STAT3 involvement in the development of renal interstitial fibrosis after unilateral ureteral obstruction

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Short title: STAT3 involvement in the development of renal fibrosis

Key words: Fibrosis, UUO, STAT3

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Abstract

Background: In vitro studies suggest that the signal transducer and activator of transcription (STAT) plays a critical role in renal fibrosis. However, the process of STAT activation in vivo remains unclear. This study in rats aimed to localize STAT3 activation within the kidney and examine the in vivo relationship between STAT3 activation and renal fibrosis.

Methods: Unilateral ureteral obstruction (UUO) was induced in the rats and the kidneys examined 3 or 7 days after obstruction. Activation of STAT3 in Western blot and immunohistochemical analyses was identified by the phosphorylated form of STAT3 (pSTAT3).

Results: Myofibroblasts were identified by α-smooth muscle actin expression and were upregulated in obstructed kidneys. pSTAT3 was localized mainly in tubular epithelial cells of collecting ducts in normal and obstructed kidneys and interstitial cells in obstructed kidneys. After UUO, Western blotting showed a 4-fold increase in pSTAT3, with a peak at day 7. Immunostaining showed a 6-fold increase in pSTAT3 at day 7 in tubular epithelial cells and a 2500-fold increase at day 7 in interstitial cells.

Conclusion: STAT3 was activated in rat tubular epithelial cells and myofibroblasts after UUO, suggesting that STAT3 may contribute to the progression of interstitial fibrosis.

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Introduction

Signal transducer and activator of transcription (STAT) 3 is a member of the STAT protein family. STAT proteins are latent transcription factors that are activated by phosphorylation. Activated STAT proteins dimerize and translocate to the nucleus where they activate specific target genes [1].

STAT3 activation has been implicated not only in cell proliferation [2, 3], but also in fibrosis [4]. In various in vitro studies, STAT3 activation has been reported in mesangial cells [2], proximal tubular epithelial cells [5], fibroblasts [3] and macrophages [6]. We have also reported mesangial cell proliferation involving STAT3 activation [7]. However, little is known concerning in vivo STAT3 activation and renal fibrosis.

Most of our understanding of the role of STAT3 in renal cell responses comes from in vitro studies using drugs that block the function of STAT3 activation. STAT3 activation plays a crucial role in the response of various cultured cells to a variety of stimuli, such as transforming growth factor 1 and angiotensin II [4, 8]. However it is difficult to relate these studies to the normal or diseased kidney since we know very little about STAT3 activation in situ. Interstitial fibrosis is a significant pathological condition after renal injury, and unilateral ureteral obstruction (UUO) is a well-characterized experimental model of tubulointerstitial injury and fibrosis. Many investigators have demonstrated increased numbers of interstitial myofibroblasts in the early phase following UUO [9]. We examined the obstructed kidney as this model features mechanical stretch and a marked proliferative response of tubular epithelial cells and fibroblast-like cells [10]. Recent work suggests that various signaling pathways may contribute to the development of renal fibrosis [9, 11], while interstitial myofibroblasts are regarded as the major effector cells of renal fibrosis [12].

The aims of this study were to identify the cell types in which STAT3 activation occurs in normal kidneys and to examine how this changed under pathological conditions. We hypothesized that

STAT3 may participate in the accumulation of interstitial cells and play a key role in the progression of renal fibrosis. To examine this hypothesis, we studied rat kidneys from animals that had undergone UUO.

Materials and Methods

Experimental model and tissue retrieval

Studies were performed on male Wistar rats (220-250 g) purchased from Shimizu Jikken Zairyou (Kyoto, Japan). The Institutional Animal Care and Use Committee at Hiroshima University (Hiroshima, Japan) approved all the animal protocols, and the experiments were performed in accordance with the National Institute of Health Guidelines on the Use of Laboratory Animals. UUO was performed using an established procedure [9].

Five groups of rats (10 rats per group) were used in the experiments. The rats were euthanized at each time point. All animals were anesthetized by intraperitoneal (i.p.) administration of ketamine (75mg/kg) and xylazine (10mg/kg) prior to harvesting of tissue samples. The kidneys of the experimental animals were removed on days 3 or 7 after UUO. For controls, a group of normal rats that had not undergone any procedures was used.

