

## Mizoribine Inhibits the Proliferation of Renal Stem/Progenitor Cells by G1/S Arrest during Renal Regeneration

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Immunosuppressive agents are generally administered to treat kidney diseases. However, it is unclear whether renal stem/progenitor cells are directly affected by the immunosuppressive agents. We used normal rat kidney cells, ureteric bud cells and rat kidney stem/progenitor cells in this study. Mizoribine (MZR), cyclophosphamide (CPA) and cyclosporine (CyA) were added to the culture media of these cells. We evaluated the effects of these immunosuppressive agents on cell proliferation using an electrical cell-substrate impedance sensing system (ECIS) and their effects on the process of renal regeneration using the ischemia-reperfusion (I/R) injury rat model. The ECIS data showed that proliferation of each of the 3 types of cells was significantly suppressed by MZR. MZR treatment enhanced renal tubular injury in ischemia-reperfusion (I/R) injured rats, and significantly decreased levels of M-phase cells and Nestin-positive cells. These results suggested that MZR inhibits the cell cycle of renal stem/progenitor cells; thus, physicians should take note that MZR might affect not only inflammation but also renal regeneration.

**Key words:** cell biology, immunosuppression, stem cells

The incidence of chronic kidney disease (CKD) has increased over the last several decades worldwide, and CKD is recognized as a risk factor of cardiovascular disease. CKD can progress to end-stage kidney disease (ESKD), which has no cure and therefore requires renal replacement therapy, namely, dialysis or renal transplantation. The costs of treating ESKD have been reported to be greater than the direct treatment costs of cancer [1]. Regenerative medicine using stem cells is one of the clinical goals for treating kidney disease.

Regarding stem cells in renal tissue, several studies have focused on the regeneration that occurs sub-

sequent to acute tubular injury. Morphological analysis of renal repair after acute tubular injury has long suggested that new tubular cells are generated by the proliferation of pre-existing tubular cells [2-4]. In addition, Sagrinati *et al.* reported that CD24+ CD133+ parietal epithelial cells in Bowman's capsules obtained from humans have self-renewal potential and a high cloning efficiency [5]. In addition, Kitamura *et al.* reported having established renal progenitor-like cells, named rKS56 cells, from the S3 segment of nephrons in the adult rat kidney. These cells exhibit the properties of self-renewal, multiplicity and the ability to repair tissue [6]. It is expected that kidney regenerative therapy could use such cells.

Chronic glomerulonephritis is the underlying disease in many cases of ESKD, and immunosuppressive

drugs are generally used to treat it. Immunosuppressive drugs act mainly on lymphocytes. These drugs suppress T-cell or B-cell proliferation by affecting DNA synthesis, control nephritis through their anti-inflammatory effects, and prevent nephropathic progression. However, it is unclear whether renal constitutive cells are directly affected by the immunosuppressants. Recently, the effects of cyclosporin (CyA) on podocyte proliferation [7] and mizoribine (MZR) on mesangial cell proliferation [8] have been reported, however the influence for renal regeneration by immunosuppressive drugs is not known clearly.

We investigated how the immunosuppressive drugs typically administered for nephritis treatment affect kidney constitutive cells and renal regeneration. We examined the effects of cyclosporin, cyclophosphamide and mizoribine on 3 types of differentiated renal cells. In addition, we evaluated whether the immunosuppressive drugs affect the regeneration process subsequent to renal damage using a kidney ischemia-reperfusion injury rat model.

## Materials and Methods

### *Cell culture and phase-contrast microscopy.*

We used NRK52E cells, UB (ureteric bud) cells, and rKS56 cells. The NRK-52E cells were purchased from Riken (Wako, Saitama, Japan). UB tissues were dissected with fine forceps from the kidneys of embryos obtained from timed-pregnant SD rats (Crea JAPAN) on embryonic day 13 (e13). The UB cells were cultured on type-IV collagen (BD Biosciences, San Jose, CA, USA) and maintained in K+J medium (a 1:1 mixture of the culture supernatant (DMEM containing 10% FCS) of mouse mesenchymal cells (MCS) and modified K1 medium (1:1 mixture of DMEM and Ham's F12 medium, supplemented with 10% FCS, 5  $\mu$ g/ml insulin, 2.75  $\mu$ g/ml transferrin, 3.35 ng/ml sodium selenious acid (GIBCO, Langley, OK, USA), 50 nM hydrocortisone (Sigma, Ronkonkoma, NY, USA), 25 ng/ml of hepatocyte growth factor (Sigma) and 2.5 mM nicotinamide (Sigma) at 37°C with 5% CO<sub>2</sub> and 100% humidity. Cell outgrowth was observed after 7 days. We harvested the cells and used the limiting dilution method to obtain single cells. Finally, we harvested a monoclonal UB cell line. We used the cells from passages 4–8 for this study. The rKS56 cells were obtained as described previously

