Gene Expression Profiles During Tissue Remodeling Following Bladder Outlet Obstruction

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Abstract

Bladder outlet obstruction (BOO) often results in lower urinary tract symptoms (LUTSs) and negatively affects quality of life. Here, we evaluated gene expression patterns in the urinary bladder during tissue remodeling due to BOO. We divided BOO model rats into two groups according to the degree of hypertrophy of smooth muscle and hypoxia-inducible factor 1α (*HIF1α*) mRNA levels in the bladder. The strong muscular hypertrophy group, which exhibited markedly increased *HIF1α* mRNA levels and bladder smooth muscle proportion compared with the control group, was considered a model for the termination of hypertrophy, whereas the mild muscular hypertrophy group was considered a model of the initiation of hypertrophy. Some genes related to urinary function showed different expression patterns between the two groups. Furthermore, we found that several genes, including D-box binding PAR bZIP transcription factor (*DBP*), were upregulated only in the mild muscular hypertrophy group. *DBP* expression was increased in urinary bladder cells in response to hypoxic stress and induced the upregulation of some genes involved in urinary function. These findings suggested that the regulatory systems of gene expression were altered during tissue remodeling following BOO. Furthermore, circadian rhythms may be involved in urinary function via regulation of gene expression following hypoxic stress.

Introduction

Bladder outlet obstruction (BOO) occurs when there is a blockage at the base or neck of the bladder that inhibits the flow of urine into the urethra [1]. BOO is often observed in cases of benign prostatic hyperplasia (BPH) and can result in lower urinary tract symptoms (LUTSs), i.e., storage disturbances (e.g., daytime urinary urgency and nocturia) and voiding disturbances (e.g., urinary hesitancy, weak stream, straining, and prolonged voiding) [1]. LUTSs affect an estimated 30% of men over 85 years old in the United Kingdom and decreases quality of life [2].

In addition to LUTSs, BOO also leads to progressive tissue remodeling of the bladder through three sequential stages: hypertrophy, compensation, and decompensation [3]. In the hypertrophy stage, mechanical stress causes hypertrophy of smooth muscle cells (SMCs) and hypoxia, resulting in stimulation of angiogenesis. In the compensation stage, bladder growth and angiogenesis are arrested. In the decompensation stage, loss of smooth muscle, deposition of extracellular matrix, and degradation of neurons occur under the sustained stress caused by obstruction. In each stage of the bladder remodeling, several signaling pathways are modulated in the various compartments of the bladder in animal models of BOO and human patients with BOO [3,4]. Alterations in gene expression at the initiation of bladder remodeling play important roles as triggers of disease progression. Hypoxia is one type of stress stimulus that induces upregulation of hypoxia-inducible factor (HIF) 1α and vascular endothelial growth factor (VEGF) in SMCs of the bladder [5]. An *in vitro* study using normal human bladder smooth muscle cells demonstrated that transcripts of *HIF1a* and *VEGF* are transiently increased in response to hypoxia in a time-dependent manner (i.e., HIF1α is transiently upregulated after 2 h, whereas *VEGF* is gradually

upregulated after 24 h) [6]. However, the mechanisms regulating gene expression at the initiation of bladder remodeling in BOO have not yet been clarified.

In mammals, various urinary functions exhibit circadian variations. Circadian rhythms are controlled by several transcriptional factors encoded by clock genes; these factors sequentially regulate the expression of target genes [7]. Previous studies using mice with knockout or mutant clock genes have suggested that circadian clock components are involved in controlling urinary functions [8,9]. However, the mechanisms through which clock components mediated urinary functions have not been fully elucidated. Furthermore, the effects of BOO on the roles of clock components in urinary functions are unclear.

In this study, we aimed to elucidate the mechanisms regulating gene expression at the initiation of bladder tissue remodeling in BOO. Our findings demonstrated that gene expression patterns were altered during BOO-dependent bladder remodeling. Furthermore, we found that D-box binding PAR bZIP transcription factor (DBP), a circadian clock component, was upregulated in response to hypoxic stress and functioned as a regulator of urinary function-related genes.

Materials And Methods

Experimental animals

Eleven-wemk-old female Sprague-Dawley rats (230–250 g, n = 9) were housed with free access to food and water in a room with a 12-g light/dark cycle. Three control rats underwent a sham operation, and 6 rats underwent urethral constriction, as described previously [10,11], with some modifications. Briefly, a midline abdominal incision was made after induction of anesthesia with a mixture of medetomidine, midazolam, and butorphanol. The proximal urethra was dissected with the bladder and surrounding tissue and was tied using silk ligature. A PE-90 catheter was placed beside the proximal urethra and was removed after the urethra was tied. Animals were housed for an additional 4 weeks and then used for histological and gene expression analyses. All animal procedures were approved by the Animal Care and Use Committee of Kyoto Prefectural University of Medicine before the experiment and performed in accordance with the Guidelines for Animal Care of Kyoto Prefectural University of Medicine.

