The combinatory effects of PPAR-γ agonist and survivin inhibition on the cancer stem-like phenotype and cell proliferation in bladder cancer cells

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Abstract. Strategies for peroxisome proliferator-activated receptor (PPAR) activation or survivin inhibition have potential for cancer therapy. However, whether the combination of these two approaches can be developed as a rational regimen with enhanced efficiency in the inhibition of tumor cells remains to be determined. In this study, the combinatory effect of PPAR-γ agonist and survivin inhibition on bladder cancer cells was investigated. T24 and 5637 cells were treated with 15d-PGJ_2 to determine whether 15d-PGJ_2 had an inhibitory effect. Cell viability and proliferation were analyzed and efficiency of survivin siRNAs was assessed using western blot analysis. The results showed that, in the human bladder cancer cell lines T24 and 5637, the natural PPAR-γ ligand 15d-PGJ_2 significantly decreased cell proliferation and loci formation. The increase in the proportion of apoptotic cells was observed in the cells 48 h after 15d-PGJ_2 treatment. Furthermore, 15d-PGJ_2 substantially inhibited the levels of stemness-related genes in these cells. The ability of sphere formation was markedly suppressed in the cells treated with 15d-PGJ_2. More importantly, the downregulation of survivin with siRNAs significantly enhanced the 15d-PGJ_2-mediated induction of cell apoptosis and inhibition of sphere formation. Accordingly, we also found that survivin inhibition significantly enhanced 15d-PGJ_2-induced production of reactive oxygen species (ROS) in bladder cancer cells. Taken together, these findings suggest that the combination of 15d-PGJ_2 and survivin inhibition play a potentially role in the therapeutical manipulation of bladder cancer.

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Introduction

The incidence of bladder cancer, a common urologic cancer, continues to increase annually, ranking as the ninth most common malignancy wordwide (1). Although treatment with systemic chemotherapy is recommended, the prognosis for patients with metastatic bladder cancer is poor. Therefore, improvement of existing therapies and development of alternative therapeutic approaches is critical. Recent advances in the study on tumor-initiating cells, a small subpopulation of tumor cells that contribute to tumor initiation, metastasis and drug-resistance (2), suggest that targeting these cells may lead to novel therapies that can be utilized in the reduction of risk of tumor recurrence.

The peroxisome proliferator-activated receptor-γ (PPAR-γ) is a member of the nuclear receptor superfamily that is activated by its ligands. The activation of PPAR-γ may lead to cell growth arrest, apoptosis, decrease of cell adhesion and migration, and particularly, result in the differentiation of cancer cells (3). The property of their antigrowth and prodifferentiation renders natural and synthetic ligands of PPAR-γ as attractive substances in cancer prevention and treatment (3-6). However, given that PPAR-γ ligands often trigger crosstalk with other signalling pathways (6-8), use of PPAR-γ agonists alone on much more common advanced epithelial malignancies has minimal clinical effect (9). Therefore, the combination of PPAR-γ agonists with other drugs, such as EGFR inhibitor (10) or AKT inhibitor (11) has been examined for cancer treatment.

One of the hallmarks of tumor cells is the ability to evade apoptosis (12). Overexpression of antiapoptotic genes is one of mechanisms to escape cancer cell apoptosis. As an important member of the inhibitor of apoptosis gene family, survivin can block the activation of effector caspases in intrinsic and extrinsic pathways of apoptosis. Survivin is absent in normal urothelium, whereas it is present in 64-100% of bladder cancers (13). Moreover, the expression of survivin is associated with high stage and grade as well as with an increase risk of recurrence for patients with bladder cancer (14-17).

Thus, survivin has been suggested as a suitable target for the development of specific treatment of bladder cancer (15). In the present study, we report that the combination of PPAR-γ activation and survivin inhibition generates a more robust suppression in the cell survival and stem cell properties of bladder cancer cells, providing a basis for future studies testing the strategy for experimental manipulation of bladder cancer.

