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Knockout of phospholipase Cε attenuates *N*-butyl-*N*-(4-hydroxybutyl) nitrosamine-induced bladder tumorigenesis

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Abstract. Bladder cancer frequently shows mutational activation of the oncogene Ras, which is associated with bladder carcinogenesis. However, the signaling pathway downstream of Ras remains to be fully elucidated. *N*-butyl-*N*-(4-hydroxybutyl) nitrosamine (BBN) is able to induce bladder cancer by driving the clonal expansion of initiated cells carrying the activated form of Ras. Phospholipase Cε (PLCε) is the main target of BBN, while the tumor promoting role of PLCε remains controversial. The present study examined the role of PLCε in BBN-induced bladder carcinogenesis of mice with genetically inactivated PLCε. Using light and electron microscopy, the present study demonstrated that PLCε$^{-/-}$ mice were resistant to BBN-induced bladder carcinogenesis. Furthermore, it was demonstrated that cyclooxygenase 2 and vascular endothelial growth factor-A were affected by the PLCε background of the mice, suggesting that the role of PLCε in tumor promotion may be ascribed to augmentation of inflammatory responses and angiogenesis. These results indicated that PLCε is crucial for BBN-induced bladder carcinogenesis as well as signaling downstream of Ras, and that PLCε is a candidate molecular target for the development of anti-cancer drugs.

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Introduction

Bladder cancer is one of the most common tumor types of the urinary system. However, its etiopathogenisis has remained to be fully elucidated. Study of the multifocal and polyclonal origins of bladder cancer as well as various experiments and epidemiological studies have shown that the persistence of carcinogenic substances in urine is an important cause of bladder cancer and also a major reason for bladder tumor recurrence after treatment (1-3).

The activation of numerous small guanine triphosphatases (GTPases) of the Ras superfamily is a crucial step in the regulation of a variety of cellular processes via complex cellular signaling networks. Phosphatidylinositol-specific phospholipase C (PLC) has emerged as one of the important signaling nodes in these complex networks, acting as a target as well as a regulator of small GTPases. Six major families of PLC (PLCβ, PLCγ, PLCδ, PLCε, PLCζ and PLCη) are known (4), which are characterized by regulatory regions unique to each family. PLCε is a recent addition to the growing list of Ras-associated effectors (5). Most suggested Ras effectors show either kinase or guanine nucleotide exchange factor activity. However, Ras has been implicated in regulating another class of enzyme, namely phosphoinositide-specific PLC, which is involved in the generation of well-characterized secondary messengers.

Studies have demonstrated that PLCε is an effector of the Ras family of small GTPases, which bind directly to the Ras-associated domain of PLCε (6-9). Further studies have revealed that PLCε is also activated by small GTPase Ras homolog gene family, member A as well as heterotrimeric G proteins Gα12 and Gβ1γ2 (9,10). Via these multiple regulatory mechanisms, PLCε mediates signals originating from a large variety of cell surface receptors (11,12). In addition, PLCε exerts a function as a guanine nucleotide exchange factor for Ras-related protein 1 (Rap1) via its CDC25 homology domain (13). Mice homozygous regarding the functionally inactivated PLCε allele (PLCε$^{-/-}$ mice) exhibit semilunar valvulogenetic defects, which lead to cardiac dilation (14-16). Bai *et al* (17) demonstrated that PLCε$^{-/-}$ mice were resistant

Table I. Statistics of control mice and mouse models of bladder cancer.

Group	Type	Treatment	Normal n (%)	Atypical hyperplasia, n (%)	Transitional cell carcinoma, n (%)	Dead animals (n)	Surviving animals (n)
A	PLCε$^{+/+}$	Control, none	24 (100)	0 (0)	0 (0)	1	23
B	PLCε$^{+/+}$	BBN (0.1 %)[a]	8 (18.18)	8 (18.18)	28 (63.64)	4	44
C	PLCε$^{-/-}$	Control, none	24 (100)	0 (0)	0 (0)	0	24
D	PLCε$^{-/-}$	BBN (0.1 %)[a]	12 (26.09)	14 (30.43)	20 (43.48)	2	46

[a]Administered via drinking water supplementation. BBN, *N*-butyl-*N*-(4-hydroxybutyl) nitrosamine; PLC, phospholipase C.

to 7,12-dimethylbenz(a)anthracene-induced skin tumor formation with 12-O-tetradecanoylphorbol-13-acetate (TPA) as a tumor promoter. In addition, PLCε$^{-/-}$ mice were void of basal layer cell proliferation and epidermal hyperplasia, suggesting the role of PLCε in tumor promotion.