[6]. We used rKS56 cells from passages 8–10. To evaluate the effect of immunosuppressive agents on NRK, UB, and rKS56 cells, they were seeded in 96-well plates coated with type-IV collagen at a concentration of  $1 \times 10^4$  cells/well. The cells were cultured in K+J medium and treated with PBS, DMSO (Sigma), MZR (Sigma) dissolved in PBS (final concentrations: 0.1, 1, and 10  $\mu$ g/ml), cyclophosphamide (CPA, Sigma) dissolved in PBS (final concentrations: 1, 10, and 100  $\mu$ M), or CyA (Sigma) dissolved in DMSO (final concentrations: 10, 100, and 1,000 ng/ml). We chose the concentrations of these drugs based on published reports [9–11]. After 7 days of incubation, we observed the cells by phase-contrast microscopy using an FSX100 (Olympus) instrument.

***Electrical cell-substrate impedance sensing system (ECIS).*** In this study, we used 8-chambered electrode wells (8W10E+ slide). Prior to seeding the cells, the electrode wells were immersed in an aqueous solution of 10 mM L-cysteine (Sigma). After being washed twice with PBS, they were coated with mouse type-IV collagen (BD Biosciences) at a concentration of 10  $\mu$ g/cm<sup>2</sup>. NRK, UB, and rKS56 cells were inoculated at a concentration of  $1 \times 10^4$  cells/well in K+J medium. We divided the cells into 4 groups, the positive control group (PC: treated with PBS, n = 4), a group treated with 0.1  $\mu$ g/ml of MZR (MZR0.1, n = 4), a group treated with 1  $\mu$ g/ml of MZR (MZR1, n = 4), and a group treated with 10  $\mu$ g/ml of MZR (MZR10, n = 4). The well station was placed in the incubator, and the well plates were placed in it. The well plates were maintained in an ordinary humidified cell culture incubator at 37°C with 5% CO<sub>2</sub> (v/v). We measured resistance and capacitance for 180 h. The ECIS system was obtained from (Applied BioPhysics, Inc, NY, USA).

### ***Effect of immunosuppressive agents for tubular regeneration in ischemia-reperfusion model rat.***

The experimental protocol was approved by the Animal Ethics Review Committee of the Okayama University Graduate School of Medicine, Dentistry and Pharmaceutical Sciences. Male SD rats (Crea JAPAN Inc., Tokyo, Japan) weighing 100–150 g were maintained under conventional laboratory conditions and given free access to water and food. They were divided into 5 groups of 3 rats each. In sham-operated group, a midline abdominal incision was made under sodium pentobarbital-induced anesthesia. In the posi-

tive control group (PC), acute renal failure (ARF) was induced by clamping the left renal artery for 40 min after removing the right kidney (ischemia-reperfusion: I/R). In the MZR group, ARF was induced with 3 mg/kg of MZR that was infused into the peritoneal cavity for 6 days post-surgery. In the CPA group, ARF was induced with 3 mg/kg of CPA that was infused into the peritoneal cavity for 6 days post-surgery. In the CyA group, ARF was induced with 3 mg/kg of CyA that was infused into the peritoneal cavity for 6 days post-surgery. At 7 days post-surgery, the rats were sacrificed. The left kidneys were removed and processed by formalin fixation followed by paraffin-embedding or frozen-sectioning. Blood samples were drawn from the vena cava inferior under anesthesia. We measured creatinine (Cre), and blood urea (BUN) as renal injury markers. We made 4- $\mu$ m-thick sections from the kidney samples. We stained the sections with anti-phospho Histone H3 antibody (Millipore) and anti-Nestin antibody (Santa Cruz) for the 1<sup>st</sup> antibody and Alexa antibody for the 2<sup>nd</sup> antibody (Invitrogen). These tissues were examined by confocal microscopy using an FSX100 (Olympus) instrument with a 20 $\times$  objective. The numbers of phosphor Histone H3-positive and Nestin-positive cells in 1 field of view were counted, and the mean numbers of positive cells from 5 fields of view for each group were used for comparison.

**Statistical analysis.** The ECIS data were analyzed by the Wilcoxon test using the JMP for Windows software package version 8.0.2 (SAS Institute Inc., Cary, NC, USA). The data for the BrdU-positive cell counts were analyzed by an ANOVA test using SPSS version 19.0J software for Windows (IBM Corporation, Somers, NY, USA). *P* values < 0.05 were considered to be statistically significant.

