .05.

Figure 3. Silencing of IDO1 inhibits cell growth of T24 and UMUC3 cells. (A, B) Cell proliferation was detected at 24, 48, 72, and 96 hours after IDO1 knockdown as determined by CCK8 assay. (C, D) The left panel is a representative image of clone formation of cells after transfection. The right panel shows the number of colonies of cloned cells (Student’s t test, *P < .05).
downregulation of E-cadherin expression and the upregulation of N-cadherin expression in prostate cancer cell lines. Chen et al. [19] also found that kynurenine, a metabolite of IDO1, causes activation of the aromatic hydrocarbon receptor, leading to degradation of E-cadherin in breast cancer. In the case of bladder cancer, despite extensive research on EMT, the effect of IDO1 on EMT has not been reported previously. This study demonstrates that IDO1 is one of the possible pathways for EMT in bladder cancer.

Meanwhile, IDO1 has been identified as an important component of the proinflammatory, tumor-promoting environment [20]. It has been shown that the expression of IDO1 affects the secretion of various inflammatory cytokines, such as IL-6 and IL-10 [21,22]. IL-6 and its receptor IL-6R, located on the cell membrane, are important inflammatory factors that are responsible for activation of the downstream JAK2/STAT3 pathway. Activation of JAK2 protein kinase catalyzes the phosphorylation of STAT3 protein in the nucleus, thereby regulating the expression of EMT-related genes and other genes [23]. Arpita et al. [21] have indicated that IDO1 may promote tumorigenesis through the enhanced expression of the proinflammatory cytokine IL-6, while the lack of IDO1 or IL-6...
expression leads to a decrease in the metastatic tumor burden and an increase in survival. In addition, it has been reported previously that IL-6 can promote cancer cell proliferation, invasion, and stem cell production [24]. In our study, ELISA experiments also confirmed that IDO1 affects the expression of IL-6. Similarly, Luo et al. have found that the IL-6/STAT3 signaling pathway affects EMT. They discovered that polyphyllin I, a natural compound, can reverse EMT by regulating the IL-6/STAT3 signaling pathway in non–small cell lung cancer [25]. Moreover, Zhang et al. have determined that EMT can be regulated in prostate cancer by the ataxia telangiectasia-mutated (ATM) protein kinase/STAT3/PD-L1 signaling pathway and that PD-L1 is significantly reduced when ATM is knocked down [26]. Asgarova et al. also observed upregulated PD-L1 during EMT and described tumor cells’ increased proliferation, infiltration, and migration capacities and greater ability to escape immune system detection during EMT [27]. Recent demonstrations showing that adjuvant combined IDO1 inhibitor and PD-L1 inhibitor therapy can greatly improve the effectiveness of immunotherapy [28–30] suggest that IDO1 and PD-L1 may be closely related. In this context, our findings suggest that they may be linked in the EMT process. Therefore, PD-L1 not only participates in tumor immune evasion but also participates in EMT. Thus, we hypothesized that IDO1 may promote EMT in bladder cancer via the IL-6/STAT3/PD-L1 signaling pathway (Figure 7).

One limitation of this study should be noted. The role of IDO1 in bladder cancer was only analyzed in vitro using cell lines. We will further validate our findings in animal models and further explore the mechanisms by which IDO1 is responsible for the development of bladder cancer.

**Conclusion**

In summary, our data reveal that IDO1 significantly promotes the EMT process during bladder cancer development. Knockdown of IDO1 effectively targets cellular EMT by inhibiting the IL-6/STAT3/PD-L1 signaling pathway. Thus, inhibitors of IDO1 may become a new approach for the treatment of bladder cancer in the future.

**Figure 6.** AG490 blocks the IL6/STAT3/PD-L1 signaling pathway and is more pronounced after knockdown of IDO1. (A) T24 and UMUC3 cells after IDO1 knockdown were treated with STAT3 specific inhibitor AG490. The protein levels of STAT3, P-STAT3, and PD-L1 were measured by Western blot analysis. (B) EMT-associated markers were measured after treatment with AG490 by Western blot analysis.

**Figure 7.** The proposed model is a graphical representation of the mechanism of interaction of IDO1 and IL6/STAT3/PD-L1 in EMT regulation in bladder tumor cells.
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Conflict of Interest Statement
The authors declared that there is no conflict of interest in this work.

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