Materials and methods

Cell culture. The human bladder cancer cell lines, T24 and 5637, obtained from the American Type Culture Collection (Manassas, VA, USA), were cultured in a maintenance medium containing DMEM with high glucose supplemented with 10% fetal bovine serum and penicillin/streptomycin [1% (v/v)] (all from Gibco, Grand Island, NY, USA). The cells were then treated with 15d-PGJ_2 (Sigma-Aldrich, St. Louis, MO, USA) for the indicated times (6 days). Cell cultures were maintained at 37˚C in a humidified atmosphere with 5% CO_2.

siRNA of survivin. Survivin RNAi oligos and negative control high GC oligo were purchased from Sigma-Aldrich. The siRNA sequences are listed in Table I. One day prior to the transfection, T24 and 5637 cells were seeded in 6-well plates without antibiotics. Using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA), the siRNAs (60 nM) were transfected into the cells according to the manufacturer's instructions.

Cell viability assay. To evaluate the effect of 15d-PGJ_2 on T24 and 5637 cell growth, cell viability was determined by the 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) (Sigma-Aldrich) assay. In brief, a total of 1,000 5637 or 5,000 T24 cells/well were seeded in 96-well plates (BD Biosciences, San Jose, CA, USA) in a volume of 200 µl. Subsequent to incubation with 0.5 or 2 µg/ml 15d-PGJ_2 for the indicated times, 20 µl MTT solution [5 mg/ml in phosphate-buffered saline (PBS)] was added to each well and incubated for an additional 4 h at 37˚C. MTT solution was aspirated off, 150 µl dimethyl sulfoxide (DMSO) was added to each well, and the absorbance was measured at 540 nm. Data were recorded on a daily basis and the growth curve was drawn.

Cell cycle analysis. Cells were collected and centrifuged at 300 x g at 4˚C for 5 min and resuspended by PBS in tubes. The abovementioned steps were then repeated. The cells were fixed in ice-cold 70% ethanol overnight. After washing with PBS twice, the cells were labelled with propidium iodide (PI) (50 µg/ml; Sigma-Aldrich) and treated with RNase A (100 µg/ml; Amresco, Solon, OH, USA) for 30 min in the dark. The cells were then analyzed using a FACSCalibur flow cytometer (BD Biosciences).

Apoptosis assay. T24 and 5637 cells were collected, centrifuged at 300 x g at 4˚C for 5 min, and washed twice with PBS containing 0.5% BSA. The cells were dissociated in 1X binding buffer and the cell concentration was adjusted to $1x10^6$/ml. Cell suspension (100 µl) was added with Annexin V-FITC (BD Biosciences) and 7-AAD (Sigma-Aldrich) according to the manufacturer's instructions, and incubated for 20 min in the dark. Following the addition of 200 µl 1X binding buffer in the tube, FACS was performed.

Table I. List of siRNA sequences.

Name		siRNA sequence
siSurvivin1	F	GUCUGGACCUCAUGUUGUUdTdT
	R	AACAACAUGAGGUCCAGACdTdT
siSurvivin2	F	CCUCUACUGUUUAACAACAdTdT
	R	UGUUGUUAAACAGUAGAGGdTdT
siSurvivin3	F	GGUUUAUUCCCUGGUGCCAdTdT
	R	UGGCACCAGGGAAUAAACCdTdT

F, forward; R, reverse.

Western blot analysis. Cells were lysed in a RIPA lysis buffer (Beyotime, Nantong, China) with Protease Inhibitor Cocktail and PhosSTOP (Roche, Monza, IT, USA). Proteins were detected using indicated antibodies: anti-PPAR-γ, anti-survivin (all from Cell Signaling Technology, Beverly, MA, USA); anti-GAPDH, anti-α-tubulin (both from Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA). The ChemiDoc™ XRS system (Bio-Rad Laboratories, Hercules, CA, USA) was used for obtaining images.

Sphere formation assay. To assay sphere formation efficiency, single cells were plated in Ultra Low Attachment plates (Costar, Corning, NY, USA) and cultured in 1:1 DMEM:F12 (Gibco) supplemented with B27 (1:50; Invitrogen), 20 ng/ml epidermal growth factor and 20 ng/ml basic fibroblast growth factor (R&D Systems, Minneapolis, MN, USA). The cells were incubated in a CO_2 incubator for 1-2 weeks, and spheres were counted under a stereomicroscope (Olympus, Tokyo, Japan).