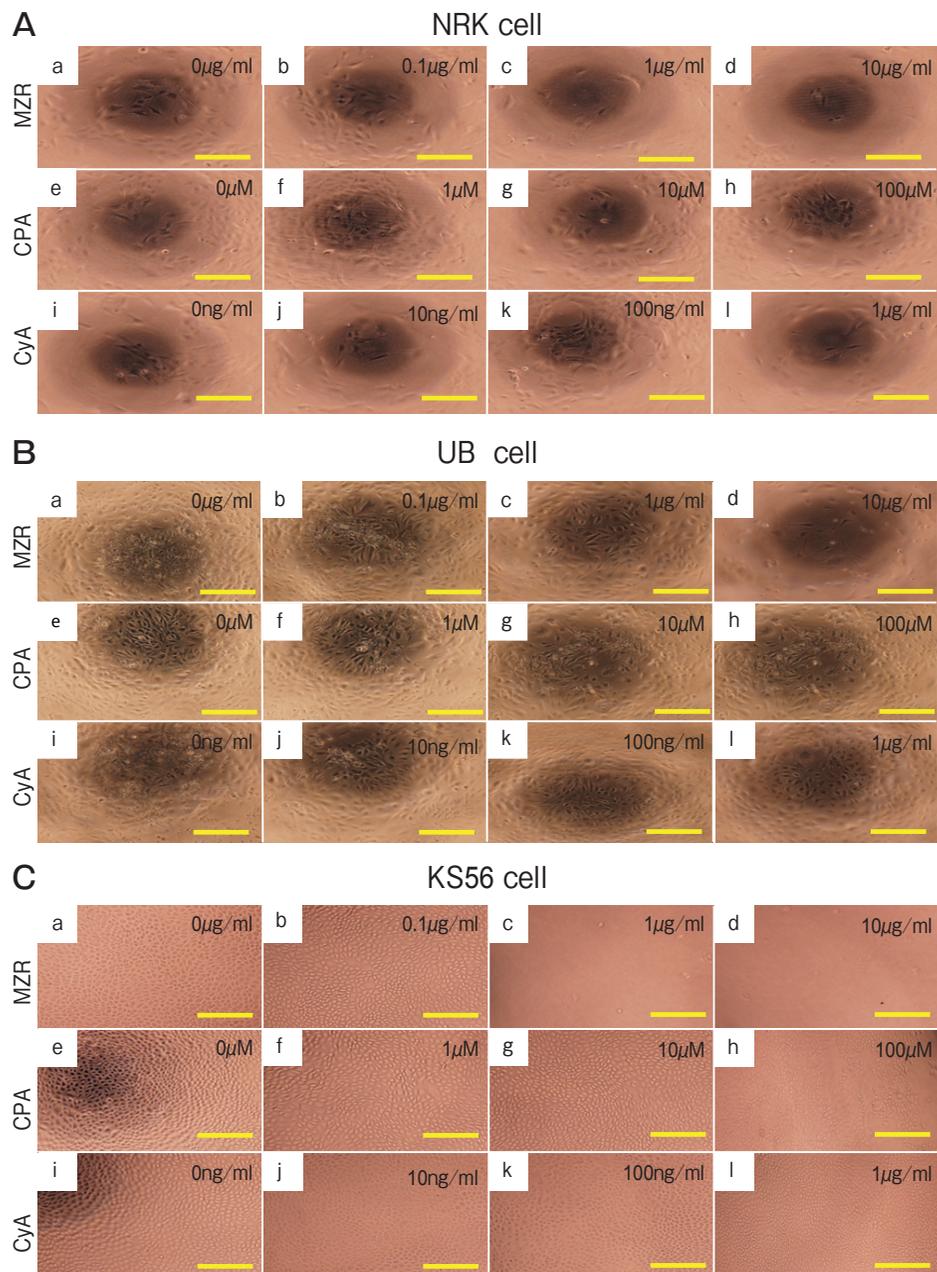
## Results

**MZR suppressed the proliferation of the three types of renal cells.** Phase-contrast image microscopy showed that the proliferation of NRK cells was suppressed by MZR at a concentration of 1 or 10  $\mu$ g/ml (Fig. 1A). The proliferation of UB and rKS56 cells was also suppressed by MZR at 1  $\mu$ g or 10  $\mu$ g/ml (Fig. 1B and 1C). However, CPA and CyA did not affect the proliferation of the 3 types of cells. We