Reagents

The mouse monoclonal antibodies (mAb) used in the study were: α-smooth muscle actin (α-SMA; Sigma-Aldrich, St. Louis, MO, USA), the macrophage and monocyte marker ED-1 (anti-CD68 antibody; Serotek, Kidlinton, UK), anti-actin (Sigma-Aldrich) and anti-bromodeoxyuridine (BrdU) antibody (Dako Cytomation, Grostrup, Denmark). Anti-non-phosphospecific STAT3 (total-STAT3: tSTAT3; rabbit polyclonal) antibody was obtained from Upstate Biotechnology (Lake Placid, NY, USA), anti-phosphospecific STAT3 (pSTAT3; rabbit polyclonal) antibody from Cell Signaling Technology (Beverly, MA, USA) and anti-aquaporin 2 (AQP-2) antibody from Calbiochem (San Diego, CA, USA).

The following secondary polyclonal antibodies were used in the study: biotinylated goat anti-rabbit

immunoglobulin G (IgG) (Zymed, South San Francisco, CA, USA), biotinylated rabbit anti-goat IgG (Zymed), goat anti-mouse IgG conjugated with horseradish peroxidase (HRP) or alkaline phosphatase (AP), goat anti-rabbit IgG conjugated with HRP (Dako), complexes of HRP-conjugated mouse, goat, or rabbit anti-HRP IgG (PAP) and complexes of AP-conjugated mouse anti-AP IgG (APAAP) (Dako).

Western blot analysis

For detection of STAT3, one half of a kidney was homogenized on ice in 1 ml of lysis buffer that consisted of 10 mM Tris-HCl, pH 7.4, 100 mM NaCl, 1 mM ethylenediaminetetraacetic acid, 1 mM ethyleneglycol tetraacetate, 1 mM NaF, 2 mM Na2VO4, 1% Triton X-100, 10% glycerol, 0.5% deoxycholate, 1 mM phenylmethylsulfonyl fluoride and 10% protease inhibitor cocktail (Sigma-Aldrich). The homogenate was incubated on ice for 10 min with regular vortexing, then centrifuged at 15,000 x g for 20 min to remove tissue debris. The supernatant was divided into aliquots and stored at -80°C.

The protein content of the cell lysate was determined by a BCA protein assay (Pierce, Rockford, IL, USA) as described previously [13]. Lysates (40 μg per lane) were electrophoresed on 8% sodium dodecyl sulfate-polyacrylamide gels and then transferred onto polyvinylidine difluoride membranes (Millipore, Bedford, MA, USA) by electroblotting in 25 mM Tris-HCl, pH 8.5, 192 mM glycine and 20% methanol overnight at 4°C using a Bio-Rad Transblot apparatus (Bio-Rad Laboratories, Hercules, CA, USA). The blots were blocked for 1 h with 5% non-fat milk powder in 20 mM Tris-HCl, pH 7.6, 137 mM NaCl and 0.05% Tween 20, and then washed 5 times in 20 mM Tris-HCl, pH 7.6, 137 mM NaCl and 0.05% Tween 20.

For positive controls of pSTAT3, we used extracted lysates of human umbilical vein endothelial

cells (American Type Culture Collection, Rockville, MD, USA) following stimulation with either Oncostatin M (R&D systems, Minneapolis, MN, USA) or interferon γ (Shionogi-Seiyaku, Osaka, Japan) [14].

The blots were incubated overnight at 4°C with anti-pSTAT3 and tSTAT3 antibodies in the blocking solution containing 5% bovine serum albumin (BSA). Following the overnight incubation, the blots were washed and incubated for 1 h with secondary antibody (HRP-conjugated goat anti-rabbit IgG at 1:5000 dilution) in binding buffer. After washing, bound antibody was detected using the enhanced chemiluminescence (ECL) Western blotting detection system (Amersham Pharmacia Biotech, Buckinghamshire, UK).