Measurement of reactive oxygen species (ROS) accumulation. ROS was monitored by FACS using dihydroethidium (DHE) (Invitrogen). Cells were incubated with 5 µM DHE at 37˚C for 30 min, and fluorescence was measured by a FACSCalibur flow cytometer.

Statistical analysis. Data are presented as the means ± SEM. Statistical analyses were conducted using SPSS 13.0 for Windows. Data between two groups were assessed using the Student's t-test. $P < 0.05$ was considered to indicate statistical significance.

Results

15d-PGJ_2 effectively inhibits cell proliferation and stem cell-like properties of bladder cancer cells. Since PPAR-γ agonists are known to inhibit cell growth in various types of cancer cells (18-20), we first detected the effect of 15d-PGJ_2, a natural PPAR-γ ligand, on the cell viability of bladder cancer cells. T24 and 5637 cells were treated with 15d-PGJ_2 at various concentrations for the indicated times (6 days). 15d-PGJ_2 efficiently suppressed T24 and 5637 cell growth (Fig. 1A). Similarly, we observed the inhibitory effect of 15d-PGJ_2 on

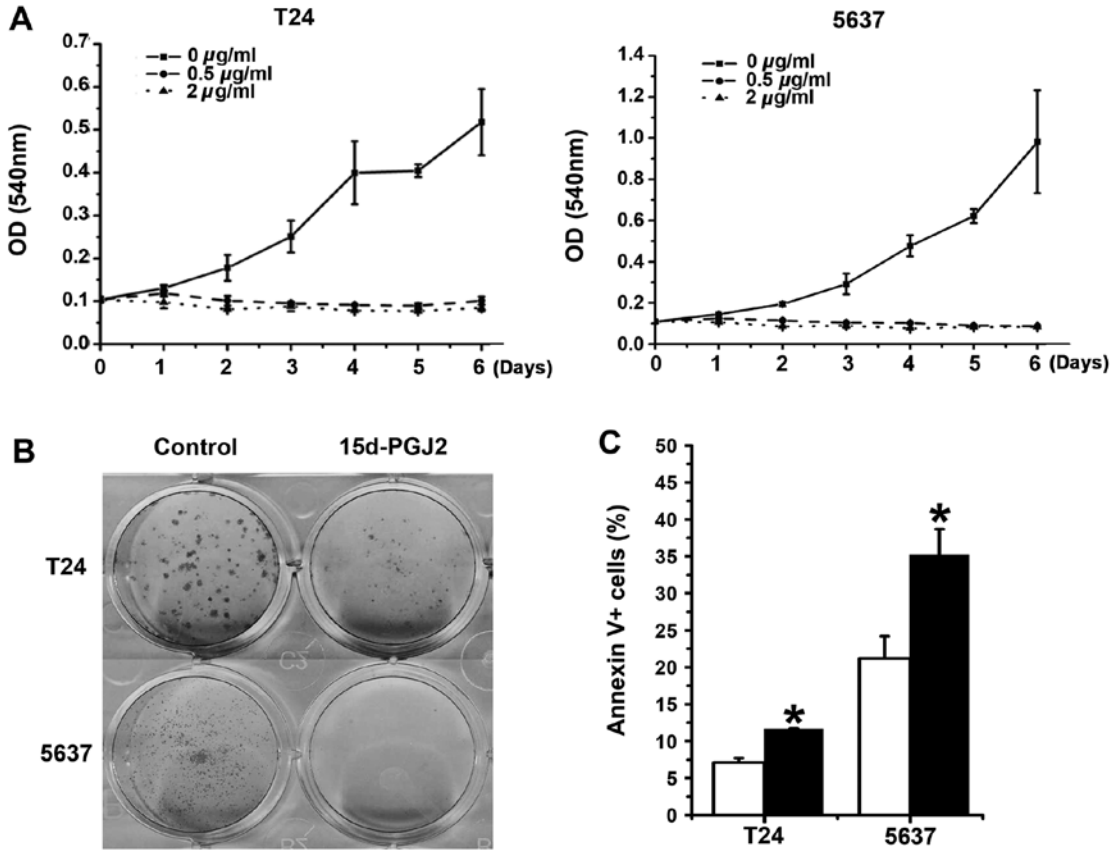

Figure 1. Suppressive effects of 15d-PGJ_2 on cell viability and proliferation of bladder cancer cells. (A) Effect of 15d-PGJ_2 on viability of T24 and 5637 cells. T24 and 5637 cells were treated with 15-PGJ_2 (0.5 or 2 μg/ml) for the indicated times. Cell proliferation was analyzed by MTT assay. Data are the means ± standard error of the mean (SEM) (n=3). (B) Representative image of dense foci formation of T24 and 5637 cells following treatment with or without 15d-PGJ_2 (0.5 μg/ml) for 7 days. (C) T24 and 5637 cells were treated with 0.5 μg/ml 15d-PGJ_2 for 48 h, and the percentage of apoptotic cells was detected by Annexin V-FITC/7-AAD staining. Quantitative bar graphs show the means ± SEM (n=3). $^*P<0.05$, compared with control cells.

bladder cancer cells by measurement of foci formation and cell apoptosis (Fig. 1B and C).

Since PPAR-γ agonists have been observed to regulate differentiation of myxoid/round cell liposarcoma (21) and inhibit tumor-initiating cells in brain and liver cancers (22), we investigated the possibility that 15d-PGJ_2 treatment affects the stem cell-like properties of bladder cancer cells. The expression of the stemness-related genes, *Oct4* and *Nanog*, was significantly downregulated in T24 and 5637 cells following