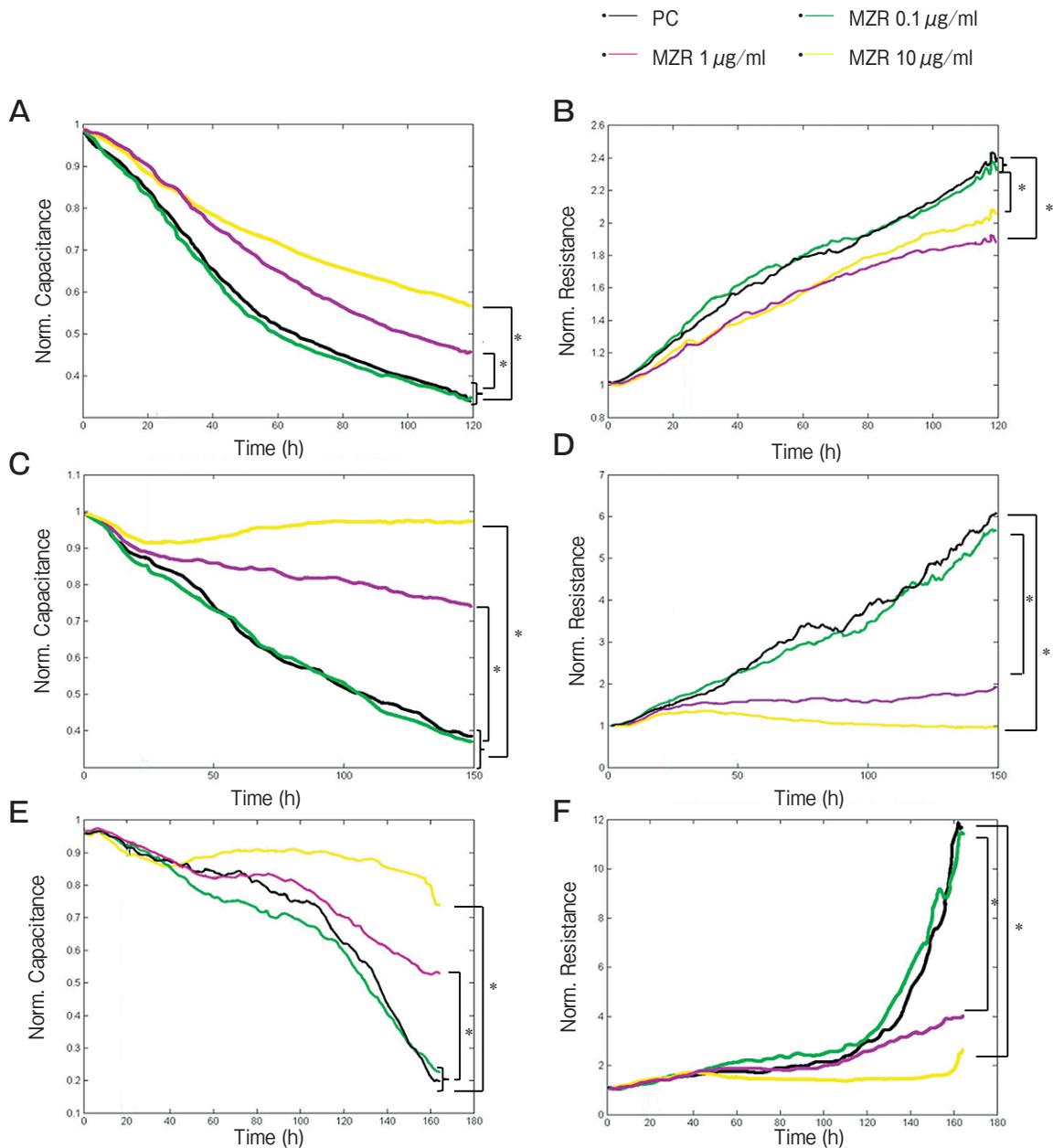
had hypothesized that immunosuppressive agents would affect the differentiation of renal cells, but we did not observe morphological changes in the cells treated with any of the immunosuppressive agents.

**Cell proliferation was suppressed by MZR in a dose-dependent manner.** Using ECIS, we could quantitatively evaluate cell proliferation. A decrease in the capacitance indicates cell proliferation and an increase in the resistance reflects cell proliferation. The ECIS data (Fig. 2) showed that the capacitance of the 3 cell types was significantly higher in the groups treated with 1 or 10  $\mu$ g/ml doses of MZR compared with the group treated with 0.1  $\mu$ g/ml of MZR and the positive control group (*p* < 0.05). For the NRK cells, the group treated with 10  $\mu$ g/ml of MZR exhibited higher capacitance compared with the group treated with 1  $\mu$ g/ml of MZR (*p* < 0.05). However, the ECIS data for the UB and rKS56 cells treated with 10  $\mu$ g/ml of MZR were not significantly different from the corresponding data for the groups of each cell type treated with 1  $\mu$ g/ml of MZR. These results suggested that MZR suppressed cell proliferation and that the proliferation-suppressive capacity of MZR is dose-dependent.

