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Nestin expression in the kidney with an obstructed ureter

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Nestin is an intermediate filament protein originally identified in neuroepithelial stem cells. This cytoskeletal-associated protein is also expressed in some non-neuronal organs including renal tubular cells and glomerular endothelial cells during kidney development. Little is known, however, about nestin expression in the kidney during injury. In this study, we find nestin expression induced in renal tubular and interstitial myofibroblasts in the adult rat kidney following unilateral ureteral obstruction. The degree of nestin expression was well correlated with the degree of tubulointerstitial fibrosis. Immunohistochemical identification of specific nephron segments showed that nestin was primarily expressed by proximal tubules, partially by distal tubules and thick ascending limbs of Henle but not by collecting ducts. The nestin-positive tubular cells also expressed vimentin and heat-shock protein 47 (HSP47) suggesting these cells reverted to a mesenchymal phenotype. Not all vimentin- or HSP-expressing cells expressed nestin; however, suggesting that nestin is distinct from these conventional mesenchymal markers. Nestin expression was also found associated with phenotypical changes in cultured renal cells induced by hypoxia or transforming growth factor-β. Nestin expression was located in hypoxic regions of the kidney with an obstructed ureter. Our results indicate that nestin could be a novel marker for tubulointerstitial injury.

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Nestin is a cytoskeleton-associated class VI intermediate filament protein, which was originally identified in neuroepithelial stem cells.[1,2] In contrast to microfilaments and microtubules, whose components are highly evolutionarily conserved and very similar within cells of a particular species, intermediate filaments display much diversity in their numbers, sequences, and abundance.[3] In humans, there are more than 60 different intermediate filament genes, which are differentially expressed in nearly all cells of the body.[4,5] Changes within the spatial and temporal expression of intermediate filament proteins were thought to regulate remodeling of the cell cytoskeleton during development.[6]

Although nestin was initially identified as a marker for neural stem and neural progenitor cells, a wider range of nestin expression has been found in non-neuronal organs than previously thought.[7] The expression of nestin was reported in developing organs in the fetus, including skeletal muscle cells,[8] cardiomyocytes,[9] pancreatic epithelial progenitor cells,[10] and vascular endothelium.[11] Compared to embryonic tissues, nestin expression is limited in normal adult tissues. However, re-expression of nestin has been reported during injury of adult organs, such as in central nervous system,[12,13] skeletal muscle,[14] liver,[15] pancreas,[16] and teeth.[17]

Recently, nestin expression in embryonic and adult kidney has been reported.[18–20] In immature glomeruli, nestin is expressed in the progenitors of glomerular endothelial cells. Nestin is also transiently expressed by epithelial cells of immature proximal tubules in the newborn kidney.[11] In contrast, in the mature adult kidney, podocytes are the only cells that exhibit persistent nestin expression.[18] However, nestin expression in injured kidneys has been poorly investigated, especially in tubulointerstitial injury.

Tubular damage and interstitial fibrosis are considered a final common pathway leading to end-stage renal disease.[21–23] Irrespective of the nature of initial renal injury, the degree of tubular damage correlates well with the decline of renal function and long-term prognosis.[21,22] Therefore, it is important to understand the molecular mechanisms underlying the progression of interstitial fibrosis. Recently, phenotypic change of renal tubular cells, so-called epithelial-

to-mesenchymal transition, was considered important in the progression of tubulointerstitial injury.[24–26] During injury, tubular epithelial cells lost the expression of epithelial cell marker, E-cadherin, and acquired mesenchymal features such as vimentin and α-smooth muscle actin (α-SMA).[25] Vimentin is a type III intermediate filament, which is found in cells of mesenchymal origin. Vimentin is known to assemble with nestin, *in vivo* or *in vitro*,[8,27–29] suggesting the possibility of nestin expression in vimentin-positive cells in injured kidneys.

In this study, we hypothesized that nestin is re-expressed in tubular cells during tubulointerstitial injury. We examined an adult rat unilateral ureteral obstruction (UUO) model and found that increased nestin expression was detected in renal tubular cells and tubulointerstitial myofibroblasts during the progression to tubulointerstitial fibrosis. We further demonstrated that increased nestin expression was associated with hypoxia and transforming growth factor-beta (TGF-β) stimulation in cultured renal cells. Hypoxia and TGF-β are recognized as important mediators for aggravating tubulointerstitial injury.[23,30–34] We propose that nestin could be a novel marker for tubulointerstitial injury.

RESULTS

Nestin expression in normal adult rat kidney and UUO kidney

We first examined the localization of nestin by immunohistochemical analysis in normal adult rat kidney. As previously reported,[18,19] nestin was predominantly detected in the glomeruli. No staining was observed in the tubulointerstitial area (Figure 1a and b). In the glomeruli, co-expression of nestin with synaptopodin, a marker for podocytes, was observed by double-immunofluorescent staining, but not with rat aminopeptidase P, a marker of endothelial cells (data not shown), consistent with the previous report that nestin was predominantly expressed in podocytes in normal adult kidney.[18,19] In contrast, nestin was strongly expressed by tubular and interstitial cells, in addition to podocytes, in the kidney at day 13 after UUO. Nestin-positive tubular cells and tubulointerstitial cells were localized mainly at the outer medulla, rather than cortex and inner medulla (Figure 1c and d). Dilated tubules tended to show nestin expression and its distribution within cells was abundant on the basolateral side and scant at the apical pole of these cells (Figure 1c–f). Nestin-positive cells were also detected in the tubulointerstitial area and most were spindle-shaped (Figure 1e and f).

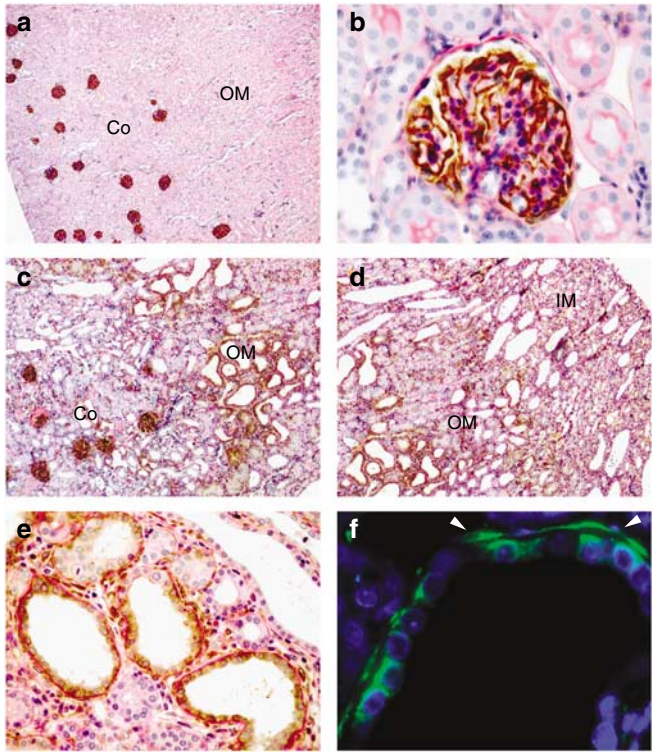

Figure 1 | Expression of nestin in the normal and UUO kidney. Nestin expression was detected by (**a**–**e**) immunohistochemical staining and (**f**) immunofluorescent staining. (**a** and **b**) Nestin expression was predominantly in glomeruli in normal adult rat kidney. (**c**–**f**) In UUO kidney at day 13, nestin expression was observed at tubular cells and tubulointerstitial cells mainly at outer medulla (OM), rather than cortex (Co) and inner medulla (IM). Nestin expression tends to be observed at the (**c**–**e**) dilated tubules and (**f**: arrowheads) spindle-shaped interstitial cells. (**f**: arrows) Nestin expression is more abundant at the basolateral plasma membrane and scant at apical pole of the tubular cells. Immunohistochemistry used counterstaining with periodic acid-Schiff. Immunofluorescence was counterstained in blue with DAPI. Original magnifications: (**a**) ×100, (**b** and **e**) ×400, (**c** and **d**) ×200, and (**f**) ×1000.

