European Review for Medical and Pharmacological Sciences 2020; 24: 1712-1717

PKCα and Netrin-1/UNC5B positive feedback control in relation with chemical therapy in bladder cancer

J. LIU, J. LI

Department of Urology, The First Affiliated Hospital of China Medical University, Shenyang, China

Abstract. – OBJECTIVE: To explore the cisplatin medical tolerance mechanism affecting bladder cancer cells.

MATERIALS AND METHODS: Bladder cancer cells were treated with protein kinase C α (PKCα) stimulation and inhibition agents. The small-interfering RNA (siRNA) inhibitory technique was used to differentiate and remove Netrin-1 from UNC5B. Cells treated by cisplatin were processed by the MIT method to estimate cell death and growth rate. The Western blot method was utilized to analyze PKCα, netrin-1, and UNC5B in bladder cancer cells, used in the relative control sample. Co-immunoprecipitation was employed to analyze PKCα, netrin-1, and UNC5B combination effects.

RESULTS: PKCα high activity, netrin-1 high expression, and UNC5B with low expression can enhance bladder cancer cells cisplatin medical tolerance. PKCα low activity, netrin-1 low expression, and UNC5B with high expression can also enhance bladder cancer cells sensitivity to chemical therapeutic treatments. PKCα high activity with enhanced netrin-1 reduced UNC5B expression, and also enhanced netrin-1/UNC5B combination. It inhibits and/or deletes PKCα, Netrin-1 lower stream extracellular regulated protein kinases (ERK) signal, deletes UNC5B, PKCα, and lowers stream (ERK) signaling from activity.

CONCLUSIONS: PKCα and netrin-1/UNC5B form a positive feedback control loop in relation to the regulation of cisplatin in bladder cancer cells.

Key Words:
Bladder cancer, Medical tolerance, Netrin-1, PKCα, Positive feedback, UNC5B.

Introduction

Bladder cancer is a form of malignant tumor occurring in the bladder mucosa and is one of the common forms of malignant urological tumors[1]. Bladder cancer is a complex, multi-factor, and multi-stage pathological condition. Current clinical treatment of bladder cancer is mainly surgical and supplemented by chemical therapy. With the combination of well-managed chemical therapy and planning, there can usually be enhanced results in reducing anticancer medication toxicity to bladder cancer cells. Still, bladder cancer recurrence rate remains high, which may result from the cancer cell's high medical tolerance to anticancer medications[2-4]. Tolerance to anticancer medications has been one of the primary obstacles in bladder cancer treatment, therefore researching the mechanism of its medication tolerance is our main objective.

Materials and Methods

Cell Culture and Processing

T24 and 5637 cell sequence was acquired in the U.S Standard Culture Storage Center. Culture agents Roswell Park Memorial Institute-1640 (RPMI-1640; Hyclone, South Logan, UT, USA) were used, including 10% fetal bovine serum (FBS; Hyclone, South Logan, UT, USA), 100 μg/mL streptomycin and 100 UI/mL penicillin (Beyotime, Shanghai, China). Culture was conditioned at 37°C, 5% CO^2, and cultivated for two days. (1) PMA at 5 μg/mL was used for 48 h in the cell process. (2) Calphostin C at 5 μg/mL was used for 48 h in the cell process. (3) Cisplatin at 10 μm was used 24 h in the cell process.

Cell Interference

T24 and 5637 cell cultivation was in great condition. Once concentration reached around 70%, cell interference was started. Interference agents used were SMARTpool siRNA netrin-1,

Corresponding Author: Jun Li, MD; e-mail: mskongchuize@sina.com

SMARTpool siRNA UNC5B, and negative comparison agent Non-targeting siRNA, acquired from Dharmacon (Shanghai, China). Interference agents Dharmafect 1 reagent, also came from Dharmacon (Shanghai, China). The incubation condition was at 37°C, 5% CO_2, for 48 h, until it was ready for collection.

MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyl Tetrazolium Bromide) Experiment

Cell cultivation was conducted in the 96-hole cultivation dish, with a density of $5x10^3$ per hole, for 5 days. Afterwards, each hole was filled with 20 μm 5 mg/mL MTT solutions (Beyotime, Shanghai, China), and continued to be cultivated for 4 h. The cultivated base was then removed and filled with 150 μL of dimethyl sulfoxide (DMSO; Beyotime, Shanghai, China), with a wavelength result of 490 nm.