Immunohistochemistry

Immunohistochemical staining was performed on tissues fixed in 10% formalin and embedded in paraffin, as described previously [9]. Tissue sections were placed in 0.01 M citrate buffer, pH 6.0, and heated for 10 min in a microwave oven. This treatment was used for detection of pSTAT3 (Tyr705), macrophages (ED-1) and BrdU, but was not used for detection of either AQP-2 or α-SMA. Sections were blocked in 10% fetal calf serum (FCS), 5% BSA and 10% normal goat serum in phosphate-buffered saline (PBS) for 30 min, and then incubated overnight at 4°C with primary antibody in 10% normal goat serum and 5% normal rat serum. After washing, endogenous peroxidase was blocked by incubation in 0.6% H_2O_2 in methanol for 20 min. Some sections were incubated sequentially with HRP or AP-conjugated goat anti-mouse or goat anti-rabbit IgG, followed by mouse PAP or APAAP or rabbit PAP for 45 min each. Other sections were blocked in Avidin-Biotin blocking kit (Vector Laboratories, Burlingame, CA, USA) and then incubated with biotinylated goat anti-rabbit IgG for 45 min, followed by incubation with the Vectastain Elite kit

(Vector Laboratories). The sections were developed with diaminobenzidine (Sigma) to give a brown color, or with Vector SG (Vector Laboratories) to give a blue/gray color for α-SMA, AQP-2 and ED-1 or with Fast Blue BB salt (Sigma) for BrdU. Some sections were counterstained with PAS reagent (Muto Kayaku, Kyoto, Japan) without hematoxylin.

Sections that were double labeled were microwaved to prevent antibody cross reactivity, blocked as described above, and then incubated with primary antibody overnight at 4°C. After washing, sections were incubated with HRP or AP conjugated goat anti-mouse IgG or goat anti-rabbit IgG, followed by mouse PAP or APAAP or rabbit PAP for 45 min each and then developed with Vector SG or Fast Blue BB salt. The specificity of immunohistochemical staining was demonstrated by pre-incubation of the antibodies with the immunizing peptide (pSTAT3 blocking peptide) to completely block the respective immunostaining signal.

Quantification of immunohistochemical staining

Immunostaining for pSTAT3 and BrdU was quantified as follows: 10 pre-determined high power fields (x400) of the renal cortex were scored for the number of tubular epithelial cells or interstitial cells stained with either pSTAT3 or BrdU.

Statistical analysis

Statistical differences were analyzed by one-way analysis of variance (ANOVA) using the Dunnett multiple comparison post-hoc test on Stat View version 5.0 statistical software (Cary, NC, USA). Data were expressed as mean ± standard deviation (SD) for each group of ten animals. Values were considered statistically significant when $P < 0.05$.

Results

STAT3 activation in obstructed rat kidneys

Western blot analysis identified pSTAT3 in the lysates of kidney tissue (Fig. 1A). The Western blotting panel showed a peak of STAT3 activation at day 7 (Fig. 1B). The specificity of the Western blotting results was confirmed by incubation of the primary antibodies with their respective blocking peptides, which prevented detection of the bands (data not shown). Quantification of protein expression by densitometric analysis showed a 4-fold increase in STAT3 activation during the seven-day time course (Fig. 1B).

Immunohistochemistry showed staining for pSTAT3 in tubular epithelial cells and interstitial cells in the renal cortex (Figs. 2A, B). Immunostaining identified a pattern of increased staining for pSTAT3 in both tubular epithelial cells and interstitial cells on day 3 (Fig. 2B) compared with day 0 (Fig. 2A). There was an increasing pattern of STAT3 activation in both tubular epithelial cells and interstitial cells during the seven-day time course (Fig. 3). Staining for pSTAT3 was abolished by incubation of pSTAT3 antibody with the pSTAT3 peptide antigen (Fig. 2C).

Cortical tubular epithelial cells stained positive for pSTAT3 (Fig. 3 black bars ■), reaching a peak on day 7. Meanwhile, there was a 2500-fold increase in the number of pSTAT3-positive (Fig. 3 white bars □) interstitial cells over the seven-day time course.

Tubular alteration and interstitial lesions in obstructed rat kidneys

Post-obstruction immunohistochemistry showed an area in the interstitium that stained positive for α-SMA (Figs. 4A - C) and BrdU positive cells (Figs. 4D – F). Increased immunostaining for α-SMA as well as increased numbers of BrdU positive cells were seen during the seven-day time course following obstruction.

Cell proliferation was assessed by BrdU incorporation. Quantification of positively immunostained cells showed time-dependent induction of BrdU after UUO in both tubular epithelial cells (black bars ■) and interstitial cells (white bars □) in the renal cortex of the kidneys (Fig. 5).

