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BIOCHEMISTRY, BIOPHYSICS, AND MOLECULAR BIOLOGY

Punica granatum Extract Inhibits Bladder Cancer Cell Viability, Invasion and Migration through Down-Regulation of HOXD10 Signalling Pathway

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Abstract—The present study investigated *Punica granatum* extract (PGE) as potential proliferation inhibitory agent for bladder cancer cells and elucidated the possible mechanism. PGE reduced viabilities of HT-1197 and RT4 cells in concentration-based manner at 72 h. Colony forming potential of HT-1197 and RT4 cells was also significantly ($p < 0.05$) inhibited on exposure to 2 and 12 mg/mL PGE. Exposure to 12 mg/mL PGE for 72 h significantly ($p < 0.05$) decreased miR-10b expression and suppressed migration potential of HT-1197 and RT4 cells. In PGE exposed HT-1197 and RT4 cells, invasiveness was reduced to 30.25 and 33.47%, respectively. PGE treatment of HT-1197 and RT4 cells caused a significant ($p < 0.05$) elevation in HOXD10 protein and mRNA levels compared to control. The miR-10b mimic transfection in HT-1197 and RT4 cells reversed inhibitory effect of PGE on cell viability. Thus, PGE exhibited cytotoxicity and anti-invasive effect on HT-1197 and RT4 cells through targeting miR-10b and up-regulation of HOXD10 expression. Thus, PGE may be developed as therapeutic agent for treatment of bladder cancer.

Keywords: *Punica granatum* extract, bladder cancer, viability, migration, oncogenes

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INTRODUCTION

Bladder cancer is the second commonly diagnosed cancer of urogenital tract in USA and the fourth highest malignancy in males [1]. In more than 50% of the patient's bladder cancer invasion has been reported to muscles followed by metastasis to pulmonary tissues and liver [2]. Metastasis to various organs is responsible for poor prognosis and 5 year survival rate of less than 60% in bladder cancer patients [2]. In spite of extensive advancement in treatment techniques like chemo, radiotherapies and surgical procedures the prognosis of bladder cancer patients is very poor while as mortality rate is very high [3]. In 2017 the estimated bladder cancer cases diagnosed were ~80000 and deaths ~17000 in USA alone [3]. The major hindrance to chemotherapy at present is the high resistance and low sensitivity of bladder cancer [4]. The compounds which demonstrated efficient anti-tumor properties against bladder cancer during preclinical trials but were not effective at advanced stage include Balversa, APL-1202, etc. [4]. Thus, discovery of novel therapeutic agents is urgently required for bladder cancer treatment.

MicroRNAs (miRNAs) comprised of short sequence of 18–25 nucleotides are non-coding RNAs involved in regulation of gene expression through interaction with 3'-untranslational regions (UTRs) of mRNAs [5]. Abnormality in miRNAs expression leads to growth and progression of different types of cancers in human beings [6]. During human malignancies like bladder cancer miRNAs either act as oncogenes or suppressors of tumor growth [7]. Up-regulation of miR-373 and miR-21 has been reported during blad-

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der cancer and act as oncogenes through apoptotic p53-pathway suppression [8]. The miR-137 up-regulation elevates bladder cancer growth through promotion of progestin and adipoQ receptor-3 expression [8]. The miR-31 serves as suppressor of tumor growth and induces sensitivity of bladder cancer cells to mitomycin-C by integrin α5 down-regulation [9].

Punica granatum plant attains a height of 12–16 feet, has spiny branches and grows for many years. The plant is known for anti-carcinogenic, inflammation inhibitory and antioxidant properties [10]. The present study investigated *Punica granatum* extract as potential proliferation inhibitory agent for bladder cancer cells and elucidated the possible mechanism.

MATERIALS AND METHODS

PGE Preparation

Punica granatum plant material was collected, shade dried for 72 h and then grinded using mechanical grinder to prepare its powder. The powdered material was subjected to solvent extraction in ethanol for 48 h in a solvent extractor. Solvent was then decanted and subsequently evaporated under vacuum to prepare the PGE extract.

Cell Culture

HT-1197 and RT4 cell lines were provided by the ATCC (Manassas, VA, USA) and were cultivated in DMEM (Invitrogen) supplemented by 10% fetal bovine serum (FBS, Gibco). The medium also contained 100 U/mL penicillin, 100 mg/mL streptomycin. Cells were cultured at 37°C in an incubator under 5% CO_2.