The Ras gene is one of the most common genes associated with bladder cancer. It has been confirmed that PLCε is an effector of Ras and is in turn regulated by Ras in a GTP-dependent manner. The association between bladder transitional cell carcinoma and PLCε has not been fully elucidated. The present study hypothesized that PLCε has a significant role in the development of bladder transitional cell carcinoma. In order to verify this hypothesis, the effects of PLCε knockdown on *N*-butyl-*N*-(4-hydroxybutyl) nitrosamine (BBN)-mediated induction of bladder cancer were investigated in mice. BBN is able to induce bladder cancer by targeting PLCε and activating Ras to drive the clonal expansion of initiated cells (18). The present study suggested that PLCε has a crucial role in the development of cancer downstream of Ras signaling.

Materials and methods

Animals. PLCε$^{-/-}$ mice were obtained from the Laboratory of Experimental Molecular Biology at Kobe University (Kobe, Japan) and maintained at the 302 Hospital of the Chinese People's Liberation Army (Beijing, China). PLCε$^{-/-}$ homozygous mice and wild-type (PLCε$^{+/+}$) littermates were obtained by crossing C57BL/6J PLCε$^{+/-}$ and 129S4 PLCε$^{+/-}$ mice. Mice carrying inactivated PLC allele (PLCε^{-}) were generated using in-frame deletion of an exon encoding the catalytic X domain, as described previously (14). All animals were housed under standard conditions (22±1°C; 12-h light/dark cycle; 50-55% humidity) with free access to food pellets and tap water. Experiments were performed on 6-8 week-old male mice, and a total of 72 PLCε$^{+/-}$ and 72 PLCε$^{+/-}$ mice were used in the present study. The mean weight of the animals was 20.3±0.1 g for the PLCe^{+}/$^{+}$ mice and 20.3±0.15 g for the PLCe^{-}/$^{-}$ mice. Disparate groups of mice were used in each experiment. Animal experiments were approved by the Animal Care and Use Committee of The 463 hospital of Chinese People's Liberation Army (Shenyang, China).

Materials. Antibodies used included anti-mouse PLCε antibody (Dako Cytomation, Copenhagen, Denmark), rabbit anti-mouse polyclonal anti-vascular endothelial growth factor-A (VEGF-A) antibody (cat. no. ab51745; Abcam, Cambridge, MA, USA), rabbit anti-mouse polyclonal anti-GAPDH antibody (cat. no. sc-25778; Santa Cruz Biotechnologies, Dallas, TX, USA), rabbit anti-mouse anti-cyclooxygenase-2 (COX-2) antibody (cat. no. 160126; Cayman, Ann Arbor, MI, USA) and goat anti-rabbit immunoglobulin G-horseradish peroxidase conjugated secondary antibodies (Santa Cruz Biotechnologies).

BBN-induced mouse model of bladder cancer. A BBN-induced mouse model of bladder cancer was prepared according to the method described by Vecchione *et al* (19) with certain modifications. Briefly, six-week-old male PLCε^{-}/$^{-}$ mice (n=72) and PLCε^{+}/$^{+}$ mice (n=72) were sub-divided into BBN treatment groups (n=48) and control groups (n=24) without treatment of BBN (Table I). BBN-treated mice were given tap water containing 0.1% BBN for 12 weeks. Thereafter, they had access to tap water without BBN. Control mice were given water without BBN throughout the experiment. Mice were sacrificed at 8, 12 and 18 weeks after the cessation of BBN treatment. Bladder specimens were harvested and analyzed for pathology [hematoxylin-eosin (HE; Beyotime Institute of Biotechnology, Shanghai, China) staining and ultrastructural assessment] and protein (western blot and immunofluorescence).