Time-course study of nestin expression and interstitial fibrosis after UUO

We then examined the temporal expression of nestin after UUO. At day 3 after UUO operation, only a small number of interstitial cells expressed nestin and no tubular cells were positive for nestin (Figure 2a–d). At day 7, nestin-positive interstitial cells were markedly increased and some nestin-positive tubular cells were detected. At day 13, the number of nestin-positive tubular cells increased as well as that of interstitial cells. As shown in Figure 2e and f, the numbers of nestin-positive interstitial cells and tubular cells were significantly correlated with the tubulointerstitial fibrosis score. The changes of nestin expression were further determined by Western blotting using whole kidney lysate after the UUO procedure (Figure 2g). Nestin expression was faint at day 0. Then it increased gradually in the UUO kidney, whereas no increase was noted in the contralateral kidney. As podocytes express nestin in the normal kidney[18,19] and their nestin expression is reported to be increased during puromycin aminonucleoside nephropathy,[19] we used kidney lysates without glomeruli, and performed Western blot analysis. Increased nestin expression was clearly confirmed in kidney lysates in which glomeruli were eliminated by sieving (Figure 2h).

Distribution of nestin-positive tubular cells in UUO

To determine which segment of tubular cells express nestin in UUO, we performed double immunostaining using several markers of nephron segments (Figure 3). Many nestin-

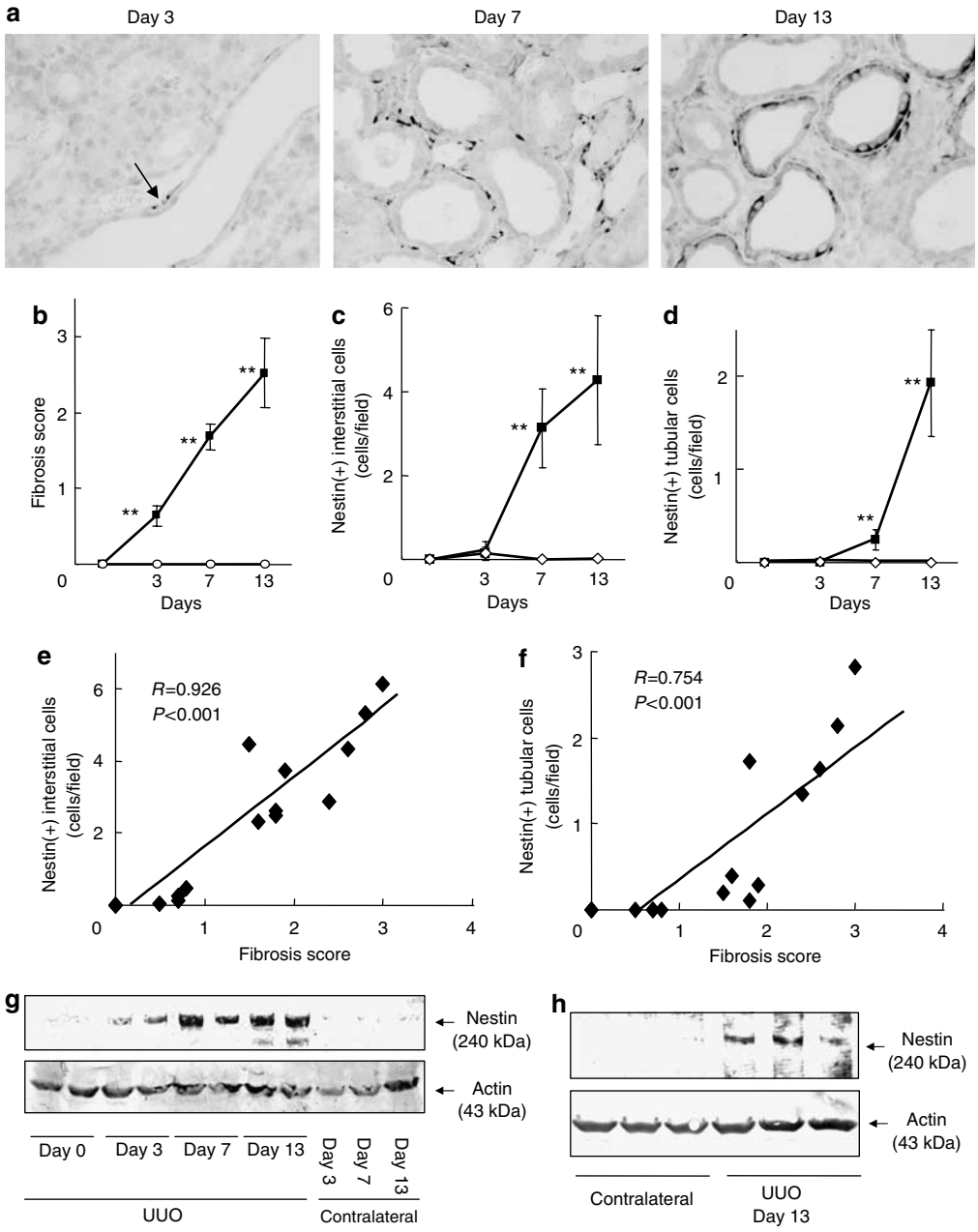

Figure 2 | Temporal expression of nestin in UUO. (**a**) Immunohistochemical nestin staining in UUO kidney. Nestin-positive interstitial cells were already observed at day 3 in UUO (arrow). (**b**) Tubulointerstitial fibrosis score, (**c**) number of nestin-positive interstitial cells, and (**d**) number of nestin-positive tubular cells were examined in UUO (-■-) and contralateral (-◇-) kidneys, as described in Materials and Methods. Significant positive correlation was observed between tubulointerstitial fibrosis score and (**e**) the number of nestin-positive interstitial cells and (**f**) nestin-positive tubular cells. Upregulation of nestin protein expression was confirmed by western blotting of the (**g**) whole kidney lysate and (**h**) kidney lysate without glomeruli. Actin was used as a loading control.

positive tubular cells were co-stained with aquaporin (AQP)-1, a marker of proximal tubules. Some nestin-positive tubular cells were found in tubules labeled with peanut agglutinin, a type of lectin that labels distal tubules and collecting ducts. However, nestin-positive cells were never detected in tubules expressing AQP-2, a marker of collecting tubules, indicating that nestin was expressed by some distal tubules, but not by collecting tubules. Of note, at distal tubules, nestin-positive cells themselves were not labeled with peanut agglutinin, suggesting that phenotypic changes developed in nestin-positive distal tubular cells. Some nestin-positive tubules were also co-stained with Tamm Horsfall glycoprotein, which is expressed in the thick ascending limb of Henle. Accordingly, in the UUO kidney, nestin expression was primarily detected in the proximal tubular cells. Some nestin-positive cells were also detected in the distal tubules and the thick