Cell Death Rate Experiment

Cells were collected and phosphate-buffered saline (PBS) solution was used to neutralize the cells. A 250 μL buffer solution was added to fill the test tube. Next, 5 mg/mL of propidium iodide (PI) and Annexin V/FITC (Beyotime, Shanghai, China) were added. Under darkroom conditions, 15 min later, the cell death rate was recorded.

Western Blot

Cells were collected and added to the radioimmunoprecipitation assay (RIPA; Beyotime, Shanghai, China) lysis buffer, ice chilled for 10 min, low-temperature and high-speed centrifugation (4°C, 12 000 s/min) for 30 min. Serum was collected from the total cell protein (TCP). TCP undergo dodecyl sulfate, sodium salt-polyacrylamide gel electrophoresis (SDS-PAGE) electrophoresis, print on polyvinylidene difluoride (PVDF; Thermo Scientific, Waltham, MA, USA). 5% bovine serum albumin (BSA; Beyotime, Shanghai, China) was used in the enzyme-linked immunosorbent assay (ELISA; Novus Biologicals, Littleton, CO, USA).

The antibodies used in this assay are netrin-1 (1:1000, sc9292, Santa Cruz Biotechnology, Santa Cruz, CA, USA), and from Cell Signalling Technology, (Danvers, MA, USA), UNC5B (1:1000, 13851), p-ERK (1:1000, 9101), PKCα (1:1000, 59754), Glyceraldehyde-3-Phosphate Dehydrogenase (GAPDH) (1:1000, 2118) were all obtained from Abcam (Cambridge, MA, USA). The incubation condition was at 4°C overnight. Then, they were compared with incubation condition at 37°C for 2 h, post-incubation enhanced chemiluminescence (ECL; Thermo Scientific, Waltham, MA, USA) and electrophoresis analysts.

Co-Immunoprecipitation

Cells were collected and added to RIPA lysis buffer, ice chilled for 10 min, low-temperature high-speed centrifugation (4°C, 12 000 s/min) for 30 min. The serum was collected and ready for Western blot analysis. Lug corresponding antibody lysis buffer was added to the remaining serum, at 4°C, and slow sway incubated overnight. 10 μL of protein A agarose was rinsed with lysis buffer, and added to the serum that was prepared overnight, and again incubated for 4 h to fuse the agarose and antibody together. It was then centrifuged at (4°C, 3 000 s/min) for 3 min, the substrate of agarose was at the bottom of the test tube, and the filtered serum was discarded. The agarose was rinsed with lysis buffer, 15 μL 2×SDS buffer added and then boiled for 5 min. A Western blot analysis was completed to confirm the conjugate protein.

Statistical Analysis

Statistical Product and Service Solutions (SPSS) 16.0 (SPSS Inc., Chicago, IL, USA) software was used to analyze the data collected. The Student's *t*-test analytical methods were used to analyze the data from the control and experimental groups. The clinical significance between the two groups was demonstrated as $p<0.05$.

Results

PKCsα, Netrin-1, and UNC5B Were Utilized to Test Tolerance in the Bladder Cancer Cells

To investigate whether PKCα had an impact on cisplatin medications, we first induced apoptosis of bladder cancer cells with cisplatin. Then, the apoptosis-induced bladder cancer cells were treated with PMA and calphostin C, respectively.

The results showed that the induction of apoptosis induced by cisplatin was weakened when the activity of PKCα was increased. After PKCα activity decreased, the apoptosis-inducing effect