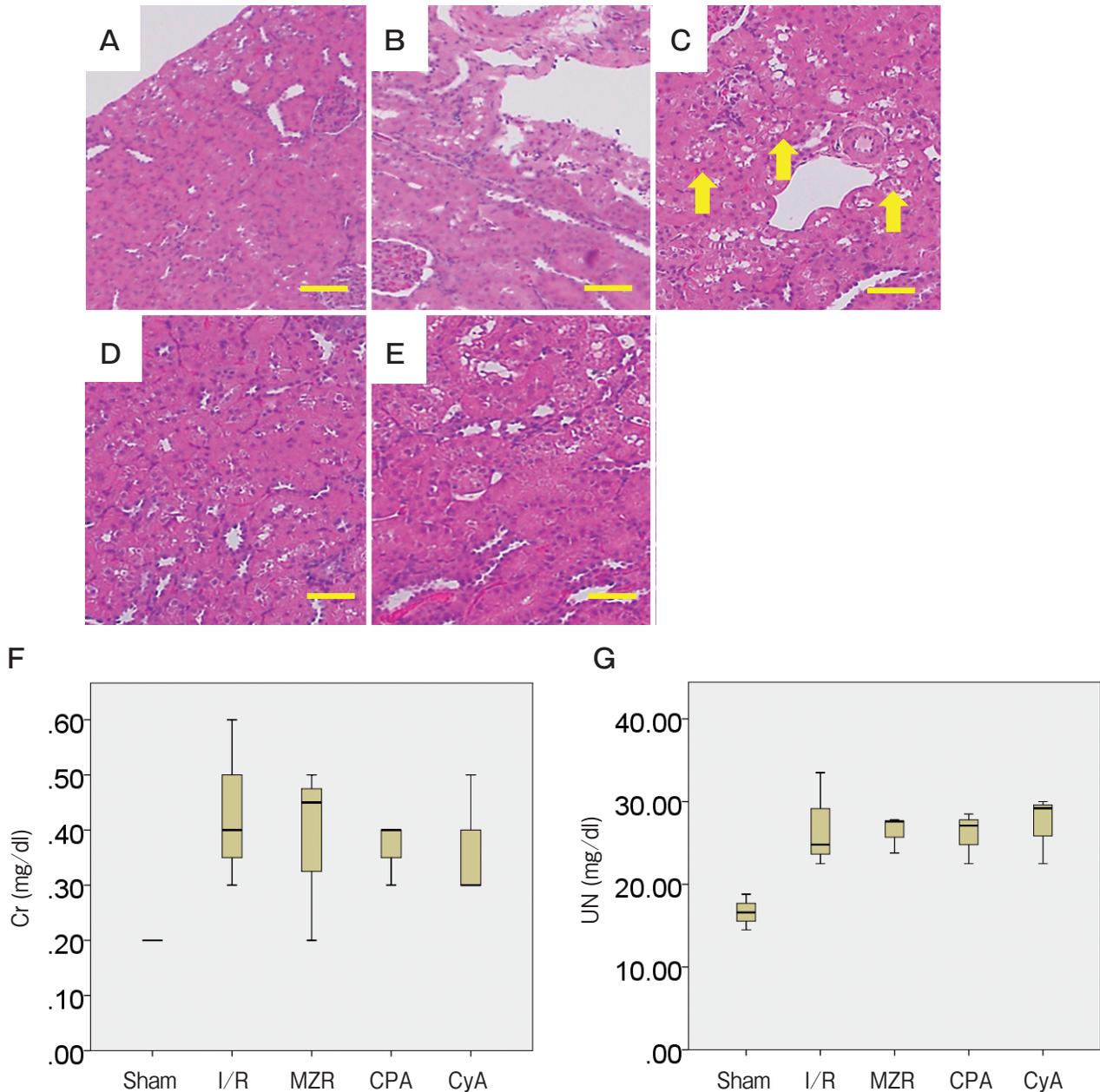
**MZR may inhibit renal regeneration after ischemia-reperfusion tubular injury.** To determine whether MZR affects renal regeneration following tubular injury due to cell cycle arrest, we evaluated tubular damage and the content of M-phase renal cells in the renal ischemia-reperfusion rat model. Two weeks after ischemia-reperfusion injury, tubular damage was not observed in the positive control group (Fig. 3B). Moreover, tubular damage was not observed in the CPA- (Fig. 3D) and CyA- (Fig. 3E) treated groups. However, vacuolar degeneration of tubular epithelial cells was observed in the MZR-treated group (arrows in Fig. 3C). In the groups treated with MZR, CPA and CyA, the Cre and BUN levels were slightly higher than those of the sham-operated group. However, we could not observe any significant differences among the I/R-, MZR-, CPA- and CyA-treated groups (Fig. 3F and G). Additionally, we stained with Phospho Histone H3 to detect M-phase cells (Fig. 4 A-E, and F). In the groups treated with immunosuppressive agents without MZR, the numbers of M-phase cells were drastically increased compared with those of the sham-operated and PC groups. Regarding the immunosuppressive agents, the number