the treatment of 15d-PGJ_2 (Fig. 2A). Of note, the decreased expression of *Oct4* and *Nanog* genes was observed starting ~1 h after treatment with 15d-PGJ_2 in T24 cells, suggesting the key role of 15d-PGJ_2 on the repression of the stem-like phenotype of bladder cancer cells. We also performed a speroid formation assay. The results showed that 15d-PGJ_2 treatment at low concentrations had no significant effect on the spheroid formation of T24 or 5637 cells (Fig. 2B). Only the treatment with a high dose of 15d-PGJ_2 (up to 5 μg/ml) decreased the spheroid number that bladder cancer cells formed (Fig. 2B), suggesting that the treatment of 15d-PGJ_2 alone is not sufficient to prevent bladder cancer.

Survivin inhibition accelerates the suppressive effect of 15d-PGJ_2 on cell proliferation and the stem cell-like properties of bladder cancer cells. The anti-apoptotic protein survivin has been demonstrated as a promising biomarker for detection and prognosis in bladder cancer. Thus, we hypothesized whether the combination of 15d-PGJ_2 and survivin inhibition may more efficiently inhibit cell growth and the stem-like phenotype of bladder cancer cells as compared to the single treatment of 15d-PGJ_2. We first evaluated the expression of survivin in 15d-PGJ_2-treated bladder cancer cells. The results showed no significant difference in the expression of survivin between the cells treated with or without 15d-PGJ_2 (Fig. 3), suggesting that downregulation of survivin may increase the efficiency of 15d-PGJ_2 treatment. Survivin expression was depleted with specific siRNAs in the T24 and 5637 cells and the effectiveness of survivin siRNAs was validated by western blotting (Fig. 4A). In the presence of 15d-PGJ_2, we found that inhibition of survivin expression by specific siRNAs increased cell apoptosis induced by 15d-PGJ_2 (Fig. 4B). Moreover, siRNAs against survivin strengthened the suppressive effect of 15d-PGJ_2 on the spheroid formation of T24 cells (Fig. 5A). Notably, the downregulation of survivin by siRNA did not facilitate 15d-PGJ_2-mediated inhibition of the stemness-related genes in 5637 cells, even if survivin depletion alone affected the expression of Oct4 and Nanog (Fig. 5B), suggesting that survivin inhibition by siRNAs exacerbated the inhibitory effects of 15d-PGJ_2 on bladder cancer cells by directly inducing cell death.