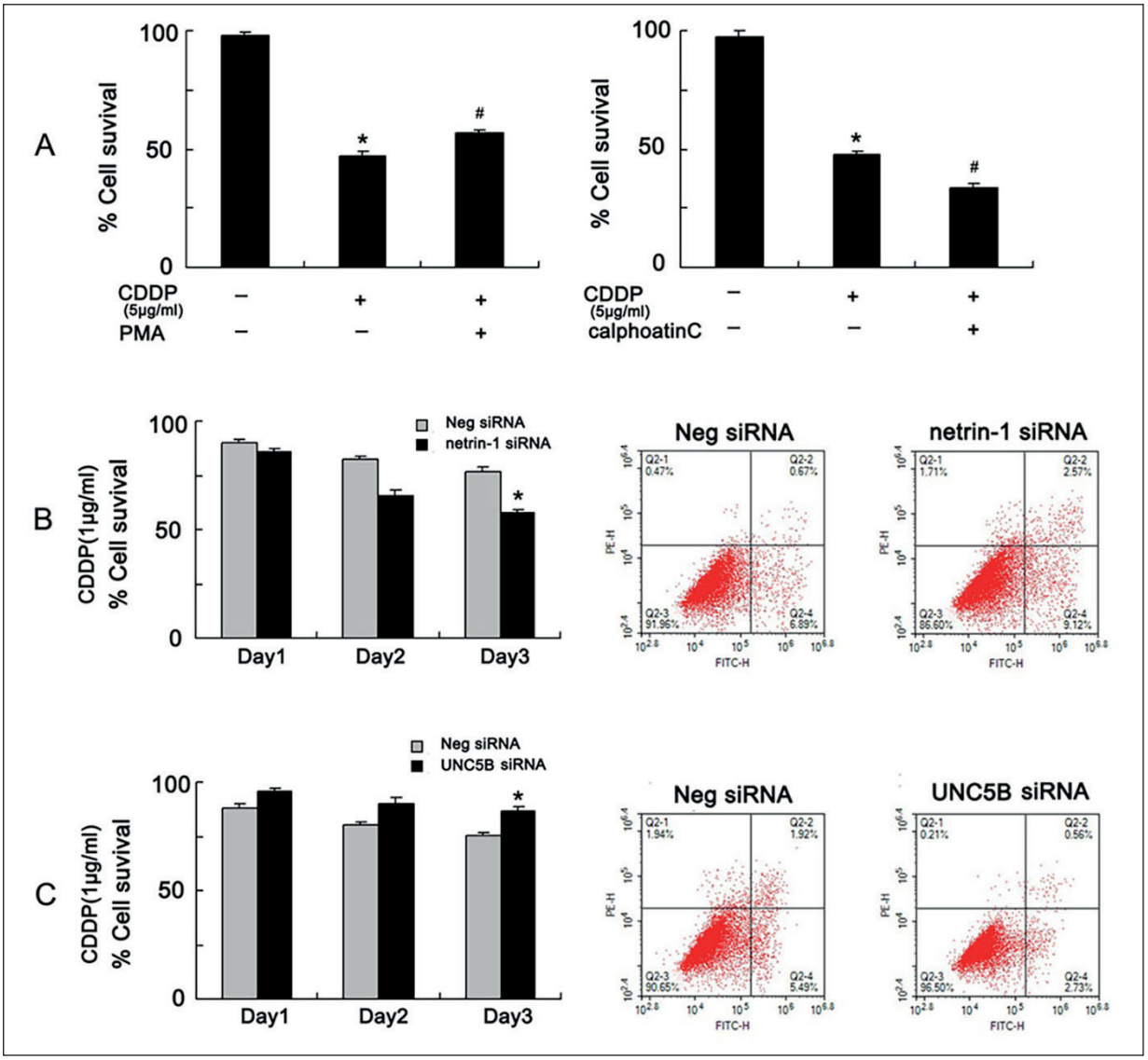

Figure 1. Effects of PKCα, netrin-1 and UNC5B on chemoresistance of bladder cancer cells. **A,** *Indicates (CDDP+/PMA- *vs.* CDDP-/PMA-) or (calphoatin C+/PMA- *vs.* calphoatin C-/PMA-); #Indicates (CDDP+/PMA+ *vs.* CDDP+/PMA-) or (calphoatin C+/PMA+ *vs.* calphoatin C+/PMA-). **B,** *Indicates netrin-1 siRNA *vs.* Neg siRNA; **C,** *Indicates UNC5B siRNA *vs.* Neg siRNA.

of cisplatin on cancer cells was further strengthened (as shown in Figure 1A). Similarly, we knocked out netrin-1 and UNC5B in the cancer cell lines and found that, after netrin-1 knockdown, the level of apoptosis induced by cisplatin was higher than that of control group, and the cell survival rate was lower than that of control group (as shown in Figure 1B).

After the knockdown of UNC5B, the level of apoptosis induced by cisplatin was lower than that of control group, and the cell survival rate was higher than that of control group (as shown in Figure 1C). These experimental results show that the level of PKCα, netrin-1, and UNC5B in bladder cancer cells can regulate the resistance of those cancer cells to cisplatin.

