ERK5 regulates tobacco smoke-induced urocystic epithelial-mesenchymal transition in BALB/c mice

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Abstract. Tobacco smoke (TS) is an important risk factor of bladder cancer. Epithelial-mesenchymal transition (EMT) is involved in the initiation and development of cancer. The role of extracellular signal-regulated kinase (ERK) 5 in regulating TS-induced EMT remains to be elucidated. The aim of the present study was to investigate the regulatory role of ERK5 in TS-triggered EMT in the bladder of mice. BALB/c mice were used for an *in vivo* TS exposure model. Mice were treated for 6 h a day for 12 weeks. The results demonstrated that mice exposed to TS had decreased mRNA and protein expression levels of the epithelial markers E-cadherin and zonula occludens-1, whereas expression levels of the mesenchymal markers Vimentin and N-cadherin were increased. Treatment with XMD8-92, a highly specific ERK5 inhibitor, effectively abrogated TS-triggered activation of ERK5, activator protein-1 and EMT alterations in the bladder of BALB/c mice. The data suggested that ERK5 regulates TS-mediated urocystic EMT. These findings provide insight into the molecular mechanisms of TS-associated bladder tumorigenesis.

Introduction

Bladder cancer (BC) is the fifth most common type of malignant tumor worldwide (1) and one of the most frequently occurring in the urinary system. It is predominantly present in Europe, North America and Australia, with approximately 420,000 newly diagnosed cases each year and a leading cause of cancer-associated mortality (2,3). Of all urinary malignancies, BC is the primary cause of mortality in China (4,5).

A number of studies have revealed that tobacco smoke (TS), the environment and diet are primary risk factors for BC, in addition to drinking water contaminants, including chlorinated byproducts and arsenic, the use of pioglitazone, obesity, hypertension and diabetes (2,6,7). A previous study reported that 23% of female and 50% of male BC cases have been attributed to TS (8). It has been reported that current tobacco smokers have a fourfold greater risk of developing BC, compared with non-smokers (9). Progress in the understanding of the molecular mechanisms underlying the initiation and progression of BC has been made; however, the molecular pathogenesis remains to be fully elucidated.

Epithelial-mesenchymal transition (EMT) is an important mechanism in embryonic progression and cancer development (10). Cells progressively lose their epithelial characteristics and acquire mesenchymal features during the process of EMT (11). In addition to facilitating tumor invasion and metastasis, EMT is also involved in the initiation of tumorigenesis by promoting cell malignant transformation. TS has been previously demonstrated to promote the initial progression of EMT (12-14). TS-triggered EMT has been revealed to regulate early events in carcinogenesis, including downregulation of E-cadherin, loss of cell-cell adhesion and increased mobility of cells. However, the underlying molecular mechanisms by which TS induces EMT remain to be fully elucidated.

Extracellular signal-regulated kinase (ERK) 5 is the least studied member of the mitogen-activated protein kinase (MAPK) family, and is implicated in important cellular processes, including gene expression, proliferation, apoptosis, angiogenesis, cell motility and differentiation (15-18). A number of studies have investigated the functional role of ERK5

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in cancer oncogenesis; however, its role in EMT regulation requires further investigation. It has been reported that ERK5 promotes EMT (19-21) and triggers a motility and invasive phenotype of cells (22-24). Additional studies have suggested a differential regulatory role of ERK5 in EMT (25,26).

The present study, to the best of our knowledge, is the first to investigate the role of ERK5 in TS-induced urocystic EMT *in vivo*. The study was designed to investigate ERK5 regulation of TS-induced EMT in bladder tissue of mice, using *in vivo* TS exposure models. The findings of the present study suggest that ERK5 may regulate TS-mediated urocystic EMT and provide insight into the underlying molecular mechanisms of TS-associated bladder tumorigenesis.

Materials and methods

Chemicals and reagents. XMD8-92 was purchased from Tocris Bioscience (Bristol, UK). Primary antibodies, phosphorylated (p)-ERK5 (cat. no. 3371S), p-c-Jun (cat. no. 9164S), p-c-Fos (cat. no. 5348), E-cadherin (cat. no. 3195S), N-cadherin (cat. no. 4061) and Vimentin (cat. no. 3932S) were all obtained from Cell Signaling Technology, Inc. (Danvers, MA, USA). Antibodies for zonula occludens (ZO)-1 (cat. no. sc-8146) and GAPDH (cat. no. sc-20357) were from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA). Primers for E-cadherin, ZO-1, N-cadherin, Vimentin and GAPDH were synthesized by Invitrogen; Thermo Fisher Scientific, Inc. (Waltham, MA, USA).