15d-PGJ_2 enhances survivin inhibition-induced production of ROS in bladder cancer cells. Since oxidative stress is one of the most important regulatory mechanisms for cell apoptosis and

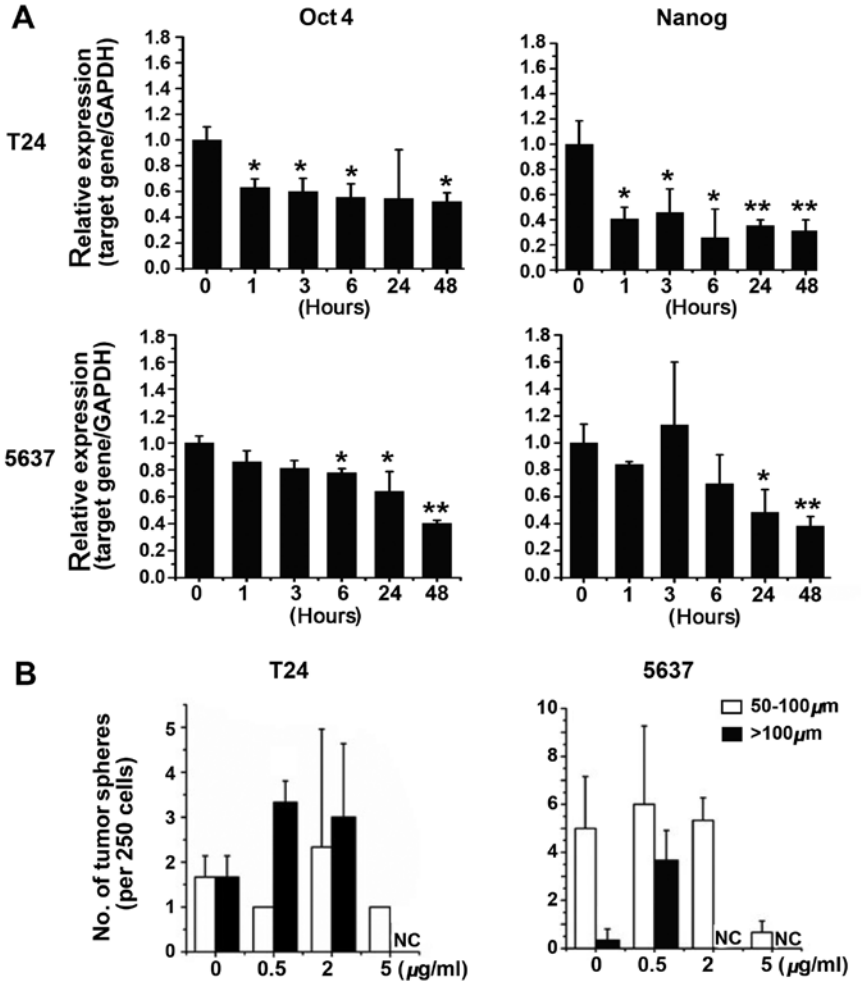

Figure 2. 15d-PGJ_2 inhibits the stem cell-like properties of bladder cancer cells. (A) T24 and 5637 cells were treated with 0.5 μg/ml 15d-PGJ_2 for the indicated times. The expression of stemness-related genes was detected by quantitative RT-PCR analysis. Quantitative bar graphs are shown [means ± standard error of the mean (SEM), n=3]. $^{*}P<0.05$ and $^{**}P<0.01$, compared with the control cells. (B) T24 and 5637 cells were cultured in spheroid-forming medium containing various concentrations of 15d-PGJ_2 (0.5 μg/ml) for 7 days. Data are the means ± SEM (n=3).

differentiation (23-25), we evaluated the generation of ROS in bladder cancer cells treated with 15d-PGJ_2 or transfected with survivin-specific siRNAs. Depleting the survivin expression significantly induced the production of ROS in the T24 and 5637 cells, and 15d-PGJ_2 further facilitated the generation of ROS (Fig. 5C and D). The upward trend in ROS was consistent with an increase of cell apoptosis induced by 15d-PGJ_2 and/or survivin inhibition (Fig. 4B), suggesting that generation of ROS may be responsible for the inhibition of cell proliferation-mediated 15d-PGJ_2 and/or survivin in bladder cancer cells.