Localization of STAT3 in obstructed rat kidneys

In the cortex, pSTAT3 was present in the cytoplasm and nucleus of tubular epithelial cells (Figs. 6A-C). In tubulointerstitial lesions, pSTAT3 (Fig. 6D) was present on myofibroblasts, as spindle-like shapes (arrows). There was little evidence of glomerular activation of pSTAT3 (Fig. 6F), whereas pSTAT3 (Fig. 6E) was detected in vascular smooth muscle cells.

Double immunostaining with AQP-2, a marker of collecting ducts, demonstrated that pSTAT3 (Figs. 6A, B) was restricted to the collecting duct epithelial cells on day 0. The staining of pSTAT3 and AQP-2 was not uniform along the collecting duct, but was present in occasional cells in the cortical collecting duct (Figs. 6A-C). Double staining for α-SMA with pSTAT3 (Fig. 6D) showed that pSTAT3 was also occasionally activated in α-SMA positive myofibroblasts. However, double staining for ED-1 with pSTAT3 (Fig. 6E) showed that pSTAT3 was only occasionally activated in ED-1 positive macrophages. Double staining for BrdU with pSTAT3 (Fig. 6F) was also only observed in the occasional interstitial cell.

Discussion

This study has localized STAT3 activation within the normal kidney and also in the pathologic state associated with an obstructed kidney. The findings relating to STAT3 activation in normal and obstructed kidneys are discussed below, followed by consideration of the role of myofibroblast accumulation in the development of renal fibrosis.

We demonstrated that upregulation of STAT3 was present in the early phase of tubular epithelial cell proliferation and renal fibrosis after UUO. In normal kidneys, we detected the presence of pSTAT3 by Western blot analysis and by immunostaining. pSTAT3 was localized mainly on collecting duct cells. We also found significant upregulation of pSTAT3 following UUO using Western blot analysis. These patterns of upregulation were found primarily on tubular epithelial cells of collecting ducts by immunohistochemistry. An in vitro study has reported that osmotic stress activates STAT3 [15]. Thus, the co-localization of STAT3 activation and AQP-2 expression supports the hypothesis that osmotic stress may be a key regulator of STAT3 activation in normal renal physiology.

We also demonstrated distinct upregulation of STAT3 in cortical interstitial cells. Previous reports described an accumulation of myofibroblasts in the interstitium in the initial phase following UUO [16]. Myofibroblasts are known to express STAT3 [17]. In accordance with these previous reports, we documented the presence of co-localization of STAT3 activation and myofibroblasts in the interstitium after UUO, and demonstrated an increase in myofibroblasts and BrdU over time. Indeed, the patterns of increase for macrophages, myofibroblasts and BrdU were comparable, and resembled the pattern seen for fibronectin immunostaining, a marker of renal fibrosis [12, 16]. This result followed increases in the number of myofibroblasts associated with tubular epithelial cell and interstitial cell proliferation in UUO. We used double immunostaining to examine which interstitial

cells were responsible for the increase in pSTAT3 positive staining. By using double immunostaining for α-SMA with pSTAT3, we found that diffuse numbers of double-positive myofibroblasts were present within the interstitium. We found only a few double-positive macrophages exhibiting staining for ED-1 or BrdU with pSTAT3. This was an unexpected finding since proliferation of macrophages, tubular epithelial cells and myofibroblasts in vitro is STAT3 dependent. It is possible that mitogenic stimuli in vitro may induce either a very transient or a prolonged period of STAT3 activation. Thus, transient STAT3 activation in macrophages or BrdU positive cells may not be detected by immunostaining, whereas prolonged STAT3 activation in myofibroblast would be detectable. These results suggest that STAT3 activation plays an important role in incremental accumulation of myofibroblasts in the early phase after UUO.