PKCα Regulated the Level of Netrin-1 and UNC5B in Bladder Cancer Cells

If PKCα, netrin-1, and UNC5B are able to regulate bladder cancer cells resistant to cisplatin, then what is the relation between them? To verify the regulation of netrin-1 and UNC5B expression by PKCα, we detected the protein expression of netrin-1 and UNC5B in bladder cancer cells by Western blot. Results showed

that netrin-1 was up-regulated and UNC5B was down-regulated in cells after the PKCα activity was enhanced. However, the expression of netrin-1 was down-regulated and the expression of UNC5B was up-regulated when PKCα activity was inhibited (Figure 2A).

PKCα Promotes the Binding of Netrin-1 to Its Receptor UNC5B in Bladder Cancer Cells

It has been reported that netrin-1 and its receptor UNC5B affect tumorigenesis and development through the interaction of netrin-1 and its receptor in many malignant tumors. However, whether PKCα affects cell resistance to cisplatin by regulating the interaction between netrin-1 and its receptor UNC5B it remains unknown. We analyzed it with co-immunoprecipitation. The results show that enhanced PKCα activity can promote netrin-1 and its receptor UNC5B mutual binding; and PKCα activity decreased netrin-1 and UNC5B binding (Figure 2B).

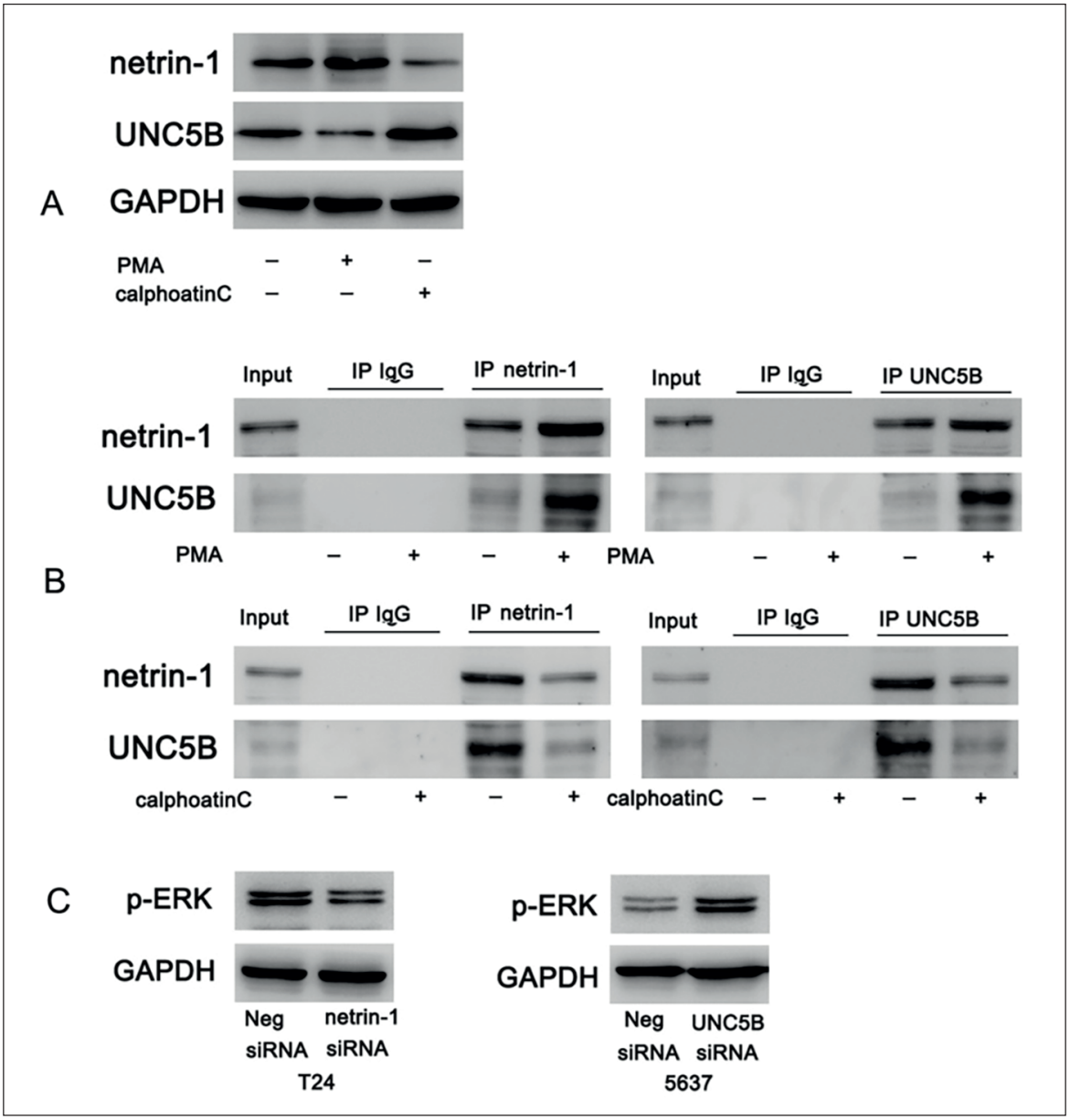

Figure 2. A, PKCα regulated the level of netrin-1 and UNC5B in bladder cancer cells. **B,** PKCα promotes the binding of netrin-1 to its receptor UNC5B in bladder cancer cells **C,** Netrin-1 and its receptor UNC5B regulated the activity of PKCα in bladder cancer cells. PKCα, netrin-1 and UNC5B mutual regulation.

Netrin-1 and Its Receptor UNC5B Regulated the Activity of PKCα in Bladder Cancer Cells

It is noteworthy that the expression levels of netrin-1 and its receptor UNC5B in bladder cancer cells can, in turn, regulate the activity level of PKCα and its downstream ERK1/2 signaling pathway in cells. Western blot results showed that the levels of PKCα and p-ERK1/2 in cells were decreased after the knockdown of netrin-1 in bladder cancer cells. However, the knockdown of UNC5B in cells resulted in increased levels of PKCα and p-ERK1/2 (Figure 2C).

Discussion