Immunohistochemistry

Bladder tissue was dissected from 3 bladders in the control group and 6 bladders in the BOO groups. Frozen sections were prepared by standard methods. Tissues were stained with hematoxylin and eosin stain. Immunohistochemistry was performed using Dako LSAB + system-HRP (Agilent, CA, USA) according to the manufacturer's instructions. Rabbit anti-DBP antibodies (cat. No. 12662-1-AP; Proteintech, IL, USA; 1:50) were used.

RNA-seq

Total RNA from 3 bladders in the control group and 6 bladders in the BOO groups was isolated using ISOGEN (Nippon Gene, Tokyo, Japan) according to the manufacturer's instructions. RNA quality was checked using a NanoDrop (ThermoFisher, MA, USA), agarose gel electrophoresis, and a 2100 Bioanalyzer (Agilent). Sequencing libraries were built, and the quantified libraries were fed into Illumina sequencers.

RNA-seq analysis was performed using CLC Genomics Workbench 12.0.2 (Qiagen, Limburg, Nederland). Transcript expression levels are shown as transcripts per million (TPM) counts. Data were used for Gene Ontology (GO) analysis with the PANTHER Gene List Analysis tool (http://www.pantherdb.org/).

Cell culture and transfection

J82 cells were provided by American Type Culture Collection (Manassas, VA, USA) and cultured in RPMI1640 (Nacalai Tesque, Kyoto, Japan) with 10% fetal bovine serum at 37 °C in an incubator containing 5% CO_2. To induce hypoxia in cell culture, $COCl_2$ (Nacalai Tesque) was used at a final concentration of 100 or 500 mM in culture medium. Cells were incubated in $COCl_2$-containing medium for 24 h at 37 °C in an atmosphere containing 5% CO_2.

To knockdown DBP expression, transfection of siRNA targeting DBP (cat. no. SI00359751; Qiagen) and siRNA negative control (cat. no. 452002; Invitrogen, CA. USA) was performed using Lipofectamine RNAiMAX (Invitrogen) for 3 days.

Reverse transcription quantitative polymerase chain reaction (RT-qPCR)

The following experiments were performed using kits according to the manufacturer's instructions. Total RNA was isolated using ISOGEN (Nippon Gene). RT was performed using PrimeScript RT Master Mix (Takara, Shiga, Japan). cDNAs were quantified by real-time PCR using SYBR qPCR mix (Toyobo, Osaka, Japan) and a Thermal Cycler TP800 (Takara). Primer sets for PCR are shown in Table S2.

Western blotting

Whole-cell lysates were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis and then western blotting using standard methods [12]. Primary antibodies targeting DBP (cat. no. 12662-1-AP; Proteintech; 1:500), HIF1α (cat. nos. H1a67, 400080; Calbiochem, MA, USA; 1:500), and β-actin (cat. no. A5441; Sigma, MO, USA; 1:1000) were used.

Statistical analysis

Correlation analysis was conducted using Pearson's correlation coefficient. Statistical significance was assessed by one-way analysis of variance. Results with *P* values of less than 0.05 were considered significant.

Results

Expression levels of genes involved in bladder function depended on the degree of tissue remodeling

To investigate gene expression profiles of bladder remodeling due to BOO, we generated an experimental BOO model in rats. The proportion of bladder smooth muscle increased markedly in all rats exposed to experimental partial outlet obstruction compared with those in control rats. Variations in the degree of muscular hypertrophy were observed among rats, and rats were categorized into two groups according to the degree of muscular hypertrophy: the strong muscular hypertrophy (SH) group and the mild hypertrophy (MH) group (Fig. 1A). We then analyzed total gene expression patterns in the groups by RNA-seq (control group, n = 3; BOO group, n = 6; Table S1). We first confirmed the mRNA expression levels of the hypoxia markers *Vegf* and *Hif1a*. *Vegf* levels were significantly higher in all BOO rats than in control rats and were positively correlated with the thickness of the detrusor muscle (r^2 = 0.9023, Fig. 1B). In contrast, *Hif1a* levels increased about 4-fold only in SH group rats (Fig. 1C). HIF1α protein is known to be transiently upregulated in hypoxia, whereas *HIF1a* mRNA is frequently downregulated [13–15]. The reduction of *HIF1a* mRNA expression in hypoxia is an important component of the cellular adaptation to hypoxia. Therefore, SH group rats exhibiting high levels of *Hif1a* mRNA were presumed to be in the termination of hypertrophy stage and to have lost protection against hypoxia. In contrast, MH group rats exhibiting low levels of *Hif1a* mRNA were thought to be in the initiation of hypertrophy stage.