Myofibroblasts play a crucial role in the progression of kidney fibrosis [12]. Macrophages present within the inflamed renal interstitium are derived from the circulation, but the origin of myofibroblasts is unclear. Recently, it has been proposed that myofibroblasts originate from tubular epithelial cells by a process termed epithelial-mesenchymal transition (EMT) [18]. Many cytokines and signaling pathways such as transforming growth factor-beta [19], MAPK/ERK kinase (MEK) 1 and extracellular signal-regulated protein kinase 1 and 2 (ERK1/2) [20] are known to facilitate EMT. Jain et al. described crosstalk between the JAK2/STAT3 and MAPK/ERK pathways [21]. In this study, we demonstrated that pSTAT3 expression by tubular epithelial cells was largely restricted to the cortical collecting ducts of normal rat kidney. After ureteral obstruction, pSTAT3 exhibited marked upregulation in tubular epithelial cells and myofibroblasts. There is evidence that pSTAT3 is localized mainly on collecting ducts and myofibroblasts as well as activated ERK [9]. STAT3 activation may be similar to ERK activation in obstructed kidney, with another pathway causing EMT after UUO.

In summary, our experiments suggest that STAT3 is activated in tubular epithelial cells and myofibroblasts in the early renal response to UUO, and that this activation may contribute to the progression of interstitial fibrosis.

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Figures legends

Figure 1. STAT3 activation in lysates of normal and obstructed rat kidneys. A and B: Panels and graphical presentation showing STAT3 activation. A: The upper panel was probed with an antibody recognizing pSTAT3. The lower panel shows the blot following reprobing with an antibody recognizing actin. B: Densitometric analysis presented as the ratio of activated STAT3 to actin (pSTAT3/ actin).

Figure 2. Immunohistochemical staining for STAT3. Representative micrographs showing immunohistochemical staining for pSTAT3 (A-C) in the renal cortex at day 0 (A) or at day 3 post-UUO (B, C). Section (B) is serial to section (C). A: pSTAT3 at day 0. B: pSTAT3 at day 3 post-UUO. C: pSTAT3 with blocking peptide at day 3 post-UUO.

Figure 3. Induction of pSTAT3 expression in obstructed kidneys. A: Time-dependent induction of pSTAT3 after UUO. Graphical presentation showing the number of pSTAT3-positive tubular epithelial cells (black bars ■) and interstitial cells (white bars □) in the renal cortex of kidneys after immunostaining.

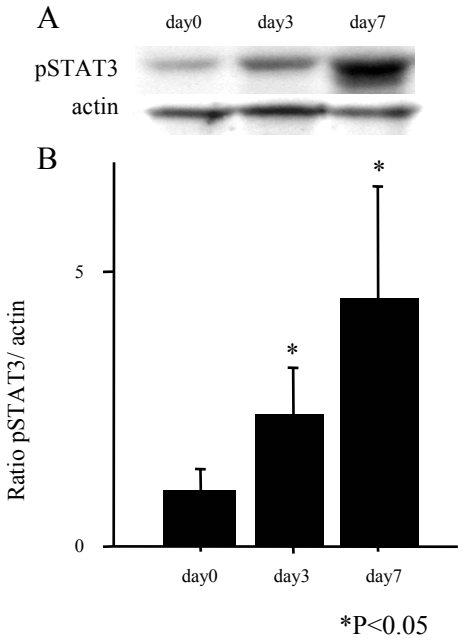
Figure 4. Immunostaining of α-SMA and BrdU in normal and obstructed kidneys. Representative micrographs showing immunohistochemical staining for α-SMA (A - C) and BrdU (D - F); day 0, normal control (A, D), day 3 post-UUO (B, E), day 7 post-UUO (C, F).

Figure 5. Induction of BrdU expression in obstructed kidneys. Graphical presentation showing the number of BrdU-positive tubular epithelial cells (black bars ■) and interstitial cells (white bars □) in

the renal cortex of kidneys after immunostaining.