Pathological analysis. The mice were anesthetized with sodium pentobarbitone (40 mg/kg, i.p.; Sigma-Aldrich, St. Louis, MO, USA) and then transcardially perfused with 10 ml 0.9% saline, followed by 30 ml 0.1 M phosphate buffer (PB; pH 7.4) containing 4% paraformaldehyde for 5 min. At necropsy, urinary bladders were removed and placed in 4% paraformaldehyde for HE staining immunofluorescence or in 2.5% glutaraldehyde (Sigma-Aldrich) for ultrastructural study. For HE staining, each bladder was dissected, processed for routine paraffin embedding, cut into 5 μm sections and mounted onto polylysine-coated slides. Sections were de-waxed in xylene (Sinopharm, Shanghai, China), re-hydrated in a descending series of ethanols and processed for routine HE. For ultrastructural study, sections were washed with 0.01 M phosphate-buffered saline (PBS), post-fixed with 10 g/l OsO_4 in PB for 45 min, de-hydrated through a graded ethanol series and propylene oxide, and flat-embedded with Epon-812 (Nanjing Tansi Technology Co., Ltd., Nanjing, China). The sections were examined under a light microscope (AE31; Motic, Xiamen, China) and regions containing all layers of the bladder were investigated under an electron

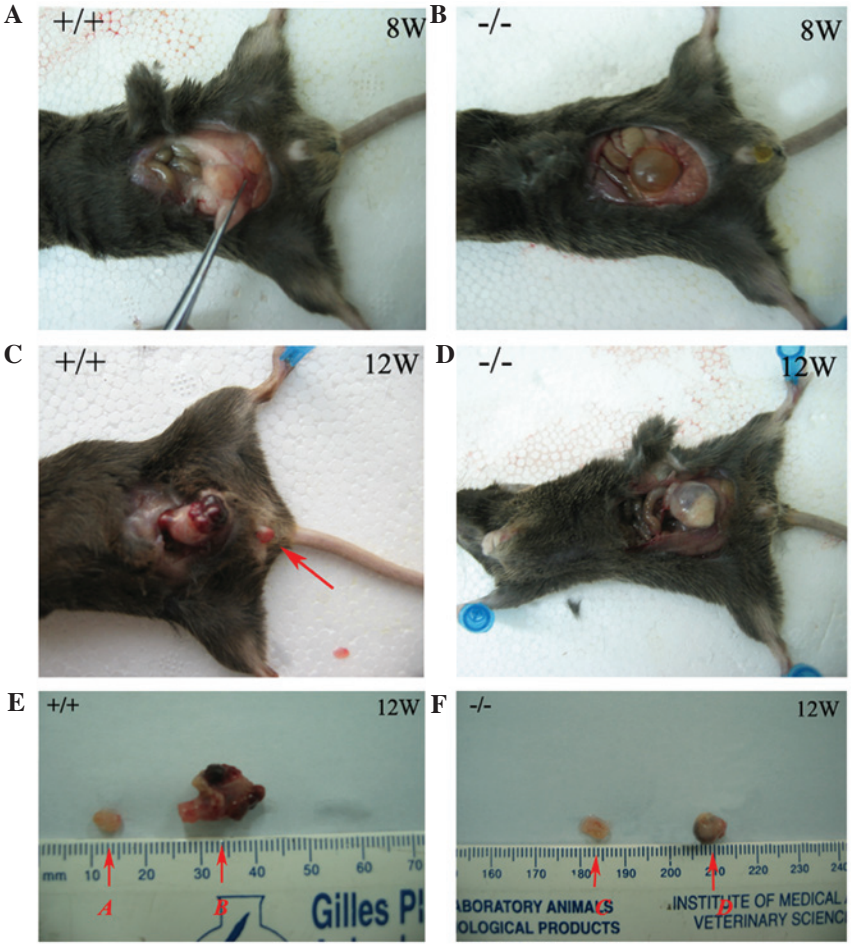

Figure 1. Bladder anatomy of mice in the experimental groups. Representative images of (A) mice in Group B at week eight, (B) mice in Group D at week eight, (C) mice in Group B at week 12 (the arrow indicates hematuria) and (D) mice in Group D at week 12. (E) Bladder tumors of Group A (left) and B (right) at week 12. (F) Bladder tumors of Group C (left) and D (right) at week 12. Groups: A, untreated PLCε$^{+/+}$ control mice; B, PLCε$^{+/+}$ mice induced with 0.1% BBN; C, untreated PLCε$^{-/-}$ mice; D, PLCε$^{-/-}$ mice induced with 0.1% BBN. BBN, *N*-butyl-*N*-(4-hydroxybutyl) nitrosamine; PLC, phospholipase C.

microscope. Tissue samples from the selected regions were cut into sections on an ultramicrotome (EM UC7; Leica, Wetzlar, Germany) and prepared for electron microscopic analysis (H-7100; Hitachi, Tokyo, Japan).