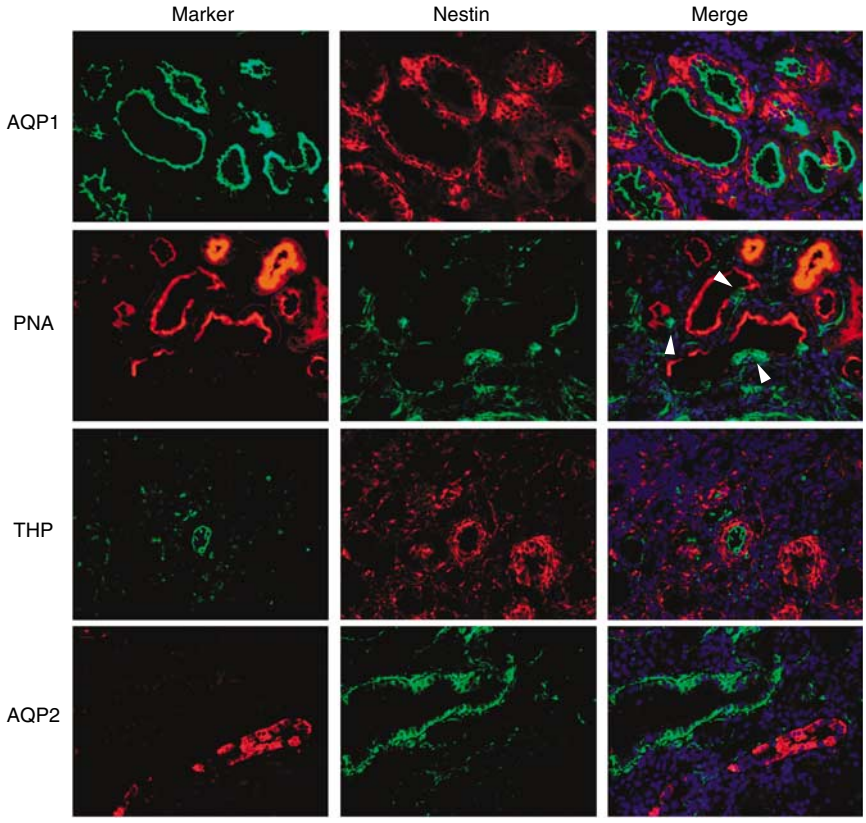

Figure 3 | Expression of nestin in each tubular segment. Double-immunofluorescent study for nestin and specific markers of each tubular segment in UUO kidney at day 13. Nestin expression was observed at tubules stained with AQP-1, peanut agglutinin (PNA), and Tamm Horsfall glycoprotein (THP). In contrast, nestin expression was not observed at tubules stained with AQP-2. Of note, nestin-positive tubular cells were not stained with PNA (arrowheads), whereas other cells in the same tubule were stained with PNA. Original magnification $\times 400$. Nuclei were stained in blue with DAPI.

ascending limb of Henle, but not in the collecting ducts. These findings agree with a previous report that nestin contributes to the differentiation of structures derived from metanephric mesenchyme in the developing kidney, but not to structures from the ureteric bud.[18]

Characterization of nestin-positive cells in UUO

Phenotypic changes of tubular cells are considered to be involved in the progression of tubulointerstitial injury.[24–26] To characterize nestin-positive tubular and interstitial cells in UUO, we performed double immunostaining using several cell-type-specific markers at day 13 after UUO (Figure 4 and Table 1). All nestin-positive tubular and interstitial cells were co-stained with type III intermediate filament, vimentin, a mesenchymal marker. This finding is consistent with the previous report that nestin requires the presence of other intermediate filament proteins to assemble into heterodimers and mixed polymers.[27–29] Nestin in tubular and interstitial cells was also co-stained with another mesenchymal marker, heat-shock protein 47 (HSP47), a chaperon protein associated with the production of collagen. However, not all vimentin- or HSP47-positive cells expressed nestin. Nearly all nestin-positive interstitial cells were co-stained with α-SMA, indicating that most were myofibroblasts. Nestin-positive interstitial cells at day 3 after UUO were also all positive for vimentin and mostly positive for α-SMA (data not shown). No nestin-positive interstitial cells were co-stained with ED1, a marker of monocyte and macrophage. In addition, nestin-positive tubular cells never co-expressed E-cadherin, an epithelial marker that is known to be expressed abundantly in the tubular segments except proximal tubule and reduced in the UUO kidney.

Induction of nestin in cultured cells after exposure to hypoxia or TGF-β

To investigate the regulatory mechanism of nestin expression, we examined the effect of hypoxia or TGF-β on nestin expression using a tubular cell line, LLC-PK1 cells, and a renal fibroblast cell line, NRK-49F cells. Hypoxia is considered to be an underlying common mechanism for the progression of renal tubulointerstitial fibrosis.[23,30–32] LLC-PK1 cells showed typical cobblestone appearance in normoxic conditions (21% O_2, Figure 5a), whereas these cells exhibited spindle-shaped morphology, when cultured in hypoxic conditions for 7 days (1% O_2, Figure 5b). In normoxic conditions, nestin expression was rarely observed by immunofluorescent study (Figure 5c). In contrast, the number of nestin-positive cells was increased in hypoxic