MTT Assay

HT-1197 and RT4 cells were distributed at 3×10^4 cells per well density in 96-well cultural plates in DME-medium. Following incubation for overnight, PGE at 2, 4, 6, 8, 10, and 12 mg/mL concentrations was added to the plates. The cells were incubated for 72 h and then MTT (5 mg/mL; 20 μL) was put into each well of the plate and incubation was continued for 5 h. To solubilize the solid material formed in the wells 150 μL DMSO was added into each well. The Microplate Reader (BioTek Instruments, Inc.) was used for measurement of absorbance at 480 nm to determine viability of the cells.

RT-qPCR

HT-1197 and RT4 cells were exposed to PGE at 2 and 12 mg/mL concentrations for 72 h. The same volume of... was added to the control wells. Total RNA from HT-1197 and RT4 cells was extracted using the commercially available TRIzol® reagent (Invitrogen). Then One Step PrimeScript® kit (Takara Bio, Inc.) was used for reverse transcription of total RNA samples into the cDNA strands as per supplier's instructions. The RT was performed under the following conditions: miRNA; 37°C for 58 min, 82°C for 5 s, and 4°C for 10 min; mRNA: 40°C for 2 min, 37°C for 12 min, 83°C for 5 s, and 4°C for 12 min. Detection of HOXD10 mRNA and miR-10b was carried out by ABI-7500 Fast RT-PCR system (Thermo Fisher Scientific, Inc.) connected to a SYBR® Premix Ex Taq™ II kit (Takara Bio, Inc.) in accordance with instructions from supplier. The conditions for thermocycling were: 93°C for 18 min, 39 cycles at 96°C for 10 s and 56°C for 58 s and 4°C for 25 min. The internal controls used for HOXD10 mRNA and miR-10b expression were GAPDH and RNA U6, respectively. The sequence of primers used was as follows: miR-10b: sense CCAGAGGTTGTAACGTTG, anti-sense TGAAGTTTTTGCATCGACC; U6: sense CTCGCTTCGGCAGCACA, antisense ACGCTTCACGAATTTGCGT; HOXD10: sense GACATGGGGACCTATGGAATG, anti-sense TGGTGGTTCACTTCTCTTTTGG and GAPDH: sense AGAAGGCTGGGGCTCATTTG, anti-sense AGGGGCCATCCACAGTCTTC.

Western Blotting

HT-1197 and RT4 cells were lysed on treatment with RIPA buffer (Beyotime) after 72 h of exposure to PGE (2 and 12 mg/mL). The same volume of was added to the control wells. Centrifugation of the cell lysate was performed at 4°C for 40 min at 13000*g* to obtain the supernatants. The protein level was quantified in supernatants using BCA assay kit (Beyotime) in accordance with the supplier's instructions. Equal protein samples (40 μg) were resolved on 12% SDS-PAGE (Beyotime) and transferred subsequently onto the PVDF membrane. Membrane non-specific sites were blocked by 60 min of incubation with 5% non-fat milk at 37°C. Incubation of the membranes with primary antibodies against HOXD10 and GAPDH (Sigma-Aldrich) was carried out for overnight at 4°C. Following TBS plus Tween-20 buffer (0.05%) washing the membranes were incubated for 2 h with anti-rabbit antibodies, conjugated with. HRP (Santa Cruz Biotechnology, Inc.). Visualization and analysis of the blots was made using Enhanced Chemiluminescence (ECL) system (Amersham Pharmacia Biotech) and Image-Pro plus software 6.0 (Cybernetics, Inc.).

Cell Migration Assay

HT-1197 and RT4 cells were exposed for 72 h to PGE (2 and 12 mg/mL) at 2×10^6 cells/well density. The same volume of... was added to the control wells. Cell monolayer after attaining 98% confluence was scraped through the middle using sterile pipette tip (100 μL) to create the wound. Then loosely bound