Western blot analysis. Protein was extracted from each sample using radioimmunoprecipitation assay lysis buffer (Beyotime Institute of Biotechnology). The concentrations of proteins were detected using the bicinchoninic acid assay (Beyotime Institute of Biotechnology). Protein samples were separated by 10% SDS-PAGE and then transferred onto nitrocellulose membranes. Then membranes were blocked with 5% non-fat milk followed by incubation with antibodies against COX2 (1:500), VEGF-A (1:5,000) and GAPDH (1:1,000), respectively, at 37˚C for another 1.5 h. Membranes were subsequently washed with Tris-buffered saline containing Tween 20 (TBST) and incubated with the corresponding secondary antibodies conjugated with horseradish peroxidase (1:5,000) for 45 min at 37˚C. Following washing with TBST, a RapidStep™ ECL reagent (Millipore, Bedford, MA, USA) and x-ray film were used to capture images of the blots.

Immunofluorescence. Detection of PLCε, COX2 and VEGF-A was performed on consecutive sections obtained from paraffin-embedded tissues using an immunofluorescence double labeling method. Sections were dried at room temperature, de-paraffinized, re-hydrated and then treated with 2% hydrogen peroxide at 37˚C for 5 min. Antigen retrieval was performed by pepsin (Biosharp, Hefei, China) treatment at 40˚C for 10 min. After blocking with 5% bovine serum albumin, the sections were incubated with anti-PLCε and COX2 (1:200) or VEGF-A antibody (1:500) overnight at 4˚C. Sections were washed with 0.01 M PBS and incubated with fluorescein isothiocyanate-labeled anti-goat immunoglobulin (Ig)G antibody (1:500; Sigma-Aldrich) for PLCε and Texas Red-labeled anti-rabbit IgG antibody (1:1,000; Molecular Probes, Breda, Netherlands) for COX2 and VEGF-A at 37˚C for 2 h. The sections were thoroughly rinsed in 0.01 M PBS between and after the incubation steps. The sections were examined using confocal laser-scanning microscopy (FV1000; Olympus, Tokyo, Japan). Digital images were captured using EZ-C1 3.50 software (Nikon).

Statistical analysis. All statistical analyses were performed using SPSS 10.0 software (SPSS, Inc., Chicago, IL, USA). The Kaplan-Meier test was used to analyze survival data. The incidence and multiplicity of urinary bladder cancer were analyzed by the Fisher's exact and Wilcoxon's rank-sum tests,