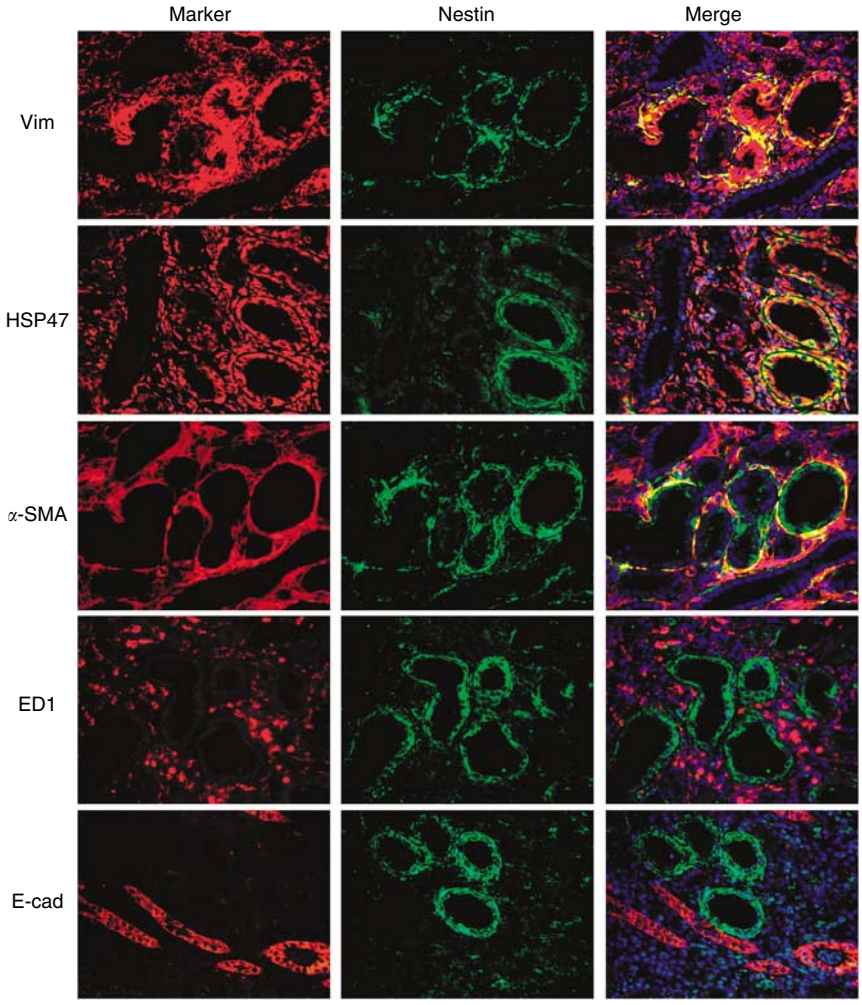

Figure 4 | Phenotypic changes of nestin-positive cells. Double-immunofluorescent study for nestin and phenotypic markers in UUO kidney at day 13. Vimentin (Vim) and HSP47 expression was widely observed at both tubular cells and tubulointerstitial cells. Some vimentin- or HSP47-positive cells were co-stained with nestin. Almost all nestin-positive cells were positive for vimentin or HSP47. α-SMA was detected in tubulointerstitial cells. Some interstitial nestin-positive cells were co-stained with α-SMA. ED1-positive cells were detected at the tubulointerstitial area. However, nestin-positive cells were distinct from ED1-positive cells. The number of E-cadherin (E-cad)-positive tubular cells was reduced in UUO kidney, compared to the contralateral kidney. Nestin-positive tubular cells were never co-stained with E-cad. Original magnification $\times 400$. Nuclei were stained in blue with DAPI.

conditions (Figure 5d). Nestin was observed mainly in spindle-shaped cells in which rearrangement of actin was demonstrated by the staining of rhodamine-conjugated phalloidin (data not shown). Increased nestin protein in hypoxic conditions was also confirmed by Western blotting, together with increased vimentin expression (Figure 6a). These results indicate that nestin is expressed in the process of hypoxia-induced cellular transformation. We then examined the effect of pharmacological modulation of hypoxia on nestin expression. Treatment with prolyl hydroxylase inhibitor cobalt chloride, which results in stabilizing hypoxia-inducible factor-α (HIF-α), induced nestin expression in normoxic conditions (Figure 6b). In addition, upregulation of nestin by hypoxia was abolished by the administration of mTOR (mammalian target of rapamycin) inhibitor rapamycin, which is known as a negative regulator of HIF-α[35,36] (Figure 6c). These results indicated that nestin protein is increased by hypoxia in LLC-PK1 cells in the process of phenotypic transformation, and HIF-α is involved in this process. TGF-β is a well-known growth factor that is deeply involved in renal tubulointerstitial injury.[33,34] Nestin expression was also increased by stimulation with TGF-β1 in NRK-49F cells along with the upregulation of α-SMA (Figure 6d).

Nestin expression and hypoxic area after UUO

As hypoxia induced increased nestin expression in cultured renal tubular cells, we investigated the relation between hypoxia and nestin expression after UUO, using hypoxia responsible element (HRE)-transgenic hypoxia-sensing rats. In HRE transgenic rats, the FLAG-tagged luciferase gene is designed to be translated in hypoxic cells and the luciferase can be detected by immunostaining with anti-FLAG anti-

Table 1 | Relationship between nestin expression and expressions of mesencymal markers in UUO kidneys

Tubular cells	
Nestin (+) cells in vimentin (+) cells	29.6±8.4%
Vimentin (+) cells in nestin (+) cells	100.0±0%
Nestin (+) cells in HSP47 (+) cells	34.4±17.6%
HSP47 (+) cell in nestin (+) cells	99.9±0.9%
Tubulointerstitial cells	
Nestin (+) cells in vimentin (+) cells	31.9±3.6%
Vimentin (+) cells in nestin (+) cells	100.0±0%
Nestin (+) cells in α-SMA (+) cells	45.2±6.2%
α-SMA (+) cells in nestin (+) cells	95.7±1.1%
Nestin (+) cells in HSP47 (+) cells	33.6±9.2%
HSP47 (+) cells in nestin (+) cells	98.1±0.8%

Cells in outer medulla of UUO kidneys at 13 days (n=5) were examined. Data are presented as the mean±s.d. (%).

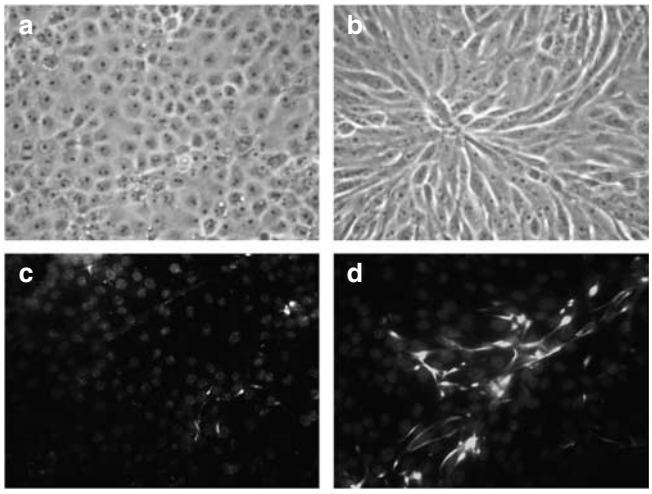

Figure 5 | Hypoxia induced morphological changes and increased nestin expression in cultured tubular cells. Phase contrast microscopic images of cultured LLC-PK1 cells (**a**) in 21% O_2 and cells cultured (**b**) in 1% O_2 for 7 days. Cells cultured in 1% O_2 became spindle-shaped compared to cobblestone appearance in 21% O_2. (**c** and **d**) Immunofluorescent staining for nestin. (**c**) Nestin expression was minimal in cells cultured in 21% O_2, (**d**) whereas increased nestin expression was observed in cells cultured in 1% O_2 for 7 days. Original magnification ×100.

body, as described previously.[37] Increased FLAG expression was demonstrated in damaged tubular cells in puromycin aminonucleoside-induced nephrotic syndrome and remnant kidney, indicating increased tubular hypoxia in these animal models.[37] HRE transgenic rats also showed interstitial fibrosis similar to wild-type Wistar rats by UUO. Interstitial fibrosis score was 2.84±0.36 (n=5) at day 13 after UUO. In the contralateral control kidney, FLAG staining was not detected in the cortex to outer medulla (Figure 7a). In contrast, in the UUO kidney, increased FLAG staining was broadly detected at tubular epithelial cells of deep cortex to outer medulla, demonstrating the existence of hypoxia (Figure 7b). In serial sections, most nestin-expressing cells were detected within the hypoxic area (Figure 7b). Double-immunofluorescent staining also reveled that some parts of FLAG-positive tubular cells were also stained with nestin (Figure 7d). In addition, we also determined hypoxic area of UUO kidney by injecting pimonidazole 1 h before killing. Serial immunohistochemical staining and double-immunofluorescent staining demonstrated that nestin-expressing tubular epithelial cells were predominantly detected within the hypoxic tubules, indicated by pimonidazole staining (Figure 7c and e). It should be noted, however, FLAG and pimonidazole staining were relatively weaker or absent in interstitial cells of UUO kidney as compared with tubular epithelial cells around them (Figure 7b–e). Consequently, nestin-positive interstitial cells were also negative or faintly stained with FLAG or pimonidazole (Figure 7d and e).