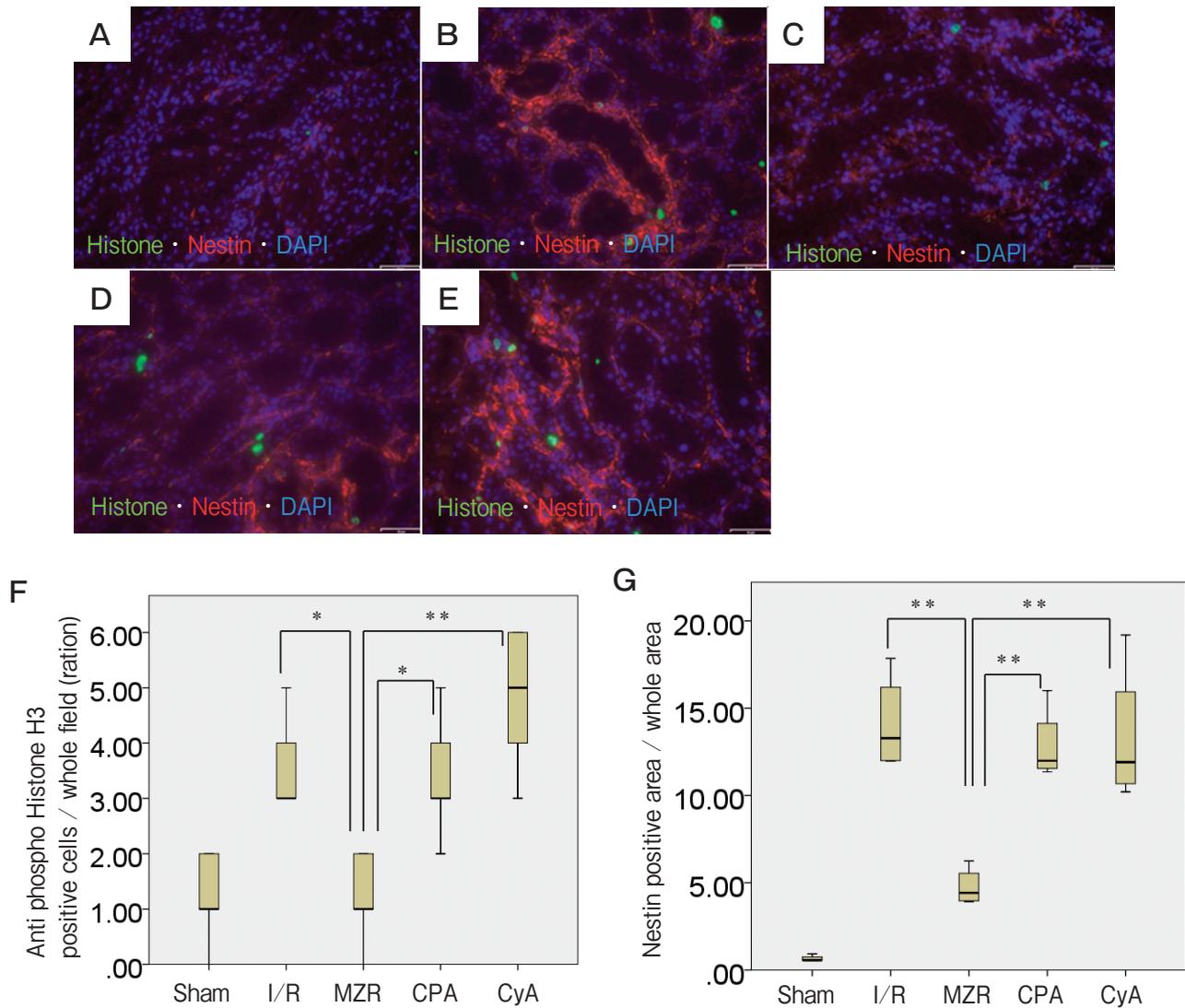
**Fig. 1** **A**, Effects of immunosuppressive agents on NRK cells. Phase-contrast microscopy showed that the proliferation of NRK cells was suppressed by MZR at concentrations of 1 (c) or 10 (d)  $\mu$ g/ml. Although we used different doses of CPA and CyA, no treatment with CPA or CyA suppressed the proliferation of NRK cells. We observed no change in the morphology of NRK cells treated with any of the immunosuppressive agents; **B**, Effects of immunosuppressive agents on UB cells. Phase-contrast microscopy showed that the proliferation of UB cells was also suppressed by MZR at 10  $\mu$ g/ml (Fig. Bd). Treatment with CPA or CyA did not suppress the proliferation of UB cells. UB cell morphology was not changed by treatment with any of the immunosuppressive agents; **C**, Effects of immunosuppressive agents on rKS56 cells. Phase-contrast microscopy showed that the proliferation of the rKS56 cells was suppressed by MZR at concentrations of 1  $\mu$ g/ml (c) or 10  $\mu$ g/ml (d). However, treatment with CPA or CyA did not suppress the proliferation of rKS56 cells. rKS56 cell morphology was not changed by treatment with any of the immunosuppressive agents. Scale bar: 100  $\mu$ m. a, negative control; b, 0.1  $\mu$ g/ml of MZR; c, 1  $\mu$ g/ml of MZR; d, 10  $\mu$ g/ml of MZR; e, PBS; f, 1  $\mu$ M of CPA; g, 10  $\mu$ M of CPA; h, 100  $\mu$ M of CPA; i, DMSO; j, 10 ng/ml of CyA; k, 100 ng/ml of CyA; l, 1,000 ng/ml of CyA. MZR, mizoribine; CPA, cyclophosphamide; CyA, cyclosporin.