Mice and TS exposure. Male BALB/c mice (weight, 18-22 g; age, 8 weeks; n=12) were purchased from the Animal Research Center of Nanjing Medical University (Nanjing, China). Mice were group-housed in polypropylene cages, maintained on a 12 h light/dark cycle, at 22±0.5°C room temperature, at 40-60% humidity with free access to water and the AIN-76A diet. Animals were handled in accordance with the recommendations in the guidelines of the Animal Care and Welfare Committee of Nanjing Medical University. The present study protocol was approved by the Committee of the Ethics of Animal Experiments of Nanjing Medical University.

The control group (n=6) was exposed to filtered air (FA), and the TS-exposure group (n=6) was exposed to TS in a smoking apparatus designed by the authors. One commercial cigarette (Hongtashan, one of the most consumed cigarettes in China, which contains 12 mg tar and 1.1 mg nicotine per cigarette) was combusted to generate TS by a smoke machine at a constant rate (each cigarette took ~5 min to burn out). The smoke was delivered to whole-body exposure chambers with a target concentration of total particulate matter (TPM) of 80 mg/m^3. Mice were exposed for 6 h daily for 12 weeks. Exposures were monitored and characterized as follows: For the control group, carbon monoxide was at 13.98±2.65 mg/m^3 and TPM was at 0 mg/m^3; and for the TS-exposure group, carbon monoxide was at 168.77±19.36 mg/m^3 and TPM was at 81.05±3.82 mg/m^3. Following the final TS exposure, mice were sacrificed by exposure to 20% CO_2 and the bladder tissues were isolated, frozen and stored at -80°C until analysis.

Delivery of ERK5 inhibitor in mice. A total of 24 mice were divided into four groups (n=6/group) as follows: i) FA-exposure group, in which mice were exposed to FA; ii) TS-exposure group, in which mice were exposed to TS; iii) TS + dimethyl sulfoxide (DMSO) group, in which mice were injected with 15 μl DMSO and exposed to TS; and iv) TS + XMD8-92, in which mice were injected with XMD8-92 and exposed to TS. XMD8-92 was reconstituted in DMSO and injected intraperitoneally (2 mg/kg body weight) every other day. Mice were weighed weekly. Following the completion of exposure, mice were sacrificed and bladder tissues were collected for analysis.

Western blot analysis. Proteins were extracted from bladder tissues (~200 mg) using a lysate buffer (5 mmol/l EDTA, 50 mmol/l Tris, 1% SDS, pH 7.5, 10 μg/ml aprotinin, 1% sodium deoxycholate, 1% NP-40, 1 mM PMSF, 1% Triton-X 100, and 10 μg/ml leupeptin). Protein concentrations were quantified using a BCA Protein Assay kit (Pierce; Thermo Fisher Scientific, Inc.). Protein (60 μg/lane) was loaded on 10% SDS-PAGE and then transferred to polyvinylidene membranes (EMD Millipore, Billerica, MA, USA). The membranes were blocked by 5% (w/v) non-fat milk for 1 h at room temperature and subsequently probed with primary antibodies p-ERK5 (1:500), p-c-Jun (1:500), p-c-Fos (1:500), E-cadherin (1:1,000), N-cadherin (1:500), Vimentin (1:1,000) and ZO-1 (1:500), GAPDH (1:5,000) overnight at 4°C, and then incubated with a horseradish peroxidase-conjugated goat anti-mouse (1:5,000) and goat anti-rabbit (1:10,000) secondary antibodies. Immunoreactive bands were detected by enhanced chemiluminescence (GE Healthcare Life Sciences, Chalfont, UK).