Figure 6. Localization of pSTAT3 in cortical tubules and interstitium of obstructed kidneys. A - C: Representative micrographs showing immunohistochemical co-staining for pSTAT3 (brown) with aquaporin-2 (AQP-2) (blue/gray) in the cortex of kidneys either at day 0 (A, B) or at day 3 post-UUO (C). A: Normal rat kidney cortex observed with a low power field (Magnification: x150). B: Magnified view of (A). Micrograph showing co-localization of cytoplasmic or nuclear pSTAT3 and cytoplasmic AQP-2 in the occasional cell in the collecting ducts. C: Nuclear or cytoplasmic staining for pSTAT3 (brown) in both dilated collecting ducts and other tubules is shown 3 days after UUO. D - F: Representative micrographs showing co-staining of pSTAT3 (brown) with α-SMA (blue/gray), ED-1 (blue/gray) or BrdU (blue)) 7 days after UUO. (D) is counterstained for PAS. D: Nuclear or cytoplasmic staining for pSTAT3 (brown) with α-SMA (blue/gray) in interstitial lesions is shown in interstitial cells (arrows, double-stained cells). E: Nuclear or cytoplasmic staining for pSTAT3 (brown) with ED-1 (blue/gray) is shown only in the occasional interstitial cell (arrows, double-stained cells). F: Nuclear or cytoplasmic staining for pSTAT3 (brown) with BrdU (blue) is shown only in the occasional interstitial cell (arrows, double-stained cells). Note that there are many cells showing co-staining with α-SMA (D), but there are only a small number of cells showing co-staining with ED-1 (E) or BrdU (F).

Figure 1.


A
day0
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Ratio pSTAT3/ actin
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Figure 2.

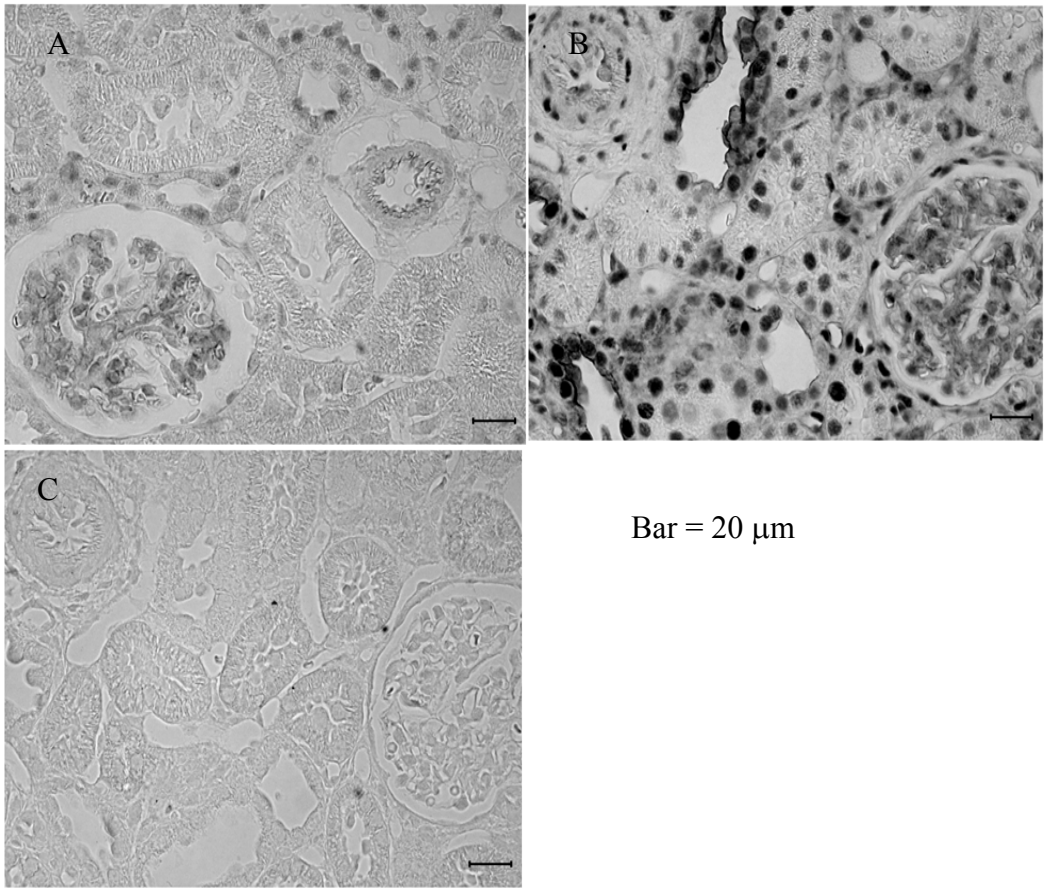
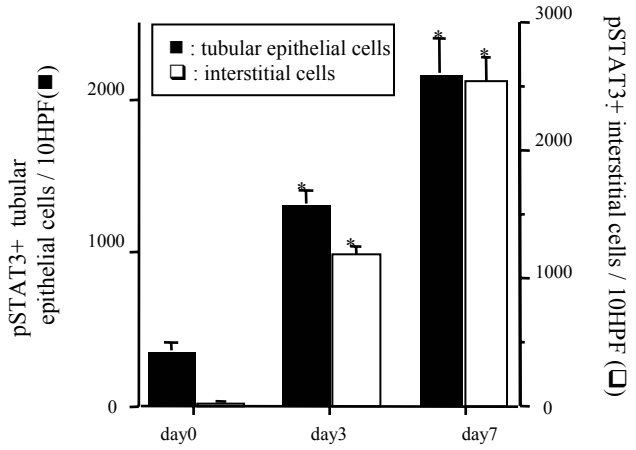