TGF-β expression in UUO kidney

We also examined the level of TGF-β in UUO kidney by real-time polymerase chain reaction (PCR) analysis, because TGF-β was shown to induce nestin expression in cultured renal fibroblasts. Increased TGF-β mRNA level was also observed in UUO kidney at day 13 compared to the contralateral control kidneys (510±220 versus 100±50%, UUO versus control, $P<0.01$).

Regulation of nestin by a proteasome-dependent protein degradation mechanism

As an increased level of nestin protein expression was observed in UUO kidney and cultured cells, we examined the nestin mRNA expression by real-time PCR. However, no enhanced level of nestin mRNA was observed in both UUO kidney and NRK-49F cells by TGF-β stimulation (Figure 8a and b, respectively). We hypothesized that the expression of nestin protein would be regulated by protein degradation via the ubiquitin–proteasome pathway rather than the transcriptional pathway. LLC-PK1 and NRK-49F cells were treated with the specific proteasome inhibitors lactacystin or MG132. As shown in Figure 9, proteasome inhibitors increased nestin protein expression in both LLC-PK1 (Figure 9a and b) and NRK-49F cells (Figure 9c and d). Thus, persistent expression of nestin protein appears to be primarily regulated by a protein degradation mechanism, but not by a transcription mechanism.

DISCUSSION

An intermediate filament protein, nestin is generally expressed in developing tissues and is downregulated with maturation.[1,8,9] In normal adult tissue, nestin expression is restricted to progenitor-like cells that are quiescent, but are capable of proliferating, migrating, and differentiating if necessary.[7] However, during injury, re-expression of nestin was observed in several adult tissues.[13–17] Recently, nestin was shown to be transiently expressed in tubular cells and glomerular endothelial cells of developing kidney.[18] In the current paper, we examined the expression of nestin during tubulointerstitial injury using a rat UUO model, and demonstrated that nestin was re-expressed in tubular cells

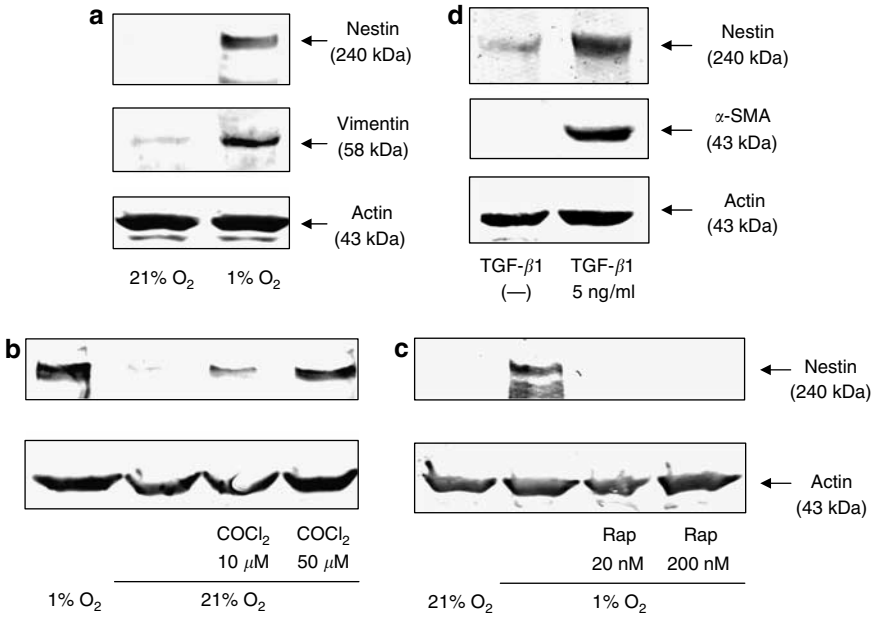

Figure 6 | Expression of nestin by hypoxia and TGF-β1 in cultured cells. Western blotting for nestin in cultured (**a**–**c**) LLC-PK1 cells and (**d**) NRK-49F cells. LLC-PK1 cells were cultured (**a**) in 1% O_2 or 21% O_2 with or without HIF-α modulating (**b**) cobalt chloride ($COCl_2$) or (**c**) rapamycin (Rap) for 7 days. (**a**) Nestin expression was increased in 1% O_2, together with vimentin expression. (**b**) Treatment with 10 or 50 μM $COCl_2$ increased nestin expression in 21% O_2. (**c**) In contrast, increased nestin expression was decreased by adding 20 or 200 nM rapamycin in 1% O_2. (**d**) NRK-49F cells were serum-starved for 24 h and treated with or without 5 ng/ml TGF-β1 for 48 h. Nestin expression was increased by TGF-β1. α-SMA was also increased by TGF-β1. Actin was used as a loading control.

and expressed in interstitial myofibroblasts. The degree of nestin expression was positively correlated with that of tubulointerstitial fibrosis, suggesting that nestin plays an essential role in the progression of tubulointerstitial injury. To our knowledge, this is the first report that nestin is expressed in renal tubular cells and interstitial myofibroblasts in adult injured kidneys.

Re-expression or upregulation of nestin after injury is considered to indicate extensive remodeling or reversion of cells to a more immature phenotype.[7] In UUO, during the progression of tubulointerstitial injury, a transition of tubular cells from epithelial to mesenchymal characteristics was observed and considered to be critically involved in tubulointerstitial injury.[33,38] We identified that nestin expression was entirely restricted to vimentin- or HSP47-positive tubular cells. Vimentin and HSP47 are markers of mesenchymal cells. Thus, nestin-positive tubular cells were considered to have undergone a transition from epithelial to mesenchymal phenotype. Double-immunofluorescent study demonstrated that nestin-positive cells never expressed E-cadherin, the marker for tubular epithelial cells, further supports our notion that these cells have lost their mature phenotype. The appearance of activated α-SMA-positive fibroblasts, also called as myofibroblasts, is another prominent feature in UUO.[39] We demonstrated that some interstitial myofibroblasts were positive for nestin. Importantly, not all vimentin- or HSP47-positive tubular cells or α-SMA-positive fibroblasts were stained for nestin, so nestin could be a marker for tubulointerstitial injury distinct from conventional markers, such as vimentin, HSP47, and α-SMA. One hypothesis is that epithelial-to-mesenchymal transition is a multistep process and therefore cells may sequentially display intermediate phenotypes characterized by different markers. To determine the functional role of nestin-positive cells among cells expressing such conventional markers would be important to understand the precise and comprehensive molecular mechanisms of tubulointerstitial injury and should be clarified in future studies.