**Fig. 2** Cell proliferation analysis using the electrical cell-substrate impedance sensing system (ECIS). The cells were divided into 4 groups, the positive control group (PC), a group treated with 0.1  $\mu\text{g/ml}$  of MZR (MZR0.1,  $n = 4$ ), a group treated with 1  $\mu\text{g/ml}$  of MZR (MZR1,  $n = 4$ ), and a group treated with 10  $\mu\text{g/ml}$  of MZR (MZR10,  $n = 4$ ). (A) The ECIS data showed that the capacitance of the NRK cells was significantly higher in the MZR1 and MZR10 groups compared with PC and MZR0.1 groups (PC vs. MZR1, PC vs. MZR10, MZR0.1 vs. MZR1, MZR0.1 vs. MZR10, MZR1 vs. MZR10,  $p < 0.05$ ). (B) The resistance of the NRK cells in the MZR1 and MZR10 groups was significantly decreased compared to those of the other NRK groups (PC vs. MZR1, PC vs. MZR10, MZR0.1 vs. MZR1, MZR0.1 vs. MZR10, MZR1 vs. MZR10,  $p < 0.05$ ). As was found for the NRK cells, the capacitance (C) and resistance (D) of the UB cells in the MZR1 and MZR10 groups were significantly different from those of the PC and MZR0.1 groups (PC vs. MZR1, PC vs. MZR10, MZR0.1 vs. MZR1, MZR0.1 vs. MZR10,  $p < 0.05$ ). The capacitance (E) and resistance (F) of the rKS56 cells exhibited the same pattern of differences (PC vs. MZR1, PC vs. MZR10, MZR0.1 vs. MZR1, MZR0.1 vs. MZR10,  $p < 0.05$ ). \* $p < 0.05$ .



**Fig. 3** Effect of immunosuppressive agents on the I/R rat kidney model. Two weeks after I/R injury, tubular damage was not observed in the positive control group, the CPA-treated group, or the CyA-treated group. However, in the MZR-treated group, vacuolar degeneration of tubular epithelial cell was observed (arrows in c). **A**, sham-operated group; **B**, positive control group; **C**, MZR-treated group; **D**, CPA-treated group; **E**, CyA-treated group. Three independent animals from each group showed similar findings. Scale bar: 200  $\mu$ m; **F**, The Cr levels were slightly higher in the ARF groups with/without immunosuppressive agents than in the sham-operated group (each group:  $n = 3$ ); **G**, The BUN levels were slightly higher in the ARF groups with/without immunosuppressive agents than in the sham-operated group (each group:  $n = 3$ ).



**Fig. 4** Phospho Histone H3 positive and Nestin-positive cells in the I/R rat kidney. Scale bar: 50  $\mu$ m. **A–E**) Phospho Histone H3, green; Nestine, red; DAPI, blue. **A**, sham-operated group; **B**, I/R injury positive control group; **C**, the group with I/R injury treated with 3mg/kg of MZR; **D**, the group with I/R injury treated with 3mg/kg of CPA; **E**, the group with I/R injury treated with 3mg/kg of CyA. Three independent animals from each group showed similar findings; **F**, Phospho Histone H3-positive cells decreased significantly in MZR-treated group compared to the CPA- and CyA-treated groups; **G**, The Nestine-positive area decreased significantly in the MZR-treated group compared to the CPA- and CyA-treated groups. Each group contained 3 animals. Each animal was evaluated in 5 fields in the sections. (\* $p < 0.05$ , \*\* $p < 0.01$ )

of M-phase cells in the renal cortex was lower in the MZR-treated group compared with the CPA- and CyA-treated groups (\* $p < 0.05$ , \*\* $p < 0.01$  for both comparisons). In addition, Nestin, which is reported as an immature cell marker, was significantly decreased in the MZR-treated group (Fig. 4A–E, and G). These results suggested that MZR inhibits cell division and

immature cell proliferation. The inhibition of cell proliferation may lead to inhibit the recovery for renal regeneration.