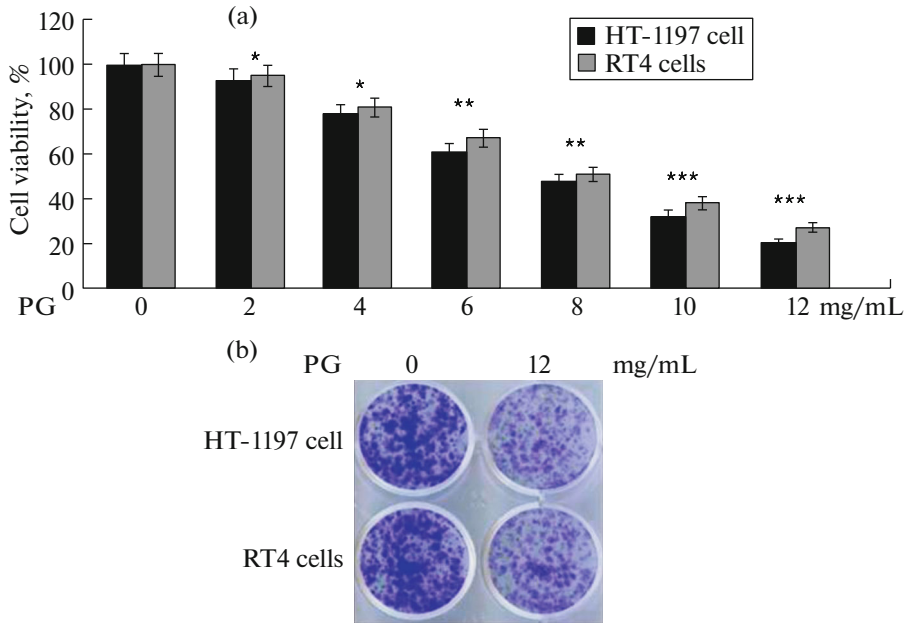

Fig. 1. Effect of PGE on HT-1197 and RT4 cell viability. (a) Exposure to PGE at indicated concentrations was followed by measurement of HT-1197 and RT4 cell viability using MTT assay. (b) Following exposure to PGE (2 and 12 mg/mL) the colony forming potential of HT-1197 and RT4 cells was detected; * $p < 0.05$, ** $p < 0.02$, and *** $p < 0.01$ vs. untreated cells.

cells were removed by washing with PBS and monolayers were subjected to incubation for 20 h in DMEM devoid of serum at 37°C. Inverted microscope (ECLIPSE TS100; Nikon Corporation) was used for measurement of wound healing at ×100 magnification.

Invasion Assay

HT-1197 and RT4 cells were exposed for 72 h to PGE (2 and 12 mg/mL) and put in upper chambers of matrigel coated 24-well Transwell chambers at 2×10^6 cells/well density in DMEM (free from serum). The lower chamber contained DMEM mixed with 10% FBS as the chemoattractant. Incubation of the cells for 72 h was followed by clearing off non-invasive cells using cotton swabs. The cells invaded to lower chamber were fixed for 35 min using 95% ethyl alcohol at 37°C and subsequently dyed for 15 min with 0.1% crystal violet. Cell invasion was observed using the light microscope (Nikon Corporation).

Cell Transfection

HT-1197 and RT4 cells were distributed at 2×10^6 cells/well density and incubated in DMEM and 10% FBS till attaining 85% confluence. The cells were then exposed for 72 h to PGE (2 and 12 mg/mL) at 37°C. The same volume of... was added to the control wells. The 100 nM miR-10b mimic and negative control (NC) of the mimic were obtained from the Guangzhou (RiboBio Co., Ltd.) and transfected to HT-1197 and RT4 cells using Lipofectamine® 2000 (Invitrogen) according to manufacturer's instructions.

Statistical Analysis

The data presented are the mean ± S.D. of three measurements performed independently. Statistical analysis of the data was made using SPSS version 19.0 (IBM Corp.) and differences were determined between groups using one-way ANOVA followed by Bonferroni's post hoc test. The $p < 0.05$ were taken to represent statistically significant differences.

RESULTS

Punica Granatum Extract Suppresses HT-1197 and RT4 Cell Viability

HT-1197 and RT4 cells were exposed to PGE at 2, 4, 6, 8, 10, and 12 mg/mL concentrations and viability changes were followed using MTT assay (Fig. 1a).

PGE reduced viabilities of HT-1197 and RT4 cells in concentration-based manner at 72 h. HT-1197 cell viability decreased to 93 and 20%, respectively on exposure to 2 and 12 mg/mL PGE for 72 h. Exposure of RT4 cells to 2 and 12 mg/mL PGE for 72 h reduced viability to 95 and 27%, respectively. PGE induced cytotoxicity for HT-1197 and RT4 cells was also confirmed using colony formation assay (Fig. 1b). Colony forming potential of HT-1197 and RT4 cells was also