Discussion

PPAR-γ participates in multiple biological pathways, such as lipid metabolism, energy homeostasis, cell proliferation, death and differentiation (26,27), and various pathogenic processes including inflammation, diabetes, atherosclerosis and cancer (28-30). However, despite extensive studies on the PPAR-γ agonists for tumor suppression, the effects of PPAR-γ agonists in tumor-initiating cells (TICs) is still poorly defined. In this study, we showed that 15d-PGJ_2, the natural ligand of PPAR-γ, impaired the maintenance and function of TICs in bladder cancer cells. Moreover, the combination of survivin inhibition and 15d-PGJ_2 yielded greater inhibition of cultured cell spheroid formation and cell growth of bladder cancer cells.

It is becoming increasingly evident that TICs overexpress multidrug resistance proteins (31,32), which provide a possible explanation for the failure of standard chemotherapy (33-36). Our results have demonstrated that 15d-PGJ_2 significantly repressed the spheroid formation of bladder cancer cells, decreased the expression of stemness-related genes, indicating that PPAR-γ agonists have a marked inhibitory effect on tumor-initiating cells of human bladder cancer. Survivin is a key biomarker for the detection of bladder cancer metastasis (13,14). When we combined 15d-PGJ_2 with survivin depletion, the cell proliferation and spheroid formation were more efficiently suppressed than either alone. These findings raise the possibility that the combination of survivin suppressants and PPAR-γ agonists is likely a new therapy for bladder cancer.

ROS play critical roles in the regulation of cell proliferation, apoptosis, and transformation (24,37). It has recently been established that 15d-PGJ_2 negatively regulates cell proliferation by eliciting the production of ROS (38-41). More importantly, previous studies have demonstrated that the level of intracellular

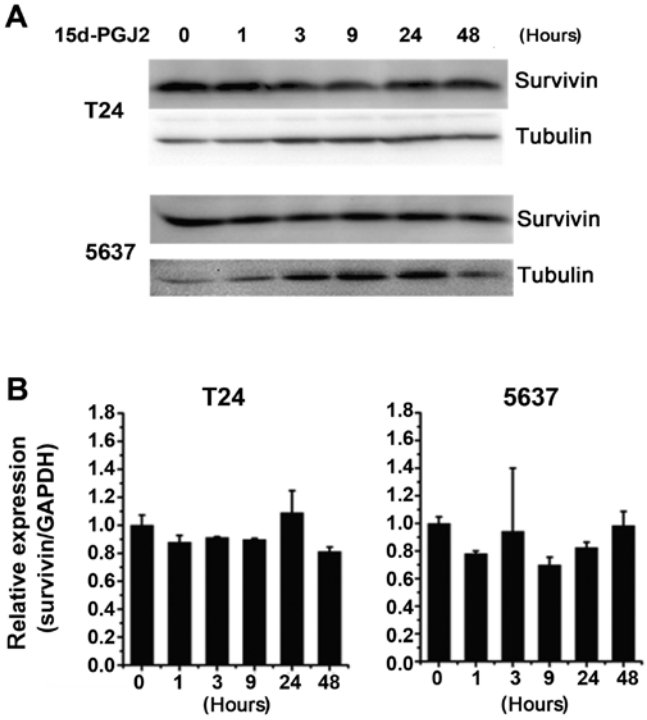

Figure 3. 15d-PGJ_2 downregulates the protein expression of survivin. (A) T24 and 5637 cells were treated with 15d-PGJ_2 (0.5 μg/ml) for the indicated times. Total protein was extracted for analysis of survivin and α-tubulin. (B) T24 and 5637 cells were incubated with 15d-PGJ_2 (0.5 μg/ml) for the indicated times, quantitative RT-PCR analysis was performed for mRNA expression of survivin. Data are shown [means ± standard error of the mean (SEM), n=3].