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). RNA was isolated from frozen bladder tissues (~100 mg) using the RNAiso Plus kit according to the manufacturer's protocol (Takara Bio, Inc., Otsu, Japan). RNA (2 μg) was reverse transcribed into cDNA using AMV Reverse Transcriptase (Promega Corporation, Madison, WI, USA). cDNA was analyzed by PCR using the Power SYBR-Green Master Mix (Takara Bio, Inc.). The thermal cycling profile for PCR was 94°C for 5 min, followed by 36 cycles of 30 sec at 94°C, with 30 sec annealing intervals at their correct temperatures (56-60°C) and 30 sec at 72°C. The primers used were as follows: E-cadherin, forward 5'-TCGACACCCGATTCAAAG TGG-3', reverse 5'-TTCCAGAAACGGAGGCCTGAT-3'; ZO-1, forward 5'-GCAGCCACAACCAATTCATAG-3', reverse 5'-GCAGACGATGTTCATAGTTTC-3'; Vimentin, forward 5'-CCTTGACATTGAGATTGCCA-3', reverse 5'-GTATCAACCAGAGGGAGTGA-3'; N-cadherin, forward 5'-ATCAAGTGCCATTAGCCAAG-3', reverse 5'-CTGAGC AGTGAATGTTGTCA-3'; and GAPDH, forward 5'-GCTGCC CAACGCACCGAATA-3', reverse 5'-GAGTCAACGGAT TTGGTCGT-3'; GAPDH served as a control. Fold alterations in gene expression were calculated by a comparative threshold cycle (Cq) method using the formula $2^{-\Delta\Delta Cq}$ (27,28).

Statistical analysis. Data are expressed as the mean ± standard deviation. Statistical analyses were performed with SPSS software, version 16.0 (SPSS, Inc., Chicago, IL, USA). One-way analysis of variance was used for comparison of statistical differences of multiple groups, followed by the least significant difference test. An unpaired Student's t-test was used for the
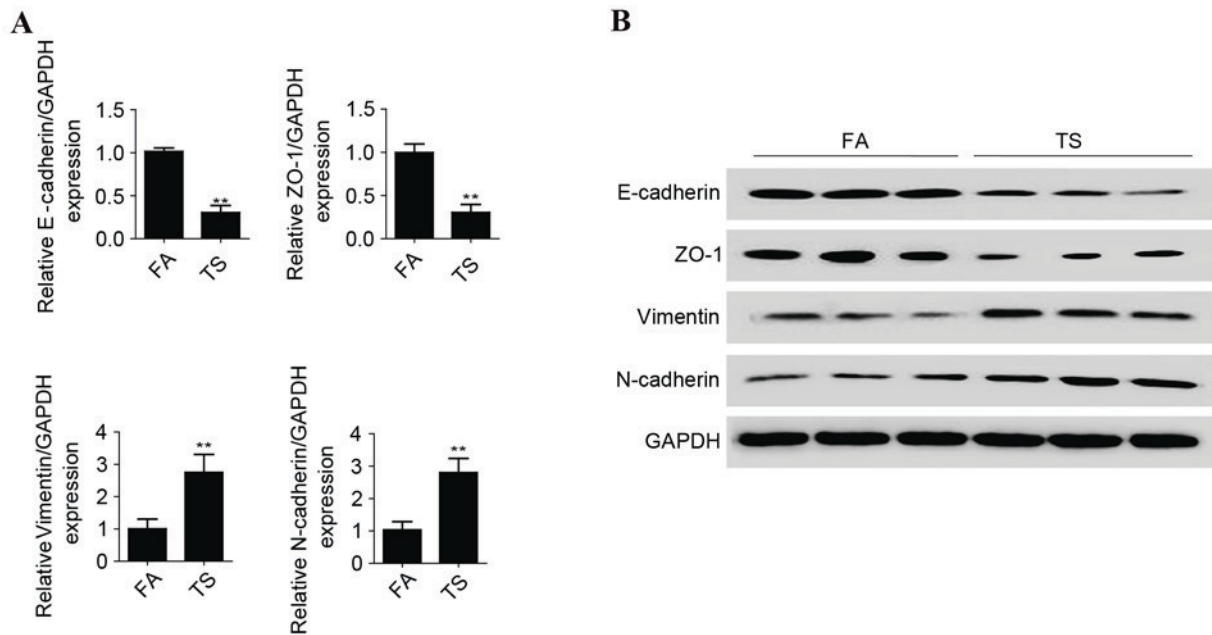
Figure 1. TS exposure alters the expression of epithelial-mesenchymal transition markers in the bladders of mice exposed to TS for 12 weeks. (A) TS exposure reduced the mRNA expression levels of E-cadherin and ZO-1, and increased the mRNA expression levels of Vimentin and N-cadherin. **$P<0.01$ vs. FA control. (B) TS decreased the protein levels of E-cadherin, ZO-1 and increased the protein levels of Vimentin, N-cadherin in the bladder tissues of mice. Data are expressed as the mean ± standard deviation. FA, filtered air; TS, tobacco smoke; ZO-1, zonula occludens.
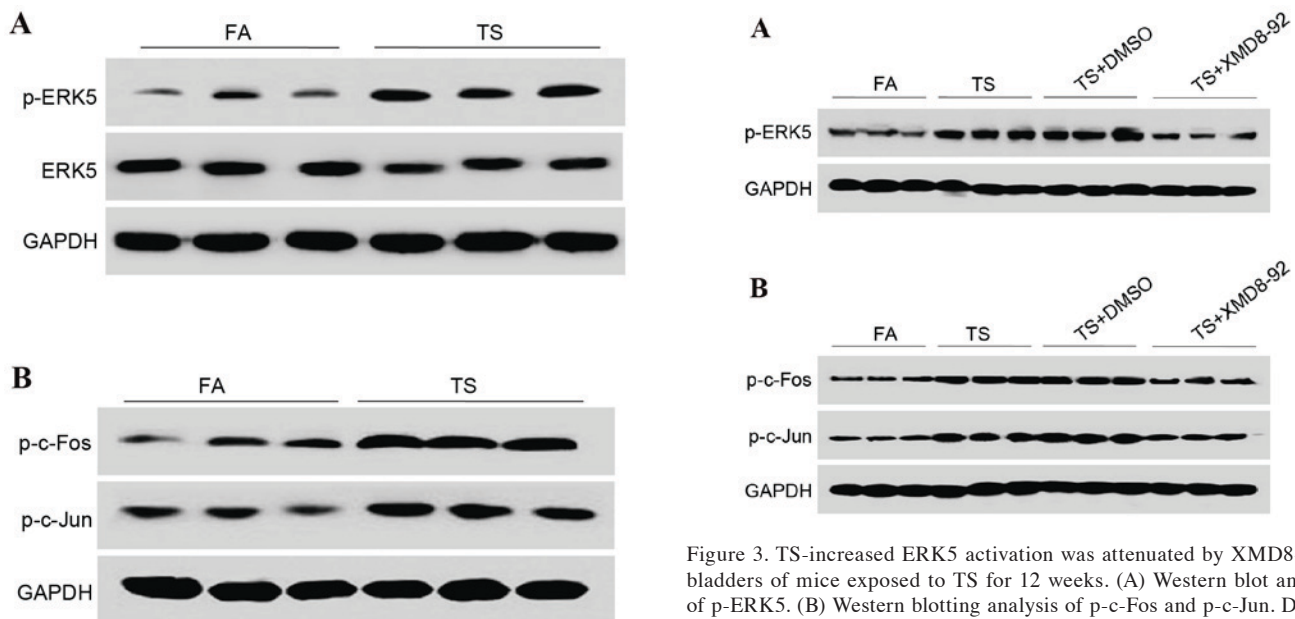