Next, we compared the expression patterns of some genes involved in bladder functions from RNA sequencing results between the three groups. Aquapolin 3 (*Aqp3*) and matrix metallopeptidase 8 (*Mmp8*) were markedly upregulated in the SH group compared with those in the control group, but only slightly increased in the MH group (Fig. 1D). In contrast, cholinergic receptor muscarinic 3 (*Chrm3*) and *Chrm2* were downregulated in the SH group compared with those in the control group and slightly decreased in the MH group (Fig. 1D). Moreover, transient receptor potential cation channel subfamily v member 4 (*Trpv4*) and nitric oxide synthase 3 (*Nos3*) were increased only in the MH group (Fig. 1D). These results suggested that the expression levels of some genes related to bladder function were altered during bladder remodeling following BOO. Furthermore, the expression patterns were different for every gene.

Total gene expression profiles in the MH and SH groups

To compare total gene expression patterns between the MH and SH groups, we further analyzed the results of RNA sequencing. In total, 4,213 genes exhibited a more than 2-fold increase in expression in the SH group compared with those in the control group, whereas 2,803 genes were increased in the MH group (Fig. 2A). Among these upregulated genes, 1,813 genes were upregulated in both the SH and MH groups (Fig. 2A). In contrast, 507 genes exhibited less than half the expression in both the SH and MH groups compared with the control group (Fig. 2A). The 1,813 upregulated genes in both the MH and SH groups were categorized using GO analysis (Fig. 2B). The results showed that many of the genes were categorized into the molecular functions category, including cytokines and cytokine receptors (Fig. 2B), consistent with the results of previous studies using BOO model rats [4,16].

Next, we focused on genes showing alterations in expression levels only in the MH group. We showed that 471 genes exhibited a more than 2-fold increase in expression in the MH group compared with those in the control and SH groups, whereas 214 genes in the MH group were expressed at levels less than half those in the other two groups (Fig. 2C). Some genes, including *Dbp*, stabilin 1 (*Stab1*), ATP binding cassette subfamily C member 5 (*Abcc5*), and neuronatin (*Nnat*) were markedly increased in the MH group compared with those in the control and SH groups (Fig. 2D). Some other genes, including apoptosis inducing factor mitochondria associated 1 (*Aifm1*) and vascular cell adhesion molecule 1 (*Vcam1*) were significantly downregulated in the MH group (Fig. 2D). Genes showing altered mRNA expression levels may play roles in bladder remodeling at the initiation of the hypertrophy stage.

Dbp was highly expressed in the MH group

The above results suggested that DBP was upregulated during the early phase of hypertrophy. DBP is a transcriptional factor belonging to the circadian clock family and plays roles in the control of circadian rhythm via regulation of target gene expression [17]. Thus, we hypothesized that DBP may regulate the expression of genes involved in bladder functions at the initiation of bladder remodeling due to BOO. First, to confirm the expression levels of *Dbp* in rat BOO models, we quantified *Dbp* mRNA levels by RT-qPCR. *Dbp* mRNA levels were higher in the MH group than in the control and SH groups (Fig. 3A). We then assessed DBP protein expression by immunohistochemistry using anti-DBP antibodies. DBP protein was detected in the nuclei of bladder smooth muscle cells in the MH group, but not in the control and SH groups (Fig. 3B).

DBP regulated *AQP3* and *TRPV4* expression under hypoxic conditions in human urinary bladder cells

Next, we assessed whether DBP was upregulated under hypoxic conditions in human bladder cells. Hypoxia was induced in J82 urinary bladder cancer cells by treatment with cobalt chloride ($COCl_2$) solution. Protein levels of the hypoxia marker HIF1α and mRNA levels of *VEGF* were increased by $COCl_2$ in a concentration-dependent manner (Fig. 4A and 4B). Under hypoxic conditions, protein and mRNA levels of DBP were higher than those under normal conditions (Fig. 4A and 4B). mRNA levels of the period circadian regulator 1 (*Per1*) gene, which is known to be transcriptionally activated by DBP, were increased under hypoxic conditions and decreased by knockdown of DBP (Fig. 4B). These results suggested that DBP was upregulated in response to hypoxia and could control gene expression via transcriptional regulation in human bladder cells. To investigate whether DBP contributed to bladder functions under hypoxic conditions, we quantified mRNA levels of genes related to bladder function using DBP-knockdown cells (Fig. 4B). AQP3 is an intrinsic membrane protein belonging to the aquaporin family and functions as a water channel in many cell types involved in fluid transport [18]. TRPV4 is a cation channel of the transient receptor potential superfamily and is implicated in sensing the filling state of the bladder [19]. NOS3 is an endothelial nitric oxide synthase that is involved in the induction of apoptosis in the bladder mucosa [20]. Rat homologs of these genes were markedly upregulated in the MH group (Fig. 1D). In human bladder cells, mRNA levels of *Aqp3*, *Trpv4*, and *Nos3* were increased by treatment with 500 μM $COCl_2$ (Fig. 4B). The increases in *Aqp3* and *Trpv4* under hypoxic conditions were blocked by *Dbp*