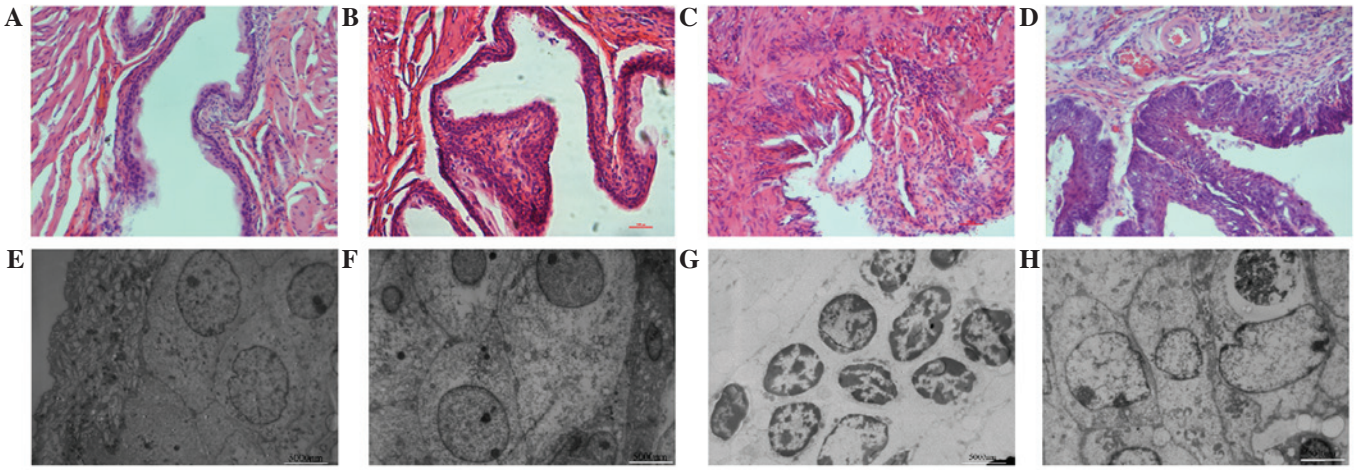

Figure 2. Representative histological HE staining images and electron micrographs of bladder mucosa of the mice in the experimental groups at 12 weeks. HE staining of normal bladder mucosa in (A) group A and (B) group C. (C) In group B, HE staining revealed significantly heterogenous bladder mucosa. (D) HE staining of bladder mucosa in group D, revealing thickened, carcinomatous bladder mucosa (magnification, x200). Ultrastructures of bladder mucosa of mice in (E) group A and (F) group C. (G) Ultrastructure of transitional epithelial membrane cells of mice of group B. The chromatin was distributed across the nuclear membrane, nucleolar hypertrophy was observed and fiber structure around the nuclei could not be distinguished. (H) Ultrastructure of bladder mucosa of mice in group D, revealing significantly heterogenous transitional cells in the mucosa. Scale bar, 5000 nm. Groups: A, untreated PLCε$^{+/+}$ control mice; B, PLCε$^{+/+}$ mice induced with 0.1% BBN; C, untreated PLCε$^{-/-}$ mice; D, PLCε$^{-/-}$ mice induced with 0.1% BBN. BBN, *N*-butyl-*N*-(4-hydroxybutyl) nitrosamine; PLC, phospholipase C; HE, hematoxylin and eosin.

respectively. Each experiment was repeated three times and representative results are presented.

Results

General observations. In the present study, one hundred and forty-four mice were used, out of which seven died during the experiment (5%; Group A, 1; group B, 4; group D, 2). These mice showed no significant difference from others in weight and activity levels prior to their death. A higher susceptibility of PLCε$^{+/+}$ mice to BBN-induced tumor development was observed. The tumors of PLCε$^{-/-}$ mice were smaller than those of PLCε$^{+/+}$ mice at all time-points examined (Fig. 1A-D). In Group B, eight mice (18.18%) had normal urinary bladder mucous membranes, eight mice (18.18%) showed atypical hyperplasia and 63.6% (28/44) presented with neoplastic lesions (with *in situ* or invasive carcinoma), and in group D, these numbers were 26.09% (12/46), 30.43% (14/46) and 43.5% (20/46), respectively (Table I). Pre-neoplastic lesions in the urothelium adjacent to advanced tumors were frequently observed. By contrast, in the control groups A and C, atypical hyperplasia and neoplastic lesions were not observed. The size of the tumors in group B was significantly larger than that in group D (Fig. 1E and F). No significant differences were observed between groups A and C.

Pathological changes. In the present study, pathological changes in the development of BBN-induced bladder carcinoma in PLCε$^{-/-}$ and PLCε$^{+/+}$ mice were monitored. The following pathological changes were observed by light microscopy: Smooth mucosa of bladder walls were present in groups A and C without the presence of ulcers, congestion or neoplasms (Fig. 2A and B). The morphology of the mucosa of the bladder walls in groups B and D changed gradually with increasing time of BBN intake. In general, tumor formation