Figure 2. TS exposure increased ERK5/activator protein 1 activation in bladder tissues of mice. (A) Western blot analyses of p-ERK5 in the bladder of mice exposed to TS for 12 weeks. (B) Western blotting analyses of p-c-Fos and p-c-Jun in the bladder of mice. ERK, extracellular signal-regulated kinase; FA, filtered air; p, phosphorylated; TS, tobacco smoke.
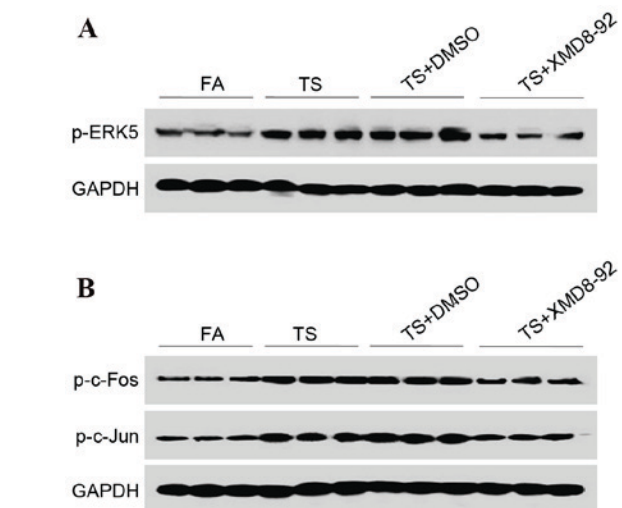

Figure 3. TS-increased ERK5 activation was attenuated by XMD8-92 in bladders of mice exposed to TS for 12 weeks. (A) Western blot analysis of p-ERK5. (B) Western blotting analysis of p-c-Fos and p-c-Jun. DMSO, dimethyl sulfoxide; ERK, extracellular signal-regulated kinase; FA, filtered air; p, phosphorylated; TS, tobacco smoke; XMD8-92, a highly specific ERK5 inhibitor.

comparison between two groups. $P<0.05$ was considered to indicate a statistically significant difference.