knockdown; however, the expression levels of *Nos3* were not affected (Fig. 4B). These results suggested that DBP upregulated *Aqp3* and *Trpv4* genes under hypoxic conditions in the human urinary bladder.

Discussion

Urinary functions are controlled by several signaling pathways. In this study, our findings suggested that BOO modulated signaling pathways by regulation of gene expression in the bladder. Gene expression patterns were altered during tissue remodeling due to BOO. Furthermore, DBP acted as a regulator of some genes involved in urinary functions in response to hypoxic stress.

HIF1α is a key regulator of cellular response to hypoxia [21]. The expression levels of HIF1α are highly regulated, and HIF1α abundance is controlled through transcriptional, post-transcriptional, and post-translational mechanisms [21]. HIF1α protein is transiently upregulated in hypoxia but rapidly degraded through von Hippel-Lindau tumor suppressor protein-mediated protein degradation in normoxia [22]. In contrast, *HIF1α* mRNA is frequently suppressed, despite expression of HIF1α protein, under hypoxic conditions [13–15]. The reduction in *HIF1α* mRNA expression in hypoxia indicates that this molecule may be important for cellular adaptation to hypoxia, and this hypothesis is supported by the observation that high levels of *HIF1α* mRNA have been observed in some cancers and are often associated with poor prognosis [23–26]. Our experimental BOO model rats were subjected to intravesical obstruction for 4 weeks, and a part of individual rats showed increased *HIF1α* mRNA levels. Their smooth muscle contents were significantly increased, suggesting that these rats were in the termination of hypertrophy stage during tissue remodeling due to BOO. Thus, these results support that high levels of *HIF1α* mRNA indicate dysfunction of the cellular adaptation system to hypoxia in bladder tissue. mRNA levels of *HIF1α* can be a useful indicator of health status due to BOO and may facilitate prediction of prognosis in patients with cancer.

Although circadian rhythms are known to affect urinary function, the roles of circadian clock components in this process are not clear [8]. DBP is involved in control of circadian rhythms via rhythmically activating the transcription of various genes through a DNA cis-element, the D-box [17]. In this study, our findings demonstrated that DBP was upregulated in response to hypoxia in our rat BOO model and in human bladder cells. Furthermore, we showed that DBP could upregulate other genes related to urinary function under hypoxic conditions. These results suggested that circadian clock components contributed to the cellular response to hypoxic stress due to BOO. Additional studies of the relationship of circadian rhythms with urinary function under hypoxic conditions and/or disease progression due to BOO may lead to the development of effective treatments for BOO. In addition, uncovering the molecular mechanisms of DBP-mediated gene expression control (e.g., direct regulation via transcriptional activation or indirect regulation) is important for improving our understanding of the roles of circadian clock components.

To identify regulators of gene expression at the initiation of adaptation to hypoxia, we performed total gene expression analysis by RNA-seq using experimental BOO model rats. Some genes, including *Dbp*, were identified as candidate regulators exhibiting alterations in mRNA expression at the early stage of

hypertrophy of SMCs following BOO. Further studies of these candidate regulators are needed to fully elucidate the molecular mechanisms involved in initiation of tissue remodeling due to BOO and to identify new therapeutic targets for management of LUTSs.

Declarations

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Contributions

S.I. wrote the main manuscript text, made substantial contributions to conception, design, acquisition of data and analysis, and gave final approval of the version to be submitted; T.N., S.I., Y.M. and H.H. made contributions to acquisition of data; T.U. wrote the main manuscript text; A.F., A.F., F,H. and O.U. participate in drafting the article.

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Ethics Declaration

Competing interests

The authors declare no competing interests.