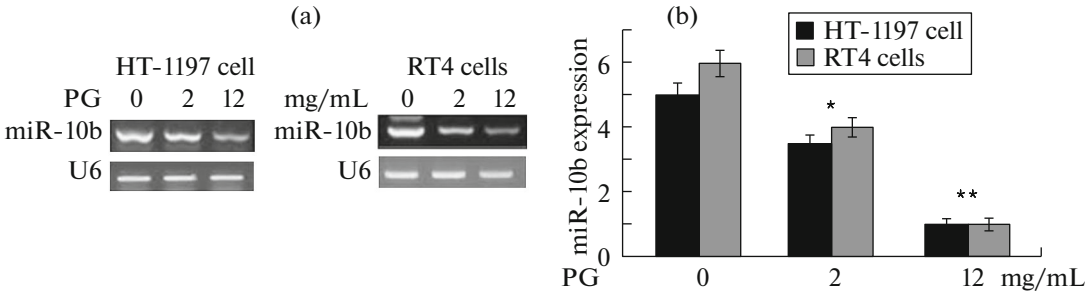

Fig. 2. Effect of PGE on miR-10b expression in HT-1197 and RT4 cells. (a) Exposure to PGE at 12 mg/mL was followed by assessment of miR-10b expression in HT-1197 and RT4 cells by RT-PCR. (b) The miR-10b expression in HT-1197 and RT4 cells; * $p < 0.05$ vs. untreated cells.

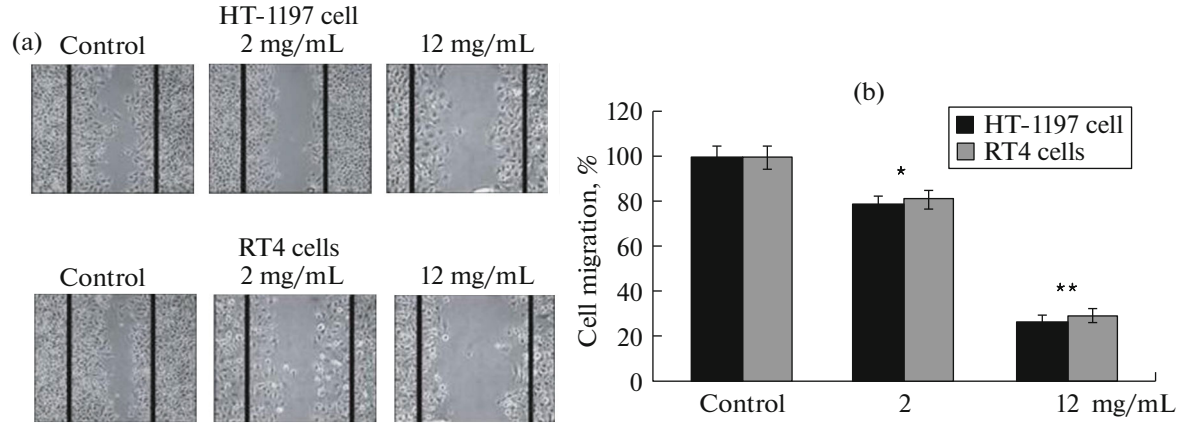

Fig. 3. Effect of PGE on HT-1197 and RT4 cell migration. (a) Exposure to PGEat 12 mg/mL was followed by measurement of HT-1197 and RT4 cell migration using wound-healing assay. (b) Quantified data; * $p < 0.05$ and ** $p < 0.01$ vs. untreated cells.

significantly ($p < 0.05$) inhibited on exposure to 2 and 12 mg/mL PGE.

Punica Granatum Extract Targets miR-10b Expression in HT-1197 and RT4 Cells

In HT-1197 and RT4 cells changes in miR-10b expression on exposure to 12 mg/mL PGE were determined by RT-PCR assay (Fig. 2). Exposure to 12 mg/mL PGE for 72 h significantly ($p < 0.05$) decreased miR-10b expression in HT-1197 and RT4 cells. Thus, PGE targeted miR-10b expression to suppress viabilities of HT-1197 and RT4 cells.

Punica Granatum Extract Inhibits HT-1197 and RT4 Cell Migration and Invasion

In HT-1197 and RT4 cells migration potential following exposure to PGE (12 mg/mL) was detected by wound-healing assay (Fig. 3).

PGE exposure at 12 mg/mL significantly ($p < 0.05$) suppressed migration potential of HT-1197 and RT4 cells. The migration ability of HT-1197 and RT4 cells was suppressed to 26.65 and 29.30%, respectively on exposure to 12 mg/mL PGE. Invasive changes induced by PGE in HT-1197 and RT4 cells were detected using Matrigel assay (Fig. 4). In PGE exposed HT-1197 and RT4 cells, invasiveness was reduced to 30.25 and 33.47%, respectively.