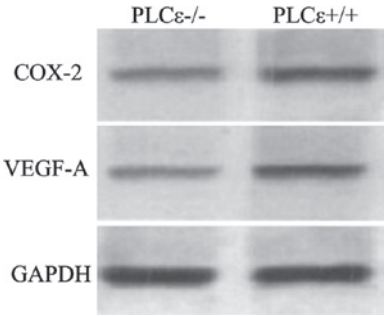

Figure 3. Western blot analysis for COX-2 and VEGF-A protein expression in bladder tumors from experimental mice at week 18 treated with *N*-butyl-*N*-(4-hydroxybutyl) nitrosamine. GAPDH was used as a reference. A representative of three blots is shown. COX, cyclooxygenase; VEGF, vascular endothelial growth factor; PLC, phospholipase C.

in PLCε$^{-/-}$ mice was delayed and its incidence was reduced compared with that in PLCε$^{+/+}$ mice. Electron microscopy further confirmed the morphological findings in the experimental groups: While no differences in morphology of the bladder transitional epithelium were observed between groups A and C (Fig. 2E and F), the following pathological changes were visible in group B at week 12: Chromatin was distributed across the nuclear membrane, nucleolar hypertrophy was present and fiber structure around the nuclei was disordered (Fig. 2G). However, heterogenous transitional cells were observed in the mucosa of mice in group D at week 12 (Fig. 2H). All of the above results demonstrated that knockout of PLCε attenuated BBN-induced bladder tumorigenesis.

Role of PLCε in BBN-induced expression of inflammatory and angiogenesis-associated molecules. In order to investigate the underlying mechanisms of the involvement of PLCε in tumor development, the present study examined