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Figures

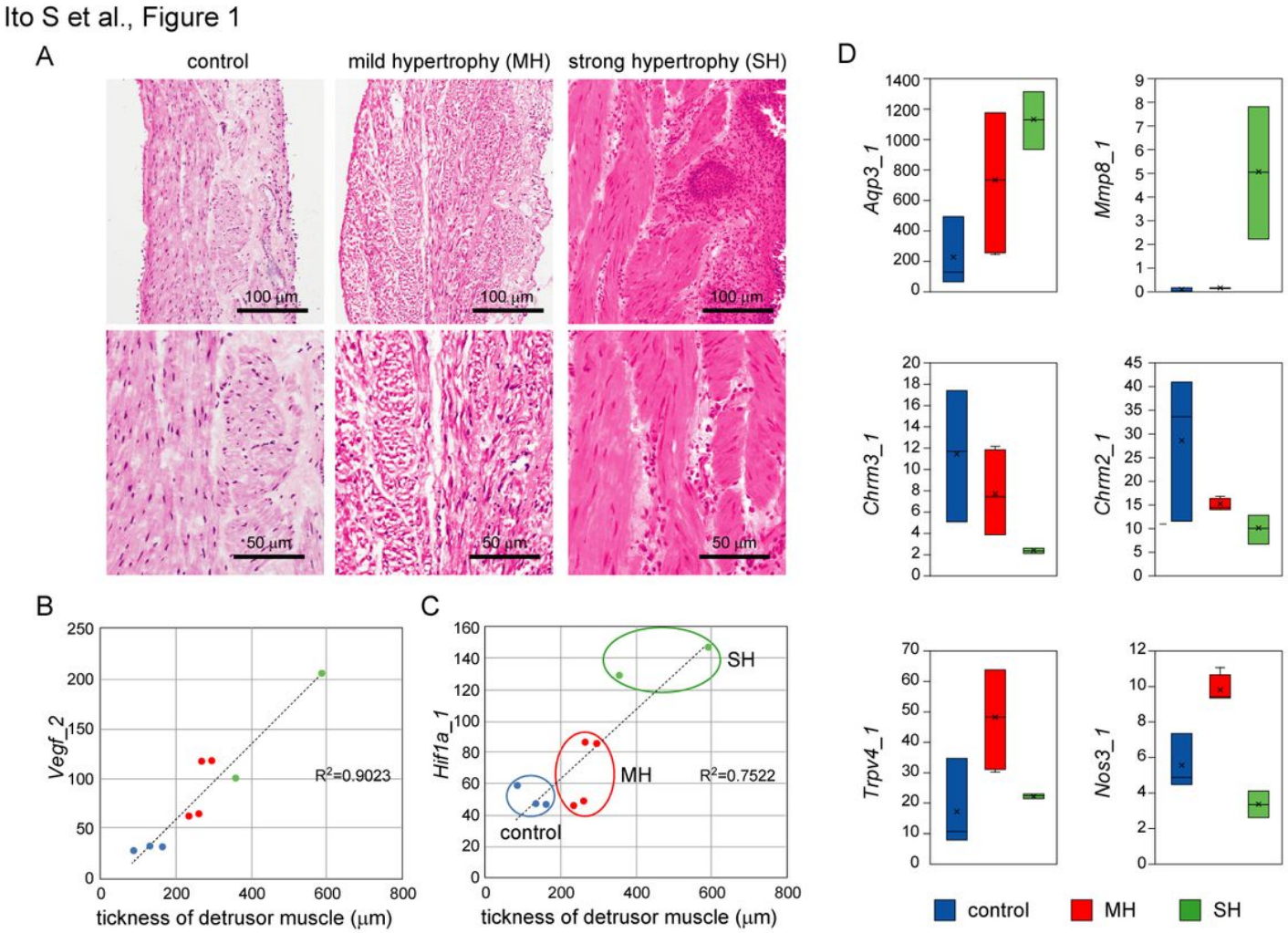

Figure 1

Expression patterns of genes related to hypoxia and urinary function in three experimental groups. (A) HE staining of the urinary bladder tissue in rats in the BOO model. Control rats underwent sham operation (left two panels). BOO model rats were categorized into two groups according to the degree of muscular hypertrophy (MH group, middle two panels; SH group, right two panels). Bottom panels show magnification of the detrusor muscle from each upper panel. (B) Correlations between Vegf levels and the thickness of the detrusor muscle in each rat (n = 9). Vegf mRNA levels were quantified using RNA-seq and normalized (TPM counts are shown on the vertical axis). The thickness of the detrusor muscle is shown as the average of 6 spots with a distance of 100 □m in the extra layer of muscle in each rat (horizontal axis). Blue dots, control group rats; red dots, MH group; green dots, SH group. (C) Correlations between Hif1α levels and the thickness of the detrusor muscle in each rat. (D) Expression patterns of genes related to urinary function in the control, MH, and SH groups. For each box plot, the box represents the 25th to 75th percentile interval, and the line represents the median and nonoutlier range.