Punica Granatum Extract Up-Regulated HOXD10 Expression in HT-1197 and RT4 Cells

The levels of HOXD10 mRNA and protein in HT-1197 and RT4 cells following exposure to PGE (12 mg/mL) were assessed by RT-PCR and western blot assays (Fig. 5). In PGE treated HT-1197 and RT4 cells a significant ($p < 0.05$) elevation in HOXD10 protein and mRNA levels was detected compared to control at 72 h.

The miR-10b Mimic Reverses Effect of PGE on HT-1197 and RT4 Cell Viability

To investigate whether PGE exhibits its effect through targeting miR-10b expression HT-1197 and RT4

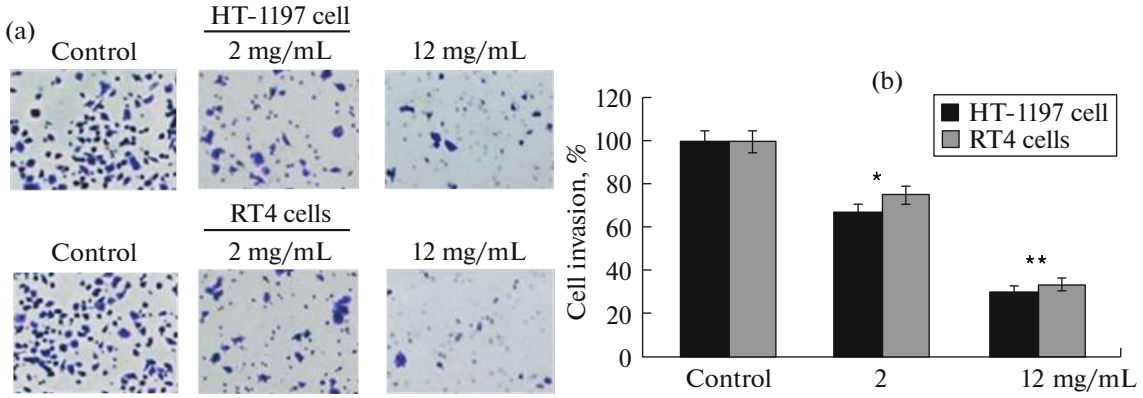

Fig. 4. Effect of PGE on HT-1197 and RT4 cell invasion. (a) After exposure to PGE (12 mg/mL) the invasive ability of HT-1197 and RT4 cells was detected using Matrigel assay. (b) Quantified data; * $p < 0.05$ and ** $p < 0.01$ vs. untreated cells.

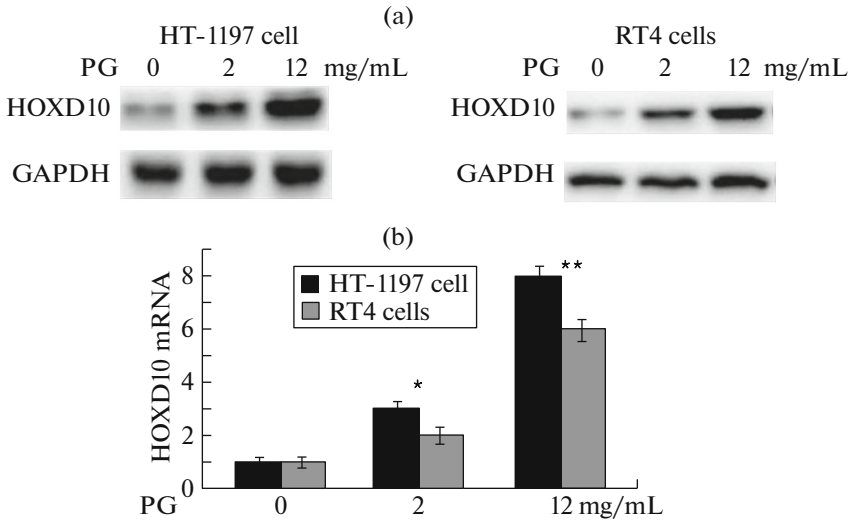

Fig. 5. Effect of PGE on HOXD10 levels in HT-1197 and RT4 cells. (a) Exposure to PGEat 12 mg/mL was followed by assessment of HOXD10 protein expression in HT-1197 and RT4 cells by western blotting. (b) The HOXD10 mRNA level was determined by RT-PCR assay; * $p < 0.05$ vs. untreated cells.

cells were transfected with miR-10b mimic (Fig. 6). The miR-10b mimic transfection also reversed inhibitory effect of PGE on HT-1197 and RT4 cell viability. The HT-1197 and RT4 cell viability decreased to 20 and 27%, respectively on exposure to 12 mg/mL PGE for 72 h. However, transfection of PGE (12 mg/mL) exposed HT-1197 and RT4 cells with miR-10b mimic increased cell viability to 98 and 99%, respectively.