Generally, proliferation, migration, and a broad differentiation potential are hallmarks of nestin-expressing cells.[7] However, the role of nestin within the cell is poorly understood. Intermediate filaments are considered to provide flexible intracellular scaffolding whose function is to organize the structure of the cytoplasm and to offer resistance against stresses externally applied to the cell.[3] Moreover, intermediate filaments are known to have pivotal roles in significant cellular functions, which range from the determination of cell shape and motility to cell-cycle control and signal transduction.[5] It is speculated that nestin is essential for maintaining the cell shape and motility of neuronal cells.[40,41] In UUO, nestin tended to be abundantly stained in dilated tubular cells or spindle-shaped myofibroblasts. So, one of the important functions of nestin may be to maintain the cell shape. We performed double-immunofluorescent staining for nestin and proliferating cell nuclear antigen (PCNA) in order to determine whether nestin expression was correlated with

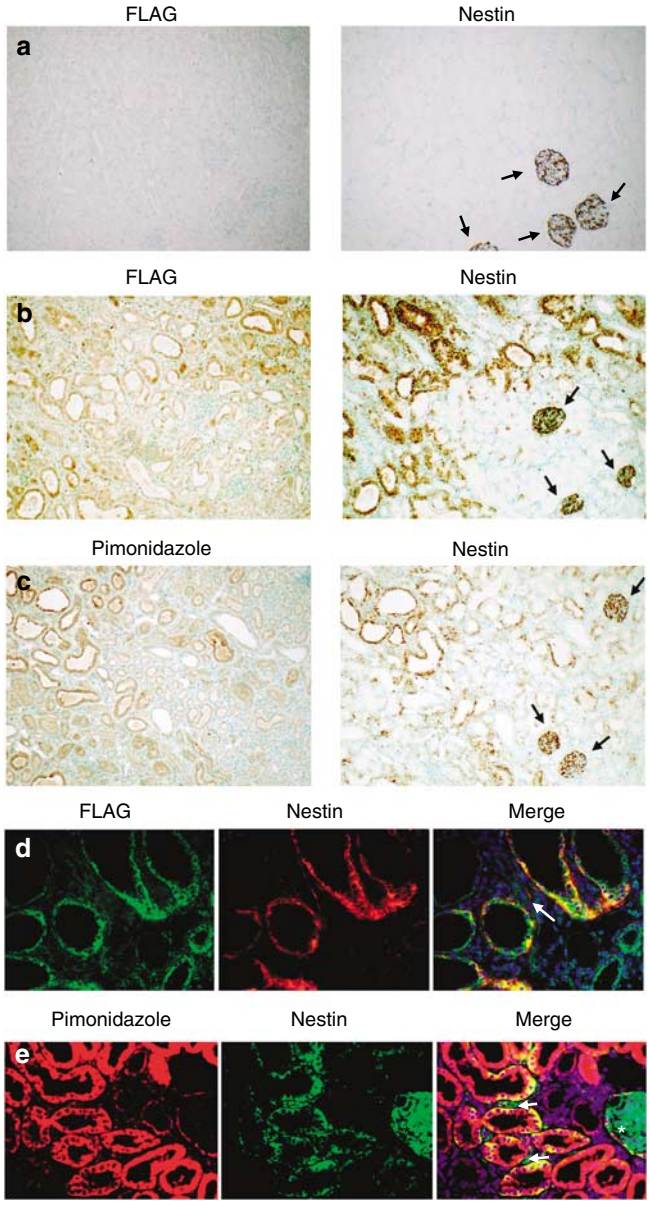

Figure 7 | Nestin expression and hypoxic area in UUO. The relationship between nestin expression and hypoxic area was determined by immunohistochemistry of serial sections using (**a** and **b**) HRE transgenic rats and (**c**) pimonidazole-injected rats. Hypoxic area was detected by (**a** and **b**) anti-FLAG antibody or (**c**) anti-pimonidazole antibody. (**a**–**c**) Nestin expression was detected by anti-nestin antibody. (**a**) In the contralateral kidney, FLAG and nestin staining were not detected in the cortex to outer medulla, except for nestin expression in glomeruli (indicated by arrows). (**b** and **c**) In contrast, FLAG or pimonidazole staining was broadly defected in tubular cells in UUO kidney at day 13. (**b** and **c**) Nestin expression was observed in some tubular cells, mostly within FLAG- or pimonidazole-positive tubules. (**b** and **c**) Nestin expression was also observed in glomeruli in UUO kidney (indicated by arrows). Double-immunofluorescent study was performed on UUO kidney of (**g**) HRE transgenic rats and (**h**) pimonidazole-injected rats. Parts of FLAG- or pimonidazole-positive tubular cells were also co-stained with nestin. However, nestin-positive interstitial fibroblasts were not stained with (**d**) FLAG (arrows) or (**e**) pimonidazole (arrows). Original magnifications: (**a**–**c**) $\times 100$; (**e** and **f**) $\times 400$. Sections were counterstained with (**a**–**c**) methyl green and (**d** and **e**) DAPI.

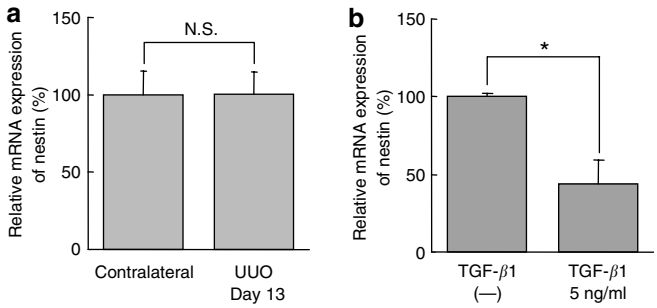

Figure 8 | Nestin mRNA expression in UUO kidney and cultured cells. The amount of nestin mRNA was measured by real-time quantitative reverse transcriptase-PCR in (**a**) UUO kidney and (**b**) NRK-49F cells stimulated with TGF-β1. (**a**) Nestin mRNA was not increased in the UUO kidney at day 13 compared to the contralateral kidney. Serum-starved NRK-49F cells were treated with or without 5 ng/ml TGF-β1 for 24 h. (**b**) Nestin mRNA expression was reduced by TGF-β1 compared to non-treated control cells. Data were normalized to β-actin mRNA, and expressed as a ratio of the (**a**) contralateral control kidney or (**b**) non-treated cells. Data are presented as the mean $\pm$ s.d. of (**a**) five independent rats and (**b**) four independent cultures. $^*P < 0.05$.

proliferation. We could not find any correlation between nestin and PCNA expression (data not shown), suggesting nestin-positive cells were not actively proliferating.

Molecular mechanisms underlying nestin upregulation during injury are not well understood. Growth factors, such as nerve growth factor, acidic fibroblasts growth factor, bone morphogenetic protein-4 are reported to regulate nestin expression.[17,42] Upregulation of nestin was also induced by changes in cell–cell contact patterns.[12] For example, loss of intercellular contacts was associated with increased nestin expression.[12] In this paper, we have demonstrated that hypoxia enhanced nestin expression in renal cells. In the UUO kidney, nestin expression was observed in the hypoxic tubules. Renal tubular cells cultured in hypoxic conditions upregulated nestin expression. However, we do not think that hypoxia directly increases nestin expression. Increased nestin expression was observed in LLC-PK1 cells at 7 days after hypoxic culture, but not notable at day 1 or day 3 (data not shown). Mimicking hypoxic conditions by modulation of HIF-α with cobalt chloride also induced nestin expression at day 7 or later. Hypoxia is reported to induce phenotypic changes of cultured tubular cells.[43] Therefore, increased nestin expression would be secondary to the phenotypic changes of tubular cells induced by hypoxia. We have also shown that TGF-β stimulated nestin expression in cultured renal fibroblasts. Increased nestin expression was observed together with α-SMA, suggesting a phenotypic transformation of the fibroblasts. Hypoxia is considered as an important factor to exaggerate tubulointerstitial injury.[23,30–32] TGF-β is also recognized as a potent growth factor that induces epithelial-to-mesenchymal transition and renal fibrosis.[33,34] Thus, nestin expression is regulated by two pivotal factors that aggravate tubulointerstitial injury; hypoxia, and TGF-β. Of note, in nestin-positive interstitial cells, FLAG or