Results

TS exposure alters the expression of EMT markers in bladder tissues of mice. TS is one of the key risk factors for BC, and TS-induced EMT is important in TS-associated malignant transformation. The present study investigated whether TS exposure induces EMT in bladder tissues. BALB/c mice were exposed to TS for 12 weeks, and the expression of the epithelial and mesenchymal markers in the bladders of mice were examined. RT-qPCR results revealed that TS exposure decreased the mRNA expression levels of E-cadherin and ZO-1. Conversely, the mRNA expression levels of Vimentin and N-cadherin were increased ($P<0.01$ vs. FA control; Fig. 1A). TS exposure reduced E-cadherin
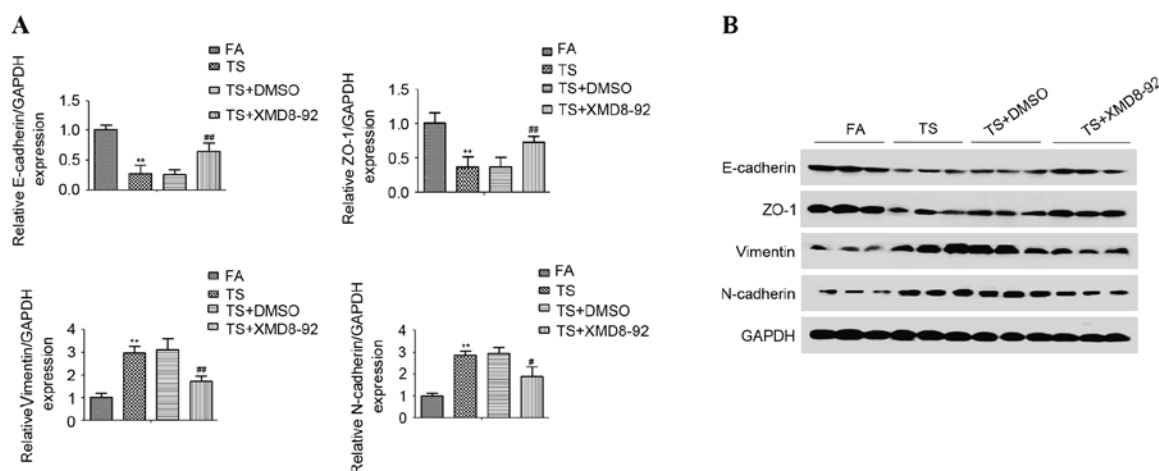
Figure 4. ERK5 suppression reverses TS-induced urocystic epithelial-mesenchymal transition alterations in mice. (A) Reverse transcription-quantitative polymerase chain reaction analyses of E-cadherin, ZO-1, Vimentin and N-cadherin mRNA. (B) Western blot analyses of E-cadherin, ZO-1, Vimentin and N-cadherin proteins. Data are expressed as the mean ± standard deviation. **P<0.01 vs. FA control; #P<0.05 vs. TS; ##P<0.001 vs. TS. DMSO, dimethyl sulfoxide; ERK, extracellular signal-regulated kinase; FA, filtered air; TS, tobacco smoke; XMD8-92, a highly specific ERK5 inhibitor; ZO-1, zonula occludens-1.

and ZO-1 protein expression levels, and elevated Vimentin and N-cadherin protein levels, as demonstrated by western blot analysis (Fig. 1B).

TS increases ERK5/activator protein (AP)-1 activation in bladder tissue. To determine if TS-induced bladder EMT alterations are associated with ERK5 activation, the expression levels of p-ERK5 were measured. It was demonstrated that TS exposure activated the urocystic ERK5 pathway (Fig. 2A). TS exposure also increased AP-1 protein expression in the bladder of mice, as indicated by elevated levels of p-c-Jun and p-c-Fos (Fig. 2B). The data therefore suggested that ERK5 activity may be important in TS-elicited EMT in bladder tissue.