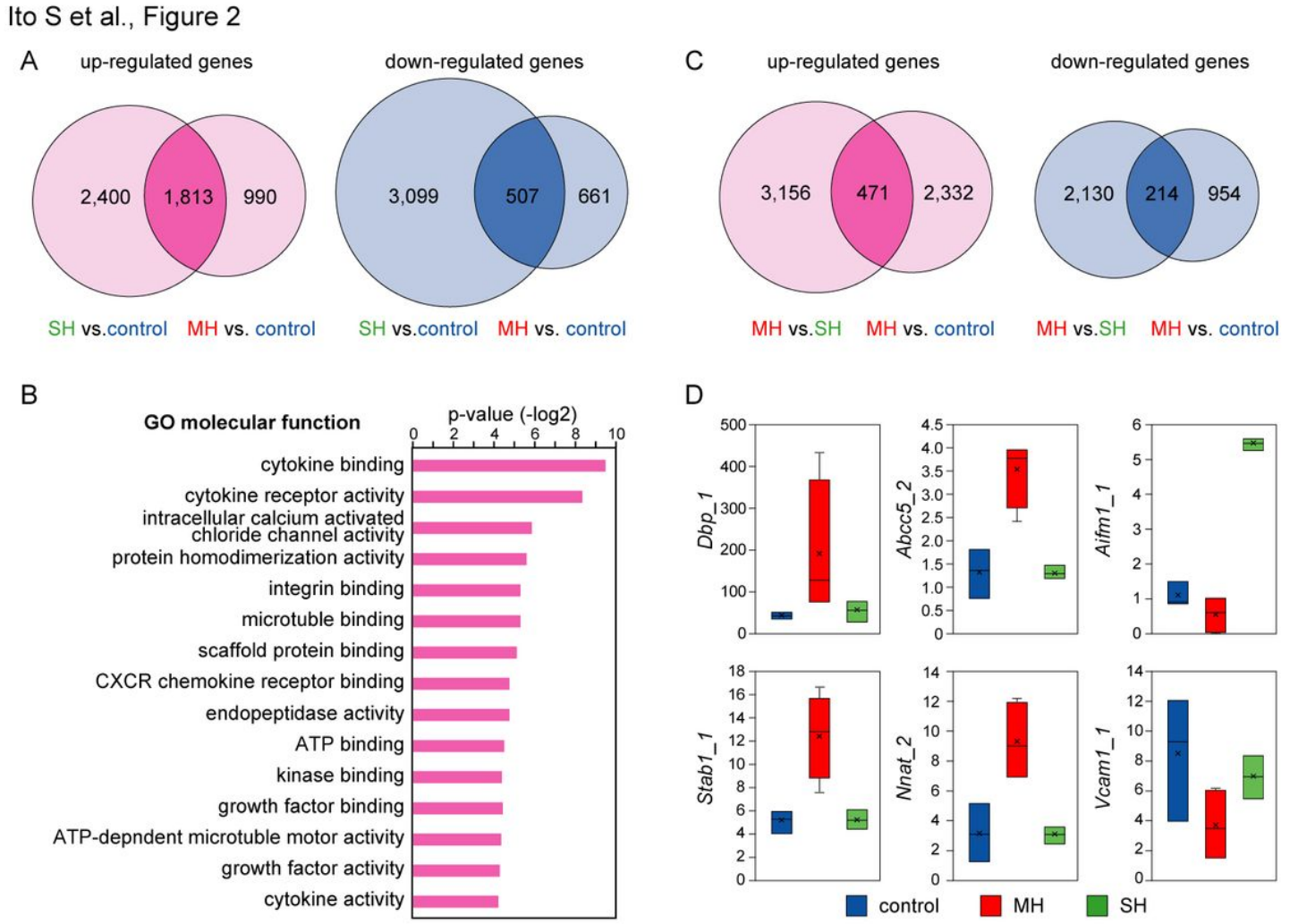

Figure 2

Total gene expression patterns in the three experimental groups. (A) Gene expression profiles of urinary bladder tissues from the control, MH, and SH groups using RNA-seq results. Comparison analysis was performed between the SH versus control and MH versus control groups. In total, 1,813 genes exhibited more than 2-fold increases in mRNA expression in the MH and SH groups compared with the control group, whereas 507 genes were downregulated by at least 50% in the MH and SH groups. (B) RNA-seq-based GO analysis of genes upregulated more than 2-fold in the MH and SH groups compared with the control group (1,813 genes shown as dark pink in A). (C) Gene expression profiles with comparisons between the MH and SH groups and the MH and control groups. In total, 471 genes exhibited a more than 2-fold increase in mRNA expression in the MH group compared with the SH and control groups. Additionally, 214 genes showed a more than 2-fold decrease in expression in the MH group compared with the other two groups. (D) Expression pattern of each gene showing marked changes in mRNA expression in the MH group compared with the SH and control groups.