## Discussion

Immunosuppressive agents have been reported to

cause renal injury. The major limiting factors for the use of CyA are its acute and long-term toxic effects on the kidney. In the short term, CyA induces a reversible reduction in renal blood flow and the glomerular filtration rate [12]. In the long term, CyA treatment leads to irreversible renal failure characterized by extensive tubulointerstitial fibrosis [13]. It was recently reported that Nox2, the classic NADPH oxidase of phagocytes, plays an important role in calcineurin inhibitor-induced renal fibrosis [14]. In addition, tubular cell injury and fibrosis are key features of calcineurin inhibitor nephrotoxicity [15]. Oxidative stress may play a role in CPA-induced renal damage. Intraperitoneal administration of CPA has been reported to increase the level of malondialdehyde, decrease the level of glutathione, and decrease the activities of antioxidative enzymes in the kidney, including superoxide dismutase, glutathione peroxidase, glutathione reductase, and catalase [16–19]. MZR may induce acute renal failure due to the hyperuricemia associated with mizoribine therapy [20]. However, the exact mechanisms of immunosuppressive agent-induced renal damage remain to be fully elucidated. There are various reports about the amount of renal damage caused by immunosuppressive agents, but how these drugs affect the regeneration process following renal damage is not known. In our study, MZR was shown to inhibit the proliferation of renal constitutive cells (Fig. 1, 2). This effect may depend on the drug dosage. Therefore, we investigated the effect of immunosuppressive agents on the cell cycle. Our flow cytometry analysis demonstrated that the proportion of G1/S phase cells was increased by MZR treatment (data not shown). Liu S *et al.* reported that MZR down-regulated the expression of Skp2, a member of the SCF<sup>Skp2</sup> ubiquitin ligase complex that is responsible for p27<sup>kip1</sup> degradation, and induced G1/S arrest in mesangial cells. In addition, they found that MZR induced G1/S arrest by inhibiting *de novo* purine synthesis. In their report, because a DNA synthesis inhibitor did not increase the level of p27<sup>kip1</sup> protein as MZR did, they concluded that the effect of MZR on the p27<sup>kip2</sup> protein level was not dependent on inhibiting DNA synthesis [8]. We did not examine these mechanisms of MZR. The effect of MZR on the renal cell cycle may not be due to inhibiting purine metabolism.

Injury models with proximal tubule damage, such

as I/R or nephrotoxin models, have been extensively used to study tubular regeneration. Kidney regeneration after I/R injury requires approximately 1 week. It was reported that proliferating tubular epithelial cells are not observed before I/R injury, but they are observed after I/R injury [21]. As previously described, MZR may block the renal cell cycle in the G1/S phase, and decrease the number of M-phase cells. In addition, the immature cells in the adult rat kidney were inhibited from proliferating in the MZR-treated group. This suggested that MZR would prolong the regeneration of renal tubule cells after I/R injury. H&E-staining confirmed that the recovery of MZR-treated rats from tubular injury was prolonged.

The rKS56 cells that we used in this study originated in the S3 segment of nephrons, and have the properties of self-renewal, multi-plasticity and the ability to repair tissue [6]. Therefore, cells similar to rKS56 cells may be involved in tubular regeneration. Our *in vitro* study demonstrated that MZR suppressed the proliferation of rKS56 cells, and in our animal study, M-phase cells in I/R and Nestin-positive cells decreased significantly with treatment by MZR compared with other immunosuppressive agents. MZR may have a more potent effect on the proliferation of cells that are involved in tubular regeneration than do the other immunosuppressive agents. Neither CPA nor CyA suppressed proliferation of rKS56 or UB cells *in vitro*, and we observed no tubular damage from treatment with these drugs *in vivo*. However, the mechanisms and the cells involved in the regeneration of renal tubules have not yet been elucidated.

In conclusion, we evaluated the effect of immunosuppressive agents on renal constitutive cells. CyA and MZR arrested the cell cycle of renal cells. Additionally, MZR may inhibit renal tubular regeneration by blocking the cell cycle of renal stem/progenitor cells.

**Acknowledgments.** A portion of this study was supported by a Grant-in-Aid for Young Scientists (B) of Japan and a grant from the Okayama Medical Foundation of Japan and a joint research grant with Organ Technologies, Inc. of Japan.

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