Figure 3.



* P<0.05

Figure 4.

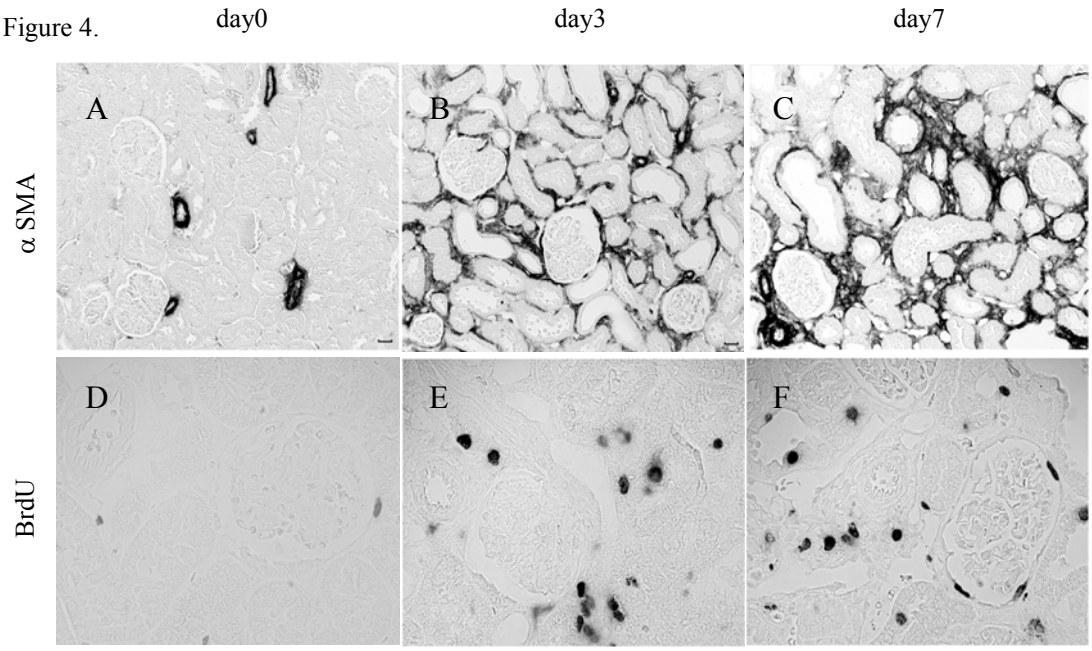


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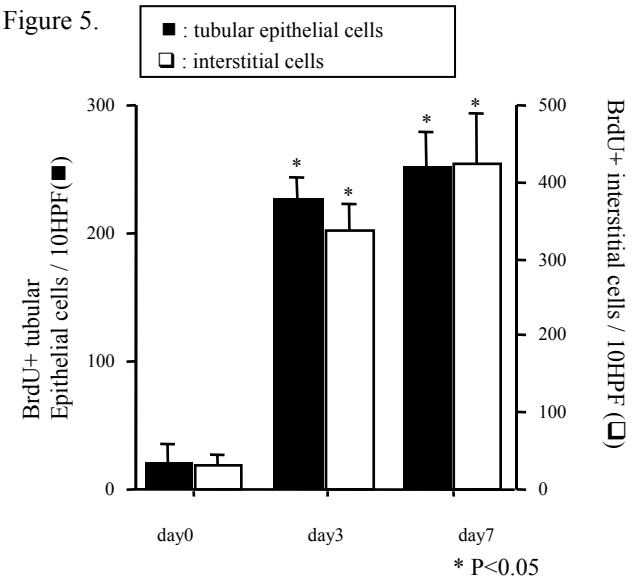
Figure 5.