Protein kinase (PKC) is an important member of the protein kinase family and is an important part of the cell growth regulatory information pathway. PKCα is one of the most common subtypes in PKC. It is a type of Ca^{2+} and phospholipid-dependent Ser/Thr protein kinase that plays an important role in transmembrane signal transduction and affects the phosphorylation of multiple proteins cell proliferation, metastasis, apoptosis, and other physiological processes[5-8]. In this study, it was found that PKCα agonist can weaken the induction of apoptosis induced by cisplatin in bladder cancer cells, while the PKCα inhibitor can further induce the apoptosis induced by cisplatin in bladder cancer cells. The high level of activity in cells increases the resistance of cells to cisplatin, whereas the low activity of PKCα enhances the sensitivity of cells to cisplatin.

Netrins family is a class of soluble secreted protein, netrin-1 and is the earliest discovered axon guidance factor, called neurotransmitter. Netrin-1 and its receptor UNC5B have been found to play an important role in cell proliferation, apoptosis, and migration[9-12]. In recent years, their role in tumorigenesis has drawn more attention. In this work, netrin-1 and UNC5B were found to be involved in the regulation of drug resistance of bladder cancer cells. After knocking down netrin-1, the apoptosis rate of bladder cancer cells induced by cisplatin was increased and the survival rate was decreased. After UNC5B knockdown, cisplatin of bladder cancer cells apoptosis rate decreased, the survival rate increased. In bladder cancer cells, netrin-1 was up-regulated and UNC5B down-regulated the cell resistance. Netrin-1 was down-regulated and UNC5B up-regulated the chemosensitivity of cells.

PKCα and netrin-1/UNC5B play a regulatory role in the regulation of bladder cancer cell resistance. We found that PKCα agonist up-regulated the expression of netrin-1 and down-regulated the expression of UNC5B. PKCα inhibitor can down-regulate the expression of netrin-1 and up-regulate the expression of UNC5B. PKCα also promotes the binding of netrin-1 to its receptor UNC5B. In our previous research[13], netrin-1 and its receptor UNC5B were found to increase PKCα activity. We speculate that PKCα and netrin-1/UNC5B form a positive feedback regulation loop in the regulation of drug resistance in bladder cancer cells. To further validate this hypothesis, we knocked out netrin-1 and UNC5B in bladder cancer cells and analyzed the activity of PKCα and its downstream ERK signaling pathway in cells. Results showed that the activity of PKCα and its downstream ERK signaling pathway were inhibited after netrin-1 knockdown, while UNC5B knocking out activated PKCα and its downstream ERK signaling pathway, indicating that netrin-1/UNC5B can, in turn, regulate the activity of PKCα in bladder cancer cells.

