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SCUBE1-enhanced bone morphogenetic protein signaling protects against renal ischemia-reperfusion injury

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ABSTRACT

We previously reported that the membrane-bound SCUBE1 (signal peptide-CUB-epithelial growth factor domain-containing protein 1) forms a complex with bone morphogenetic protein 2 (BMP2) ligand and its receptors, thus acting as a BMP co-receptor to augment BMP signal activity. However, whether SCUBE1 can bind to and facilitate signaling activity of BMP7, a renal protective molecule for ischemia-reperfusion (I/R) insult, and contribute to the proliferation and repair of renal tubular cells after I/R remains largely unknown. In this study, we first showed that I/R-induced SCUBE1 was expressed in proximal tubular cells, which coincided with the expression of renoprotective BMP7. Molecular and biochemical analyses revealed that SCUBE1 directly binds to BMP7 and its receptors, functioning as a BMP co-receptor to promote BMP7 signaling. Furthermore, we used a new *Scube1* deletion (Δ2) mouse strain to further elucidate the renal pathophysiological function of SCUBE1 after I/R injury. As compared with wild-type littermates, Δ2 mice showed severe renal histopathologic features (extensive loss of brush border, tubular necrosis, and tubular dilation) and increased inflammation (neutrophil infiltrate and induction of monocyte chemoattractant protein-1, tumor necrosis factor-α and interleukin-6) during the resolution of I/R damage. They also showed reduced BMP signaling (phosphorylated Smad1/5/8) along with decreased proliferation and increased apoptosis of renal tubular cells. Importantly, lentivirus-mediated overexpression of SCUBE1 enhanced BMP signaling and conferred a concomitant survival outcome for Δ2 proximal tubular epithelial cells after hypoxia–reoxygenation treatment. The protective BMP7 signaling may be facilitated by stress-inducible SCUBE1 after renal I/R, which suggests potential targeted approaches for acute kidney injury.

1. Introduction

Acute renal failure represents a common clinical problem associated with high morbidity and mortality [1]. Renal ischemia-reperfusion (I/R) injury is the major cause of acute renal failure after major surgery or renal transplantation [1]. Yet, the kidney has an intrinsic ability to repair and regenerate after acute kidney injury. The regenerative capacity of the kidney is multifactorial and has been studied with experimental models of I/R injury in which tubules undergo endogenous cell replacement and re-epithelization [2,3].

Bone morphogenetic protein (BMP) signaling is initiated when BMP protein binds to its receptor complex of 2 type I (BMPR-I) and 2 type II (BMPR-II) receptors that contain intracellular serine-threonine kinase domains [4]. The activated receptor complex then phosphorylates the intracellular Smad proteins, which translocate to the nucleus and activate ligand-responsive genes [5]. BMP7, which plays critical roles in nephron formation during kidney development [6,7], is induced and is involved in the proliferation and repair of tubular cells after ischemia

Abbreviations: BMP, bone morphogenetic protein; BRE, BMP responsive element; H/R, hypoxia-reoxygenation; I/R, ischemia-reperfusion; KIM-1, kidney injury molecule-1; MEFs, mouse embryonic fibroblasts; NGAL, neutrophil gelatinase-associated lipocalin; PTECs, proximal tubular epithelial cells; SCUBE1, signal peptide-CUB-EGF domain-containing protein 1; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling; WT, wild type

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via its cytoprotective and anti-apoptotic effects [8,9]. In addition, a secreted kielin/chordin-like protein enhances BMP-mediated signaling by facilitating the binding of BMP7 to its type I receptor to attenuate acute kidney injury [10,11].

SCUBE1 [signal peptide-CUB (complement C1r/C1s, Uegf, Bmp1)-EGF (epithelial growth factor) domain-containing protein 1], originally identified in endothelial cells, is the founding member of a small secreted and peripheral-membrane protein SCUBE family composed of 3 different members conserved in vertebrates [12,13]. SCUBE1 contains ~1000 amino acids and shares an organized domain structure with 5 recognizable motifs: 1) an NH_2 terminal signal peptide sequence, 2) 9 copies of EGF-like repeats, 3) a spacer region, 4) 3 cysteine-rich stretches, and 5) one CUB domain at the COOH terminus [13–17]. Our studies suggest that SCUBE1 is a multi-functional protein depending on its subcellular distribution and localization. For example, soluble SCUBE1 released from stimulated platelets is a potential biomarker of platelet activation in patients with acute coronary syndrome and acute ischemia stroke. It acts through its oligomeric EGF-like domains as a key adhesive protein by forming trans-homophilic bridges on activated platelets during thrombus formation, thus contributing to the progression of acute thromboembolic diseases [18–21]. However, membrane SCUBE1 forms a complex with BMP2/4 ligands and their receptors, possibly acting as a BMP co-receptor to augment BMP signal activity, shown to be essential for zebrafish primitive hematopoiesis [22].