■ : tubular epithelial cells
❑ : interstitial cells
BrdU+ tubular
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BrdU+ interstitial cells / 10HPF (❑)
300
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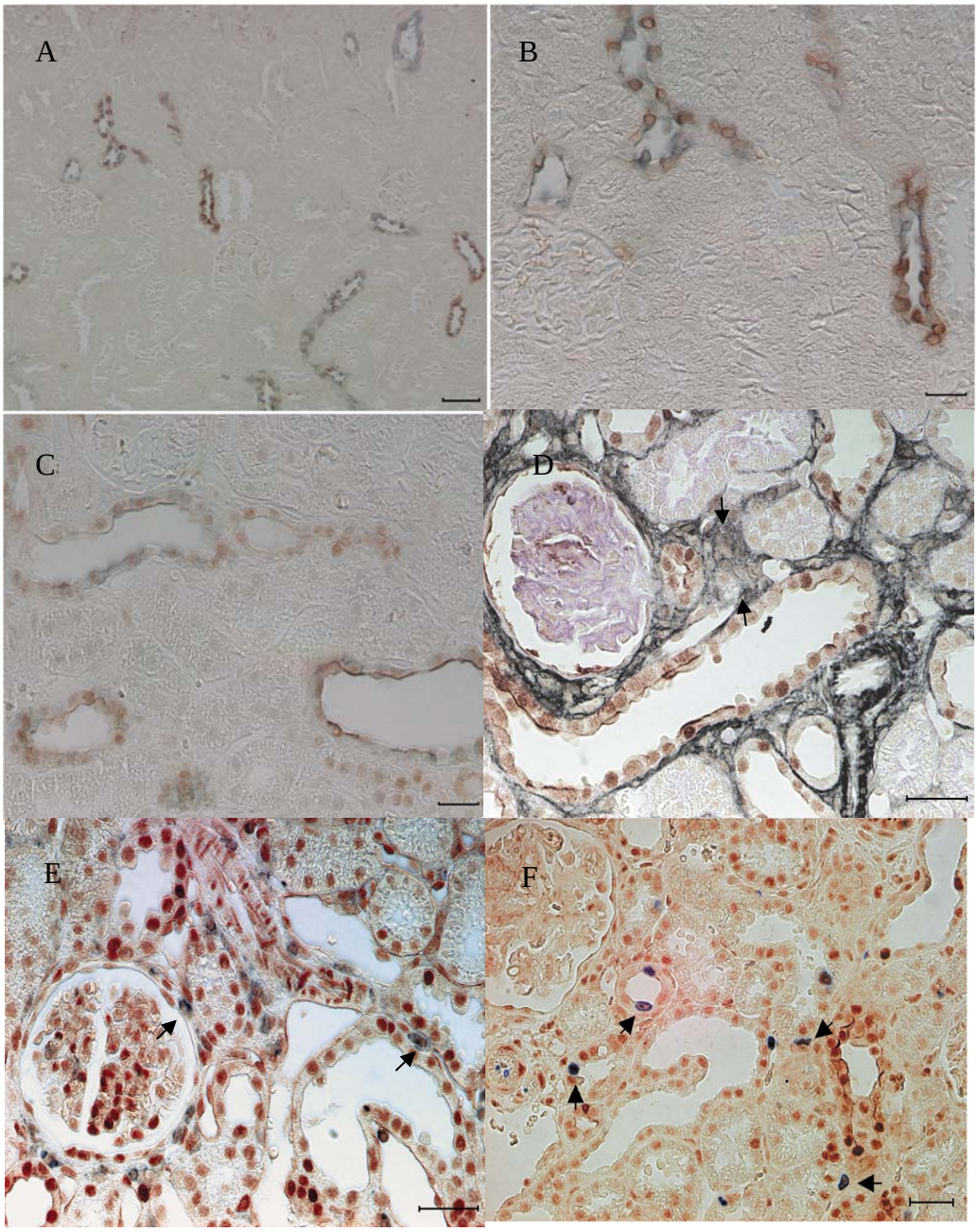
Figure 6.



A: Bar = 50 μm. B-E: Bar = 20μm.