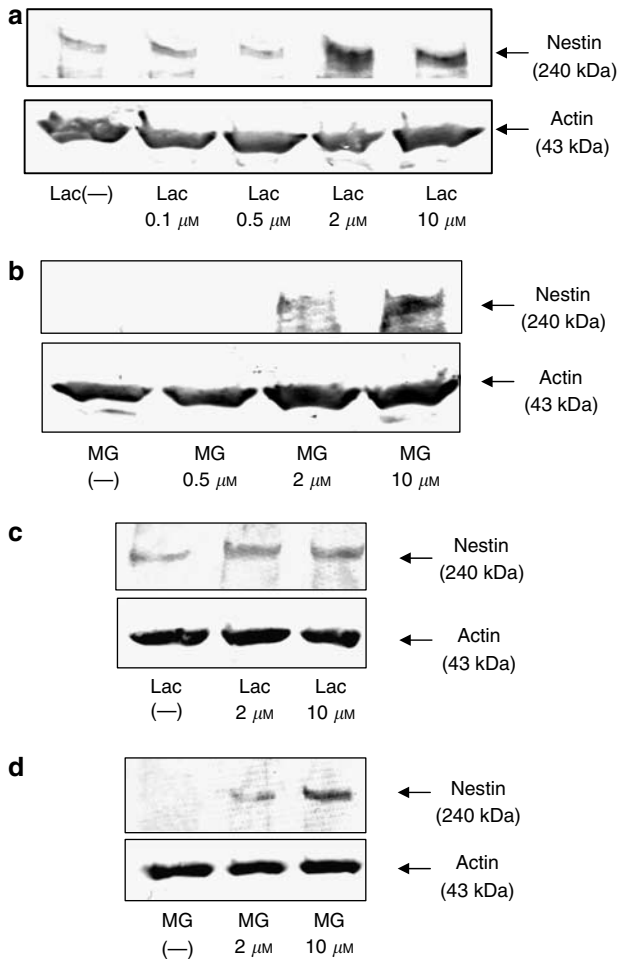

Figure 9 | Effect of proteasome inhibitors on nestin expression. Effect of proteasome inhibitors on nestin protein expression was examined by western blotting in (**a** and **b**) LLC-PK1 cells and (**c** and **d**) NRK-49F cells. LLC-PK1 cells were incubated with or without proteasome inhibitors, (**a**) lactacystin (Lac) or (**b**) MG132 (MG) for 6 h at indicated concentrations. NRK-49F cells were treated with (**c**) lactacystin for 6 h or (**d**) MG132 (MG) for 2 h at indicated concentrations. Proteasome inhibitors increased nestin protein levels in both cell types.

pimonidazole staining was weak or absent in UUO kidney, although tubular cells around those interstitial cells were strongly stained with FLAG or pimonidazole. For now, we do not know the reasons for this discrepancy. There might be a different regulation of HIF-α and different uptake of pimonidazole between tubular cells and interstitial cells. In addition, nestin was not increased by TGF-β in LLC-PK1 cells and not increased by hypoxic conditions in NRK-49F cells (data not shown). The regulation of nestin also might be different in each cell type and should be clarified in future.

Transcriptional regulation of nestin was reported in brain and spinal cord injury.[44] However, we could not find an increased nestin mRNA level in the UUO kidney, despite increased nestin protein level. In the case of other intermediate filaments, keratins, its expression is reported to be regulated by the ubiquitin–proteasome pathway.[45,46] We therefore presumed that the expression of nestin is regulated by the proteolytic pathway. Our data demonstrated that nestin protein in tubular cells is increased by the inhibition of protein degradation with proteasome inhibitors. Our finding is supported by a recent study that nestin expression in cultured neural stem cells was mediated through proteasome degradation.[47] In cultured fibroblasts stimulated by TGF-β, nestin mRNA was rather decreased, in spite of increased nestin protein. One possible explanation is that negative feedback system would reduce nestin mRNA expression against increased level of nestin protein. During the past two decades, the ubiquitin–proteasome pathway has taken center stage in understanding the control of protein turnover.[48] The ubiquitin–proteasome pathway plays essential roles in the regulation of cellular functions, ranging from the control of the cell cycle to oncogenesis.[48] Likewise, we emphasize the importance of the ubiquitin proteasome-dependent protein degradation mechanism for the nestin expression in injured kidney.

In summary, we demonstrated that intermediate filament protein nestin was re-expressed in tubular cells in adult rat kidney after UUO. Some interstitial myofibroblasts also expressed nestin. Nestin expression in tubulointerstitium is correlated with interstitial fibrosis. We also demonstrated that increased nestin expression is associated with phenotypic changes of renal cells induced by hypoxia and TGF-β, possibly through downregulation of its protein degradation. These results will provide new insights into the pathogenesis and remodeling process of tubules in renal fibrosis. In addition, nestin could be a novel marker of tubulointerstitial injury.

MATERIALS AND METHODS

Antibodies and materials

Polyclonal rabbit anti-nestin antibody was obtained from Immuno-Biological Laboratories (Gunma, Japan). Monoclonal mouse anti-nestin antibody and polyclonal anti-AQP 1 antibody were purchased from Chemicon (Temecula, CA, USA). Monoclonal mouse anti-vimentin antibody and monoclonal mouse anti-PCNA antibody were obtained from Neo Markers (Fremont, CA, USA). Monoclonal mouse anti-α-SMA antibody and polyclonal rabbit anti-FLAG antibody were obtained from Sigma (St Louis, MO, USA). Monoclonal mouse anti-rat aminopeptidase (JG12) antibody was obtained from Bender MedSystem (San Bruno, CA, USA). Polyclonal goat anti-actin antibody and polyclonal goat anti-AQP 2 were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Monoclonal mouse anti-HSP47 was purchased from Stress-gen Bioreagents (Victoria, BC, Canada). Polyclonal rabbit anti-Tamm Horsfall glycoprotein antibody was obtained from Biomedical Technologies (Stoughton, MA, USA). Monoclonal mouse anti-E-cadherin antibody was purchased from BD Bioscience Pharmingen (San Diego, CA, USA). Rhodamine-conjugated peanut lectin was purchased from EY Laboratories (San Mateo, CA, USA). Rhodamine-conjugated phalloidin was obtained from Molecular Probes (Eugene, OR, Canada).

Animals

Male Wistar rats were obtained from Nihon SLC (Hamamatsu, Japan). HRE luciferase transgenic rats were generated as described

previously.[37] The transgenic rats have a transgene consisting of three repeats of FLAG-tagged luciferase reporter gene under the combination of seven repeats of HREs and the human minimal cytomegalovirus promoter gene. Under hypoxic conditions, FLAG-tagged luciferase was expressed. Animals were housed in individual cages in a temperature- and light-controlled environment and allowed free access to chow and water. The experimental protocol was approved by the Ethics Review Committee for Animal Experimentation of Gunma University.

UUO and detection of hypoxic area

UUO was performed in 200–220 g male rats as described previously.[49] After induction of general anesthesia by intraperitoneal injection of pentobarbital (50 mg/kg), the abdominal cavity was exposed via a midline incision and the left ureter was ligated at two points with 4-0 silk. Ureteral obstruction was confirmed by observation of dilation of the pelvis and proximal ureter, and collapse of the distal ureter. Contralateral kidneys were used as controls. At the indicated times after UUO, rats were killed and the kidneys were removed to be analyzed for histologic, western blotting, and real-time reverse transcriptase-PCR examination.