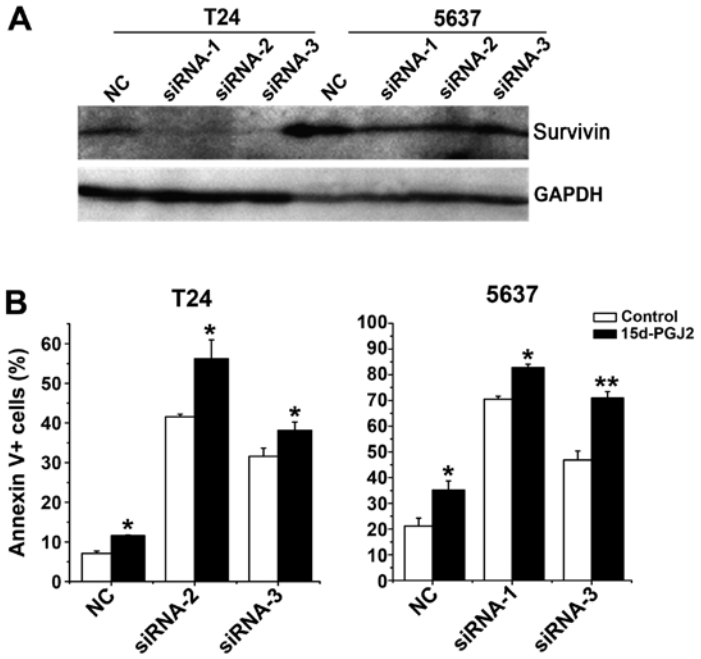

Figure 4. Inhibition of survivin facilitates the repressive effect of 15d-PGJ_2 on the cell growth of bladder cancer cells. (A) T24 and 5637 cells were transfected with survivin siRNA-1, siRNA-2, siRNA-3, or negative control siRNA. After 48 h, total protein was extracted for analysis of survivin and GAPDH. (B) T24 and 5637 cells were transiently transfected with survivin siRNA-1, siRNA-2, siRNA-3, or negative control siRNA. The cells were treated with various concentrations of 15d-PGJ_2 for 48 h and stained with Annexin V-FITC/7-AAD. Data are the means ± standard error of the mean (SEM) (n=3). *P<0.05; **P<0.01.

ROS is associated with TICs (42-46). In the present results, we demonstrated that 15d-PGJ_2 upregulated the production of ROS, and knockdown of survivin obviously enhanced the generation of ROS stimulated by 15d-PGJ_2, suggesting that 15d-PGJ_2 and/or survivin inhibition restrained bladder cancer stem-like phenotype and cell proliferation possibly by upregulating ROS production. NADPH oxidases and mitochondria are two major sources of ROS generation (47,48). A recent study has suggested that PPAR-γ agonist may involve mitochondrial function (49). Additionally, it has been shown that PPAR-γ