XMD8-92 attenuates TS-induced ERK5 activation in bladder tissue. The aforementioned results revealed that TS-induced urocystic EMT was associated with ERK5 activation; therefore, the present study aimed to further determine the role of ERK5 in urocystic EMT regulation. Mice were treated with XMD8-92 (2 mg/kg body weight), a highly specific ERK5 inhibitor that suppresses ERK5 activation. Western blot analyses revealed that XMD8-92 downregulated p-ERK5 expression levels (Fig. 3A). In addition, treatment with XMD8-92 markedly decreased TS-induced AP-1 activation (Fig. 3B).

ERK5 suppression reverses TS-triggered urocystic EMT. To further determine the role of ERK5 in TS-triggered EMT in the bladder tissue of mice, the expression of the EMT markers were examined. ERK5 suppression reversed TS-induced alterations in the mRNA levels of E-cadherin, ZO-1, Vimentin (P<0.01 vs. FA control; P<0.001 vs. TS) and N-cadherin (P<0.01 vs. FA control; P<0.05 vs. TS; Fig. 4A). Western blot analyses demonstrated that XMD8-92 treatment attenuated both the TS-induced decrease of E-cadherin and ZO-1 levels, and the increase of Vimentin and N-cadherin in the bladders of the mice (Fig. 4B). These data demonstrated the regulatory role of ERK5 in TS-induced EMT in the bladder tissue.

Discussion

BC is one of the primary causes of cancer-associated mortality, and an association between the occurrence of BC and TS has previously been established (27,29). However, the underlying molecular mechanisms by which TS results in BC development remain to be fully elucidated. The present study demonstrated that TS exposure induced EMT in the bladder of mice. To the best of our knowledge, the present study is the first to demonstrate that ERK5 regulates TS-mediated bladder EMT *in vivo*. The findings suggested that exposure to TS elevated ERK5 activation, and TS-associated bladder EMT alteration was reversed by ERK5 inhibition. These results explain the important role of ERK5 in TS-triggered urocystic EMT and furthers our current understanding of the underlying molecular mechanisms of TS-associated BC.

It has previously been demonstrated that exposure of cells to carcinogens induces EMT during tumor transformation and formation (12-14,30,31), suggesting the involvement of EMT in the initiation of tumorigenesis by promoting cell malignant transformation. The present study revealed that exposure to TS induced EMT in the bladder of mice. TS exposure altered the expression of EMT markers: Decreasing the expression levels of epithelial markers E-cadherin and ZO-1, and increasing the expression of mesenchymal markers Vimentin and N-cadherin. These results revealed that exposure to TS triggered urocystic EMT in the bladder of mice.

The underlying mechanisms of EMT induction by TS exposure remain to be fully elucidated. ERK5 is a lesser-studied member of the MAPK family. Various reports have suggested a functional role for ERK5 in cancer oncogenesis, and ERK5 has been demonstrated to promote EMT (19-21); however, its specific role in EMT regulation has not yet been verified. Previous studies have indicated that ERK5 triggers the motility and invasive phenotype of cells (22-24). Additional studies have suggested a differential regulatory role of ERK5 in EMT (25,26). These reports have suggested that ERK5 regulation of EMT may be sensitive to cell type and/or the cellular microenvironment.

The function of ERK5 in TS-induced urocystic EMT *in vivo* has not yet been investigated. The present study demonstrated that TS-induced urocystic EMT was associated with an upregulation of ERK5 activation in the bladders of mice. To determine the role of ERK5 in urocystic EMT regulation, mice were treated with XMD8-92 (2 mg/kg body weight), a highly specific ERK5 inhibitor. Inhibition of ERK5 attenuated TS-induced alterations in EMT markers, including the decreased expression levels of the epithelial markers E-cadherin and ZO-1, and the increased expression of the mesenchymal markers Vimentin and N-cadherin. These data indicated that ERK5 positively regulated TS-induced urocystic EMT.

In conclusion, the present study demonstrated that ERK5 positively regulates TS-induced urocystic EMT *in vivo*. These findings indicate the important role of ERK5 in TS-associated carcinogenesis and provide a potential strategy for the search of a novel interventional target in TS-associated bladder tumorigenesis.

Acknowledgements

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