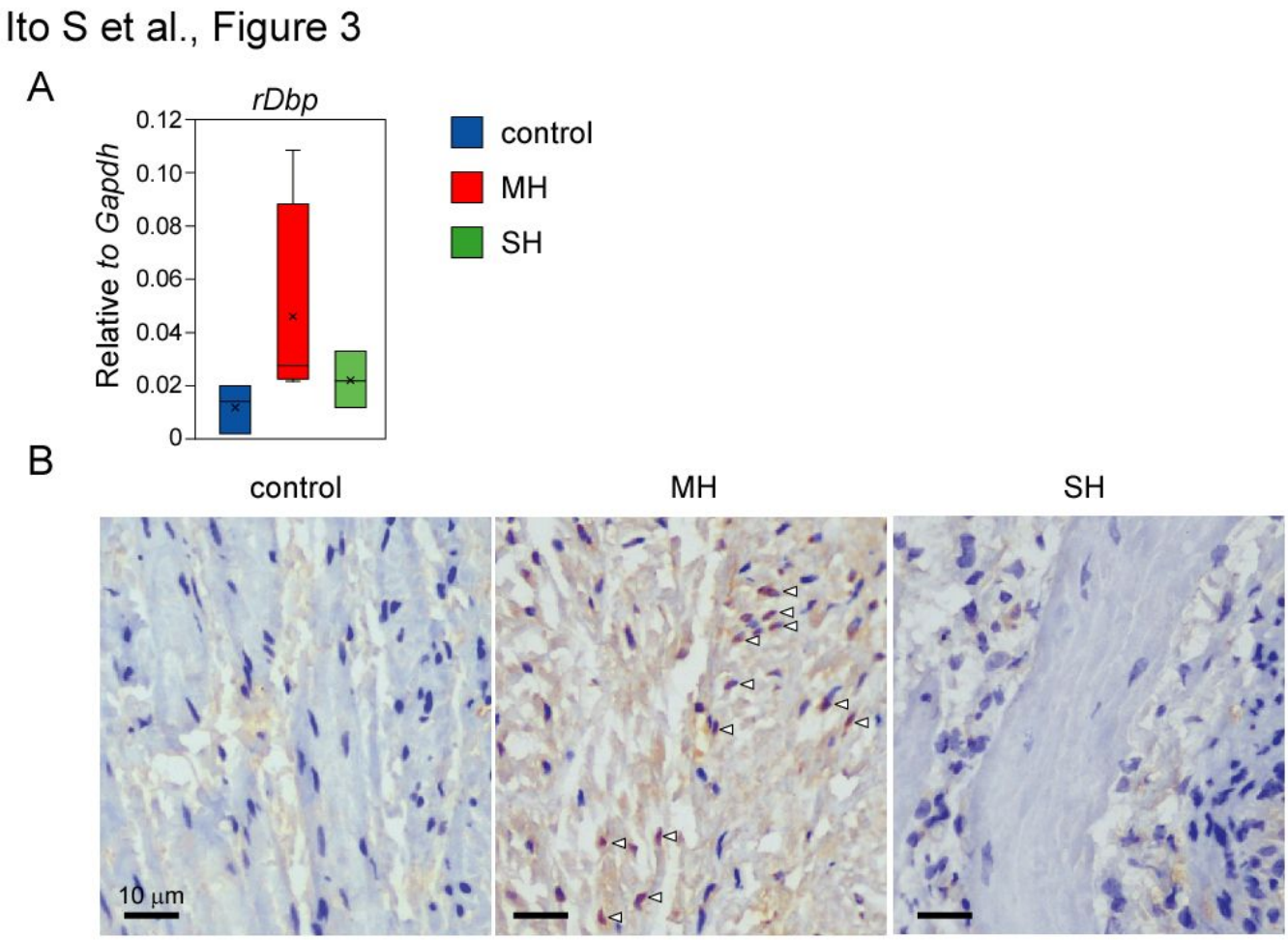

Figure 3

Expression levels of Dbp in the three experimental groups. (A) Relative Dbp mRNA levels in the rat urinary bladder in the control, MH, and SH groups. mRNA levels were quantified by RT-pPCR. Box blots show each measurement normalized to Gapdh mRNA. (B) Immunohistochemistry using anti-DBP antibodies. DBP was stained brown via DAB immunolabeling (white arrowheads). Nuclei were stained blue by hematoxylin stain. Scale bars: 10 μm.