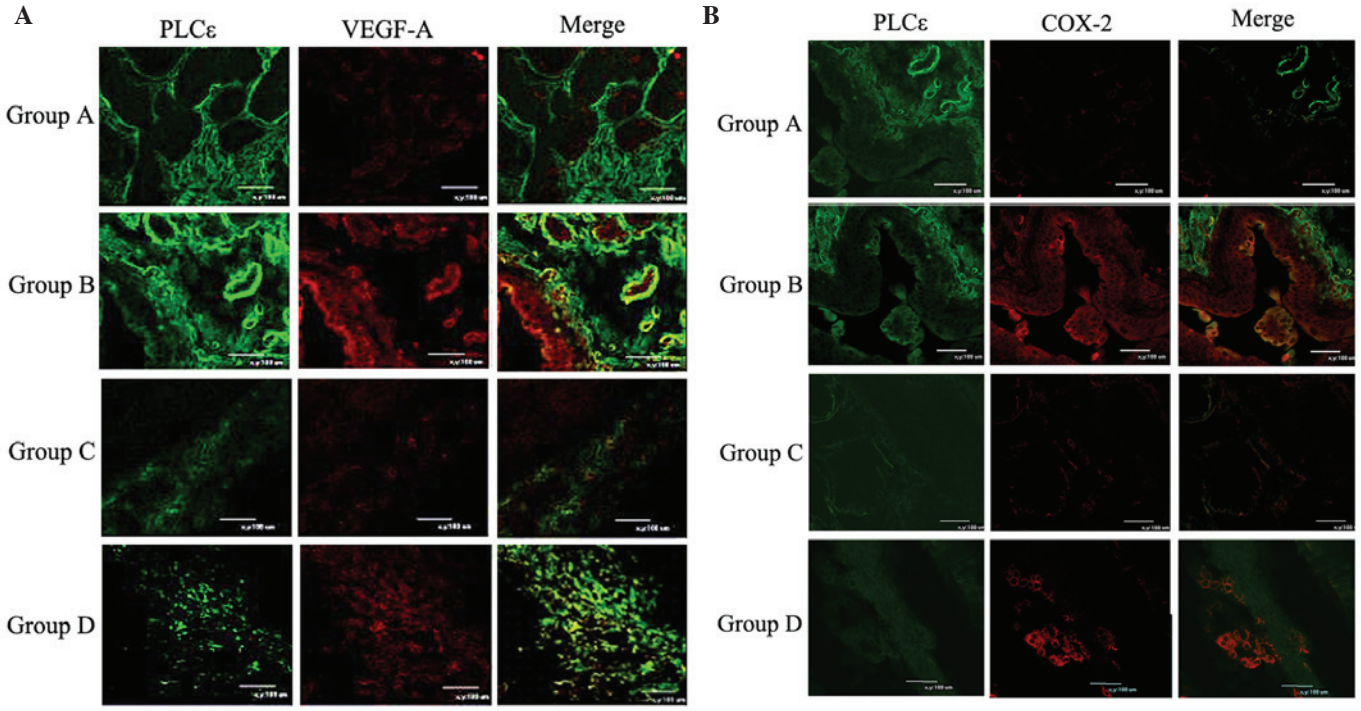

Figure 4. Representative immunofluorescence images. (A) Expression of VEGF-A in the bladder mucosa of mice in Groups A, B, C and D at week 18. Green fluorescence, PLCε; red fluorescence, VEGF-A. (B) Expression of COX-2 in the bladder mucosa of mice in Groups A, B, C and D at week 18. Green fluorescence, PLCε; red fluorescence, COX-2. Scale bar, 100 μm. Groups: A, untreated PLCε$^{+/+}$ control mice; B, PLCε$^{+/+}$ mice induced with 0.1% BBN; C, untreated PLCε$^{-/-}$ mice; D, PLCε$^{-/-}$ mice induced with 0.1% BBN. BBN, *N*-butyl-*N*-(4-hydroxybutyl) nitrosamine; PLC, phospholipase; C COX, cyclooxygenase; VEGF, vascular endothelial growth factor.

the expression of representative inflammatory and angiogenesis-associated proteins in tumors from the bladders of mice in groups B and D. Previous studies have revealed that PLCε is important in TPA-induced skin inflammation and tumor promotion (17,20). Inflammation is known to exert its highest effects on tumorigenesis in the later stage, in which tumors progress to high-grade adenomas (21-23). Therefore, the present study investigated the role of PLCε in inflammation associated with late-stage bladder tumorigenesis. In grade-matched tumors from PLCε$^{+/+}$ and PLCε$^{-/-}$ mice at week 18, the expression of COX-2 and VEGF-A was assessed; these proteins were selected to be analyzed as representative signaling molecules due to their intrinsic functions in inflammation and angiogenesis, respectively, and because they have been implicated in bladder tumorigenesis (24). Western blot analysis revealed that the expression levels of COX-2 and VEGF-A were influenced by the PLCε background of the mice, with upregulation in the BBN-treated PLCε$^{+/+}$ mice, but not in BBN-treated PLCε$^{-/-}$ mice (Fig. 3). In addition, the expression of COX-2 and VEGF-A was detected by immunofluorescence analysis. Groups A and B showed stronger green fluorescence (PLCε) than groups C and D, which was consistent with their genetic backgrounds. Consistent with the western blot results, marked increased in the VEGF-A signals in tumor epithelial cells of high-grade adenomas in PLCε$^{+/+}$ mice were observed, which were less apparent in PLCε$^{-/-}$ mice (Fig. 4A). The expression patterns of COX-2 were similar to those of VEGF-A, with increased expression in groups B and D (Fig. 4B). However, a locally elevated level of COX-2 was also identified. These results demonstrated that the protective role of PLCε knockout against bladder carcinogenesis may be associated with inflammatory responses and angiogenesis.

Discussion

Numerous chemicals are known to evoke bladder carcinoma, among which nitroso compounds, including BBN and *N*-methyl-*N*-nitrosourea (MNU), and nitrofuran compounds, such as *N*-[4-(5-nitro-2-furyl)-2-thiazolyl]-formamide (FANFT) are most potent (25,26). BBN and FANFT are indirect carcinogens through oral intake, while MNU is a carcinogen requiring direct bladder instillation. Due to its high potency to induce bladder cancer, BBN is the most suitable reagent to generate *in vivo* models of bladder cancer and to study bladder carcinogenesis. The carcinogenicity of BBN is limited to the bladders of rats, mice and dogs (27). No marked difference between the bladder cancer was observed between humans and mice, and rats and dogs. Therefore, BBN-induced bladder cancer is similar to transitional cell carcinoma in patients in both kinetic and histological features (28). PLCε is important in the development and progression of human cancer types (29). The present study used BBN to induce bladder cancer, and knockout of PLCε attenuated BBN-induced tumorigenesis of bladder cancer. This indicated that PLCε is an oncogene and may be a therapeutic target for the treatment and prevention of bladder cancer. The downstream metabolite of BBN, *N*-butyl-*N*-(3-carboxy-propyl) nitrosamine (BCPN), is subjected to urinary excretion and comes in direct contact with the urinary tract, resulting in epithelial cell DNA damage and carcinogenesis (30). Compared with a high, single dose of BBN, smaller and multiple doses of BBN induce a higher rate

of bladder cancer with larger tumor size, poorer differentiation and a higher rate of infiltration.