Conclusions

The results of this study indicated that PKCα and netrin-1/UNC5B form a positive feedback regulatory loop; PKCα high activity, netrin-1 high expression, and UNC5B low expression increased bladder cancer chemoresistance. PKCα low activity, netrin-1 low expression, and UNC5B high expression increased the chemosensitivity of bladder cancer cells.

Conflict of Interest

The Authors declare that they have no conflict of interests.

Funding Acknowledgements

The present study was supported by the National Natural Science Foundation of China (grant No. 81402088).

References

1) Yu QF, Liu P, Li ZY, Zhang CF, Chen SQ, Li ZH, Zhang GY, Li JC. MiR-103/107 induces tumorigenicity in bladder cancer cell by suppressing PTEN. Eur Rev Med Pharmacol Sci 2018; 22: 8616-8623.

2) Joseph N, Choudhury A. Adjuvant chemotherapy is more suitable than neoadjuvant chemotherapy for muscle invasive bladder cancer patients treated with radical chemoradiotherapy. Int J Radiat Oncol Biol Phys 2016; 96: 614-616.

3) Pouessel D, Bastuji-Garin S, Houédé N, Vordos D, Loriot Y, Chevreau C, Sevin E, Beuzeboc P, Taille A, Le Thuaut A, Allory Y, Culine S. Adjuvant chemotherapy after radical cystectomy for urothelial bladder cancer: outcome and prognostic factors for survival in a french multicenter, contemporary cohort. Clin Genitourin Canc 2017; 15: e45-e52.

4) Sonpavde G, Gordetsky JB, Lockhart ME, Nix JW. Chemotherapy for muscle-invasive bladder cancer: better late than never? J Clin Oncol 2016; 34: 780-785.

5) Diaz-Cueto L, Arechavaleta-Velasco F, Diaz-Arizaga A, Dominguez-Lopez P, Robles-Flores M. PKC signaling is involved in the regulation of progranulin (acrogranin/PC-cell-derived growth factor/granulin-epithelin precursor) protein expression in human ovarian cancer cell lines. Int J Gynecol Cancer 2012; 22: 945-950.

6) Karp G, Maissel A, Livneh E. Hormonal regulation of PKC: estrogen up-regulates PKCeta expression in estrogen-responsive breast cancer cells. Cancer Lett 2007; 246: 173-181.

7) Koivunen J, Aaltonen V, Peltonen J. Protein kinase C (PKC) family in cancer progression. Cancer Lett 2006; 235: 1-10.

8) Urtreger AJ, Kazanietz MG, Bal de Kier Joffé ED. Contribution of individual PKC isoforms to breast cancer progression. IUBMB Life 2012; 64: 18-26.

9) Kong C, Zhan B, Piao C, Zhang Z, Zhu Y, Li Q. Overexpression of UNC5B in bladder cancer cells inhibits proliferation and reduces the volume of transplantation tumors in nude mice. BMC Cancer 2016; 16: 892.

10) Mediero A, Wilder T, Ramkhelawon B, Moore KJ, Cronstein BN. Netrin-1 and its receptor Unc5b are novel targets for the treatment of inflammatory arthritis. FASEB J 2016; 30: 3835-3844.

11) Gopal AA, Rappaz B, Rouger V, Martyn IB, Dahlberg PD, Meland RJ, Beamish IV, Kennedy TE, Wiseman PW. Netrin-1-regulated distribution of UNC5B and DCC in live cells revealed by TICCS. Biophys J 2016; 110: 623-634.

12) Lv J, Sun X, Ma J, Ma X, Zhang Y, Li F, Li Y, Zhao Z. Netrin-1 induces the migration of Schwann cells via p38 MAPK and PI3K-Akt signaling pathway mediated by the UNC5B receptor. Biochem Biophys Res Commun 2015; 464: 263-268.

13) Liu J, Kong CZ, Gong DX, Zhang Z, Zhu YY. PKC α regulates netrin-1/UNC5B-mediated survival pathway in bladder cancer. BMC Cancer 2014; 14: 93.