DISCUSSION

Punica granatum plant has great medical significance because of diversity of biological properties including anti-carcinogenic, inflammation inhibitory, antioxidant and anti-diabetic activities [10]. The plant has shown inhibitory activity against prostate cancer growth, preventive effect against Alzheimer's disease

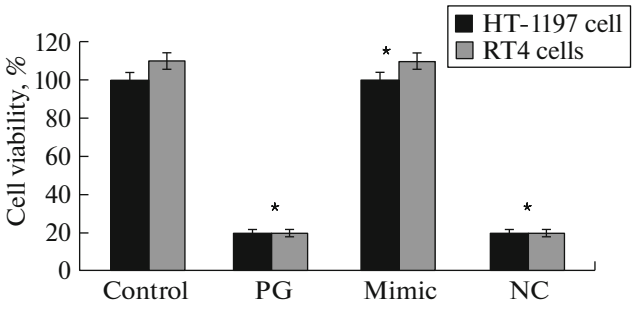

Fig. 6. Effect of miR-10b mimic on PGE induced viability changes. Transfection with miR-10b mimic and PGE treatment was followed by measurement of HT-1197 and RT4 cell viability using MTT assay; * $p < 0.05$ and ** $p < 0.01$ vs. untreated cells.

and ischemia mediated brain injury [10]. The present study investigated PGE against bladder carcinoma growth in vitro and explored the related signalling pathway. The results demonstrated that PGE induced cytotoxicity against HT-1197 and RT4 cells. Exposure to PGE reduced viabilities of HT-1197 and RT4 cells in concentration-based manner. In HT-1197 and RT4 cells PGE exposure efficiently inhibited growth of colonies compared to untreated cells.

The involvement of miRNAs is widely being investigated in the development and treatment of different kinds of cancers [6]. Many miRNAs including miR-21 and miR-372 have been found to be associated with the treatment of various cancers detected in humans [11]. Studies demonstrated oncogenic role of miR-10b in several cancers like breast, gastric, colorectal and laryngeal cancers [12]. The present study demonstrated that exposure to PGE significantly ($p < 0.05$) decreased miR-10b expression in HT-1197 and RT4 cells. Therefore, PGE-induced suppression of HT-1197 and RT4 cell viabilities involved targeting miR-10b expression. It is reported that miR-10b expression up-regulation elevates migration potential and invasive property of bladder carcinoma cells [13]. In the present study PGE exposure of HT-1197 and RT4 cells significantly ($p < 0.05$) suppressed migration ability relative to untreated cells. The invasive abilities of PGE exposed HT-1197 and RT4 cells were also efficiently reduced relative to untreated cells.

A member of homeobox (HOX) family of genes is HOXD10 which has been identified as target for miR-10b in multiple types of carcinomas including glioma, colon, ovary and gastric cancer [14]. In the present study PGE treated HT-1197 and RT4 cells showed a significant ($p < 0.05$) elevation in HOXD10 protein and mRNA levels at 72 h. In order to investigate whether PGE exhibits its effect through targeting miR-10b expression HT-1197 and RT4 cells were transfected with miR-10b mimic. The data demonstrated that miR-10b mimic transfection in HT-1197 and RT4 cells reversed inhibitory effect of PGE on expression of miR-10b. Additionally, miR-10b mimic transfection also reversed inhibitory effect of PGE on HT-1197 and RT4 cell viability. Thus, PGE suppressed HT-1197 and RT4 cell viability through down-regulation of miR-10b expression.

CONCLUSIONS

In summary, PGE exhibited cytotoxicity and anti-invasive effect on HT-1197 and RT4 cells through targeting miR-10b expression. Moreover, PGE treatment of HT-1197 and RT4 cells led to up-regulation of HOXD10 protein and mRNA levels. Thus, PGE may be developed as therapeutic agent for treatment of bladder cancer.

CONFLICT OF INTEREST

The authors declare no conflict of interest in the publication of these results.

COMPLIANCE WITH ETHICAL STANDARDS

This article does not contain any studies involving animals or human participants performed by any of the authors.

AUTHOR CONTRIBUTIONS

Rui Sun and Junjun Zhang both are the first authors, they contributed equally.

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