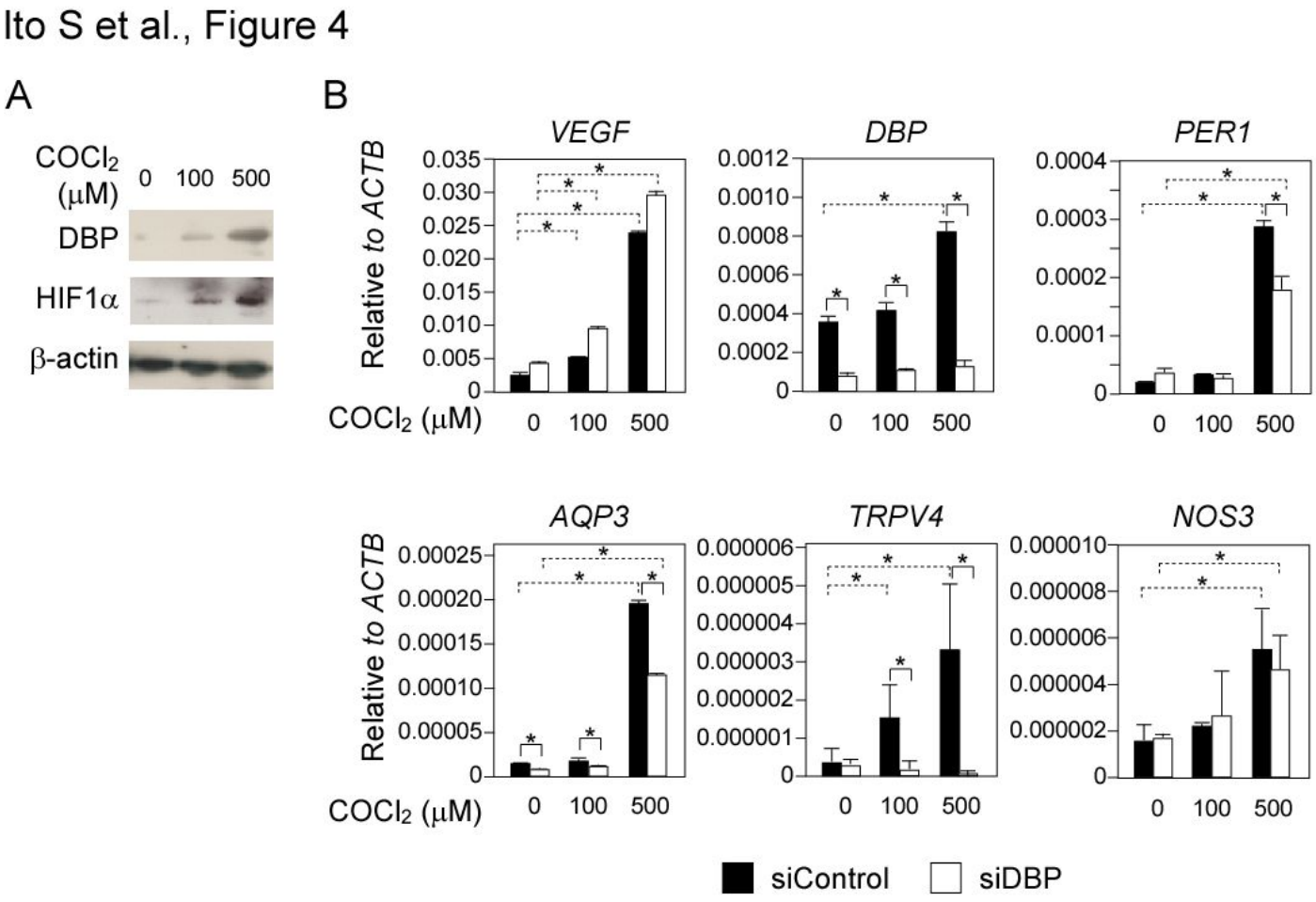

Figure 4

DBP-mediated gene expression under hypoxic conditions in human urinary bladder cells. (A) DBP protein levels in human urinary bladder cells under hypoxic conditions. J82 cells were cultured with COCl2 solution for 24 h (0, 100, or 500 □M). Western blotting was performed with anti-DBP and anti-HIF1α antibodies. (B) mRNA levels of DBP-knockdown J82 cells under hypoxic conditions. J82 cells were transfected with siDBP for 2 days and then treated with COCl2 for 24 h. RT-qPCR was used to measure gene expression. Measurements show average values of 3 independent measurements, which were normalized to ACTB mRNA expression. *P < 0.05.

Supplementary Files

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