Bladder carcinoma is the most common tumor type of the urinary system. Its occurrence and development involve numerous genes and processes, as well as congenital and acquired factors (31-33). Ras was the first oncogene identified in human bladder carcinoma and has been proved to be highly relevant to its development (34-36). PLCε, as a downstream effector protein of Ras, may have an important role in the occurrence and development of bladder carcinoma.

PLCε was first identified in *Caenorhabditis elegans* by Shibatohge *et al* (37) in 1998 as a novel sub-type of the PLC family. PLCε has been reported to act as an effector protein for the products of the oncogene Ras and the tumor suppressor gene Rap (5,7,8). In recent years, the role of PLCε in tumors has received increasing attention. PLCε$^{-/-}$ mice were successfully established in Kataoka's laboratory at Kobe University (Kobe, Japan) by Bai *et al* (17). In these PLCε$^{-/-}$ mice, the tumor incidence was significantly decreased and the progression of chemically induced skin tumors was inhibited (17), suggesting that PLCε has an important role in tumor development. Consistent with the hypothesis of the present study, Bourguignon *et al* (38) found that PLCε is involved in human head and neck squamous cell carcinoma (38); furthermore, Cheng *et al* (39) and Ling *et al* (40) reported that small hairpin RNA-mediated knockdown of PLCε inhibited bladder cancer cell proliferation and cell cycle progression *in vitro*. PLCε was also demonstrated to be association with invasion and migration of bladder cancer (41).

The present study observed almost complete squamous commitment of the neoplastic lesions. After 18 weeks of BBN treatment, 43.48% of PLCε-knockout mice developed invasive bladder cancer, which was significantly lower than the incidence observed in wild-type mice. This result is consistent with the notion that wild-type PLCε mice develop bladder tumors more rapidly and frequently after chronic intake of BBN than PLCε$^{-/-}$ mice, further suggesting that the presence of PLCε sensitizes tissues to carcinogenesis. The present study also observed that the incidence of atypical hyperplasia in PLCε$^{-/-}$ mice was significantly higher than that observed in wild-type mice. This indicates that wild-type mice are pre-disposed to developing bladder cancer. Differences in the incidence of bladder tumors between these groups of mice indicated that urothelial cells require two intact PLCε alleles in order to respond efficiently to the damaging action of chemical carcinogens.

PLCε has been demonstrated to be associated with inflammatory responses (42). Ikuta *et al* (20) have shown that two TPA targets, Ras guanyl-releasing protein 3 and protein kinase C, are involved in TPA-induced inflammation through the activation of PLCε, leading to tumor promotion. Li *et al* (43) suggested that PLCε has crucial roles in intestinal tumorigenesis through two distinct mechanisms - augmentation of angiogenesis and inflammation. In order to determine whether PLCε has a role in bladder tumorigenesis through augmentation of angiogenesis and inflammation in BBN-induced bladder cancer, the present study also examined inflammatory and angiogenesis-associated factors COX-2 and VEGF-A, respectively. The results showed that VEGF-A was upregulated by BBN treatment in the tumors of PLCε$^{+/+}$ mice, while no marked changes in PLCε$^{-/-}$ mice were observed. COX-2 showed a similar pattern to that of VEGF-A, however, with local elevation. These results suggested that bladder cancer is induced by BBN through two distinct mechanisms, augmentation of angiogenesis and inflammation, and that PLCε has a pivotal role in tumorigenesis of bladder cancer induced by BBN.

In conclusion, the present study revealed that PLCε has a crucial role in BBN-induced carcinogenesis of bladder epithelial cells, as knockout of PLCε attenuated bladder carcinogenesis induced by BBN. The present study also provided solid *in vivo* evidence for the importance of PLCε signaling in carcinogenesis. These results indicated that specific inhibitors of PLCε may be useful for the treatment and prevention of certain types of cancer.

Acknowledgements

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