In order to detect hypoxic areas, UUO was performed on HRE luciferase transgenic rats. The hypoxic area was determined by the expression of FLAG, which was detected by anti-FLAG antibody. In another experiment, pimonidazole (Chemicon) was used to detect renal hypoxia. Rats were injected with pimonidazole (60 mg/kg) via the tail vein, 1 h before killing. Pimonidazole binding was detected by immunohistochemistry with its specific antibody (Chemicon).

Immunohistochemistry and immunofluorescence

Kidneys were immersion-fixed in buffered formalin solution and embedded in paraffin. Sections (4 μm) were dewaxed and brought to water through graded alcohols. Immunohistochemical analysis was performed via an avidin–biotin coupling immunoperoxidase technique using Vectastain Elite ABC kit (Vector Laboratories, Burlingame, CA, USA) as follows. Antigens were retrieved by autoclaving samples for 20 min in 10 mM citrate buffer (pH 6.0). Subsequently, they were probed with primary antibody overnight at 4°C, with biotinylated anti-rabbit IgG (Vector Laboratories) or biotinylated anti-mouse IgG (Vector Laboratories) as the secondary antibody for 30 min at room temperature and with horseradish peroxidase–avidin (Vector Laboratories) for 30 min at room temperature. Color was developed with diaminobenzidine tetrahydrochloride solution (Nichirei, Tokyo, Japan) to yield brown staining. Sections were counterstained with methyl green or periodic acid-Schiff.

In a separate experiment, indirect immunofluorescent staining was performed. Kidneys were fixed in methyl-Carnoy's solution and embedded in paraffin. Deparaffinized and rehydrated sections were incubated with the primary antibody overnight at 4°C. Staining was detected by Alexa Fluora 488- (green: Molecular Probes) or Alexa Fluora 568- (red: Molecular Probes) conjugated anti-IgG as secondary antibody. Nuclei were stained with 4′-diamidino-2-phenylindole (DAPI).

In addition, indirect immunofluorescent staining of cultured cells was carried out. Cells were grown on coverslips in six-well dishes, fixed for 20 min at −20°C in ethanol–acetone (1:1). Cells on coverslip were incubated with primary antibody for 1 h at room temperature and detected with Alexa Fluora 488 conjugates (Molecular Probes).

Evaluation of interstitial fibrosis and nestin expression

Masson's trichrome staining was used in light microscopic evaluation for tubulointerstitial fibrosis. Interstitial fibrotic change was assessed semiquantitatively as follows: 0, no fibrosis; 1, faint; 2, mild; 3, moderate; 4, severe fibrosis. The expressions of nestin, vimentin, HSP47, and α-SMA were determined by immunohistochemical staining. The number of positive cells was counted under × 400 magnification. In each sample, 40 randomly selected fields were examined and the average number of positive cells in each field was calculated.

Western blotting

Protein was extracted from homogenized whole kidney or kidney without glomeruli, or from cultured cells using Tris-Glycine buffer (1% Triton, 10% glycerol, 20 mmol/l 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, 100 mmol/l NaCl) with protease inhibitors (Boehringer Mannheim, Indianapolis, IN, USA). Glomeruli were eliminated as follows. The cortex and outer medulla of kidneys were minced finely with a razor and treated with 2 mg/ml collagenase for 30 min at 37°C. Successively, the kidneys were passed through a 70 μm filter to remove glomeruli. The resulting suspension was centrifuged and washed twice in phosphate-buffered saline. The final pellet was suspended in Tris-Glycine buffer, centrifuged, and supernatant was collected. Cultured tubular cells were harvested by trypsin digestion, rinsed with cold phosphate-buffered saline, centrifuged, and the supernatant removed. The pellet was washed in phosphate-buffered saline, centrifuged, and extracted in Tris-Glycine buffer. After centrifugation, the supernatant was collected for examination.

Protein concentration was determined by the bicinchoninic acid protein assay (Pierce, Rockford, IL, USA) according to the manufacturer's directions. A 30–60 μg portion of protein extracts were separated on 7.5% sodium dodecyl sulfate-polyacrylamide gels and transferred to polyvinyl difluoride membrane (Immobilon-P; Millipore, Bedford, MA, USA). After blocking with Block Ace (Yukijirushi, Osaka, Japan) to reduce nonspecific antibody binding, the membrane was incubated with primary antibodies overnight at 4°C, washed with Tris-buffered saline (20 mM Tris-HCl, 150 mM NaCl, and 0.1% Tween 20), and incubated with an alkaline phosphatase-conjugated anti-rabbit IgG, anti-goat IgG, and anti-mouse IgG antibody (Promega, Madison, WI, USA) at room temperature for 1 h. After further washing, detection of the bound antibody was performed using chromogen 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium (Sigma).

Real-time PCR

The cortex and outer medulla of rat kidney were frozen with liquid nitrogen, pounded in a mortar, and suspended in RNAiso (Takara Bio, Ohtsu, Japan). After centrifugation, the supernatant was collected for analysis. In a separate experiment, cultured cells were scraped in RNAiso added directly to a culture dish, and the lysate was prepared for analysis. RNA was isolated according to the manufacturer's instructions and cDNA synthesis from total RNA was carried out with an ExScript RT reagent kit (Takara Bio). Real-time quantitative PCR with SYBR Green Real-time PCR Master Mix (Toyobo, Osaka, Japan) was performed using ABI PRISM 7700 sequence detection system (Applied Biosystems, Foster City, CA, USA). Corresponding primers were as follows: β-actin-forward, 5′-CTTTCTACAATGAGCTGCGTG-3′; β-actin-reverse, 5′-TCATGAGGTAGTCTGTCAGG-3′; nestin-forward, 5′-CTGTTCACCCATCCCAATC-3′; nestin-reverse, 5′-TCATCTGCCTCGCTTTCTTC-3′;

TGF-β1-forward, 5′-TGAGTGGCTGTCTTTTGACG-3′; TGF-β1-reverse, 5′-ACTGAAGCGAAAGCCCTGTA-3′. Relative amounts of nestin mRNA were calculated by the $\Delta\Delta C_t$ method and normalized to β-actin.

Cell culture

Pig proximal tubular LLC-PK1 cells and rat renal fibroblasts (NRK-49F cells) were obtained from the American Type Culture Collection (Rockville, MD, USA) and cultured in Dulbecco's modified Eagle's medium (Sigma) containing penicillin, streptomycin, amphotericin B, and fetal calf serum (10% for LLC-PK1 cells and 5% for NRK-49F cells). Cells were maintained under humidified 5% CO_2 at 37°C. Hypoxic conditions were provided by exposure of cells to 1% O_2 in a multigas incubator, AMP-30 (ASTEC, Fukuoka, Japan).

Cells were treated with cobalt chloride (Sigma), rapamycin (LC Laboratories, Woburn, MA, USA), lactacystin (Cayman Chemical, Ann Arbor, MI, USA), MG132 (Peptide Institution, Osaka, Japan), and TGF-β1 (PeproTech, London, UK).

Statistical analyses

Differences between the means were compared using Student's *t*-test. Relationships between variables were assessed by Pearson's correlation analysis. $P<0.05$ was considered statistically significant.

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