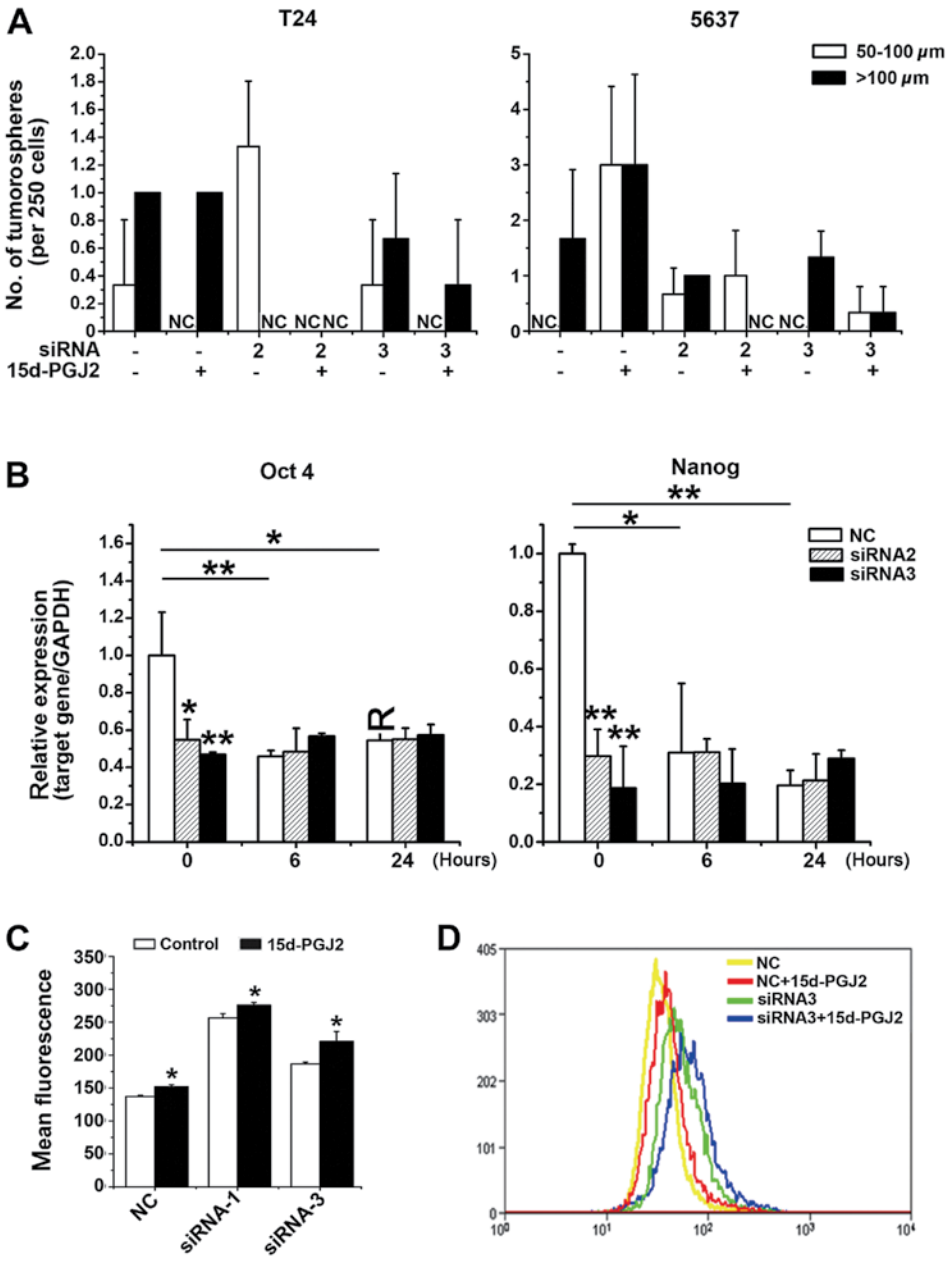

Figure 5. The effect of combination of peroxisome proliferator-activated receptor-γ (PPAR-γ) activation and survivin inhibition on the stem cell-like phenotype of bladder cancer cells. (A) T24 cells were transfected with negative control or survivin siRNAs for 24 h, after which cells were seeded on ultra-low attachment culture dishes to assay spheroid formation with or without treatment of 15d-PGJ_2 (0.5 μg/ml) for 7 days. Quantitative values are shown [means ± standard error of the mean (SEM), n=3]. (B) 5637 cells were transfected with negative control or survivin siRNAs for 24 h, and then treated with 15d-PGJ_2 (0.5 μg/ml) for the indicated times. Quantitative RT-PCR analysis was performed to determine the relative level of Oct4 and Nanog mRNAs. Data are means ± SEM (n=3). *P<0.05; **P<0.01. (C) T24 and (D) 5637 cells were transfected with negative control or survivin siRNAs for 24 h. The cells were then treated with 0.5 μg/ml 15d-PGJ_2 for another 48 h, and labeled with DHE for measurement of intracellular reactive oxygen species (ROS) generation by FACS (means ± SEM, n=3). *P<0.05.

agonists inhibit stem cell-like phenotype and cell proliferation of liver cancer cells via NOX2-mediated oxidative stress (50). Thus, the manner in which the PPAR-γ agonist is involved in the production of ROS induced by 15d-PGJ_2 and/or survivin suppression in bladder cancer cells remains to be investigated.

In conclusion, we have shown that cotreatment of 15d-PGJ_2 and survivin RNAi synergistically inhibit bladder cancer stem-like phenotype and cell proliferation *in vitro*. These observations suggest that the combined treatment with survivin inhibitor and PPAR-γ agonists may be of therapeutic importance in the clinical treatment of malignant tumors.

Acknowledgements

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