Endothelial SCUBE1 expression in glomeruli and peritubular capillaries is highly upregulated at 3 weeks after renal I/R injury [23]. Soluble SCUBE1 secreted by endothelial cells may promote epithelial cell proliferation in a paracrine manner [23]. However, whether membrane SCUBE1 expressed in tubular cells and its associated BMP co-receptor function are involved in endogenous regeneration and repair after renal I/R injury remains largely unknown.

In the present study, we used in vivo loss-of-function and ex vivo gain-of-function approaches, together with molecular and biochemical analyses, to unravel the protective role of inducible SCUBE1 after renal I/R injury. Our data suggest that I/R-induced SCUBE1 protein expression in proximal tubular epithelial cells might be a BMP co-receptor to enhance proliferating and anti-apoptotic BMP7 signaling and accelerate the repair and regeneration after renal I/R insult.

2. Materials and methods

2.1. Generation of Scube1-Δ2 mice

Scube1 gene from the mouse strain 129/SvJ was used to construct a targeting vector as described in Supplemental Fig. S1. Standard procedures were used to produce the *Scube1-Δ2* mice. Primers for genotyping are in Supplementary Table S1. The *Scube1-Δ2* allele was backcrossed into C57BL/6 mice for at least 6 generations before renal I/R experiments.

2.2. Animal model for renal I/R

Our investigation conformed to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publications No. 85-23, revised 1996). All animal experiments were approved by the Institutional Animal Care and Utilization Committee, Academia Sinica (Protocol 12-12-449). Male mice, 8 to 10 weeks old, underwent unilateral renal artery occlusion (45 min) and reperfusion for the indicated times [23]. Sham control animals underwent identical surgery except for pedicle clamping.

2.3. RNA extraction, RT-PCR and real-time quantitative PCR (Q-PCR)

Total RNA was obtained from the kidney or cells by use of TRIzol reagent (Thermo Fisher, Waltham, MA) according to the manufacturer's instructions. RNA was dissolved in DEPC-treated water and total RNA was reverse transcribed into complementary DNA (cDNA) by using the SuperScript™ First-Strand Synthesis System (Thermo Fisher, Waltham, MA). Real-time quantitative PCR analysis was performed twice in duplicate by use of the ABI PRISM 7700 Sequence Detection System (Applied Biosystems). For RT-PCR, PCR products were amplified on 1% agarose gel. RT-PCR and Q-PCR primers are in Supplementary Table S1.

2.4. Western blot analysis

Kidney and cell extracts were separated on SDS-PAGE, transferred to PVDF membranes and underwent western blot analysis with an ECL kit (Pierce). Antibodies were for SCUBE1 (homemade); phospho-Smad1/5/8, total Smad1 and active caspase-3 (Cell Signaling, Danvers, MA); FLAG, Myc and NGAL (Sigma-Aldrich, St. Louis, MO); hemagglutinin (HA; LTK Biolab, Taiwan); and tubulin (Genetex, Irvine, CA). Membranes were visualized by using the BioSpectrum Imaging System (UVP, Upland, CA).

2.5. Histology, immunohistochemical staining and TUNEL assay

Paraffin-embedded mouse kidney sections were prepared by a standard procedure. Sections were stained with hematoxylin and eosin for histology. Immunohistochemical staining was performed as described [24]. Apoptosis in renal sections was examined by TUNEL assay with an in situ Cell Death Detection kit (Roche Applied Science, Indianapolis, IN).

2.6. Cell culture, transfection and luciferase activity

Human embryonic kidney-293T (HEK-293T) cells and HepG2 cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated fetal bovine serum, 2 mM L-glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin at 37 °C in an atmosphere of 5% CO_2. Cells were transfected by using Lipofectamine 2000 (Life Technologies, Grand Island, NY). For luciferase activity, HepG2 cells transfected with the BMP-responsive luciferase reporter BRE-luc with Renilla luciferase reporter as an internal control, together with empty vector or the SCUBE1 expression plasmid. Luciferase activity was measured after 24-hr treatment with recombinant human BMP7 protein (R&D Systems, Minneapolis, MN).

2.7. Immunoprecipitation

Two days after transfection, cells were lysed in lysis buffer and clarified by centrifugation at 10,000 × *g* for 10 min at 4 °C. Samples were incubated with 1 μg antibody and 20 μl of 50% (v/v) Protein A-agarose (Thermo Fisher, Waltham, MA) at 4 °C for 2 h on the rotator. After a washing, precipitated complexes were boiled for 5 min in Laemmli sample buffer and detected by western blot analysis.

2.8. Isolation of primary proximal tubular epithelial cells (PTECs)

Primary PTECs were obtained from 10-week-old wild-type (WT) and Δ2 mice as described [25]. In brief, kidneys were dissected into 2 halves, and the medulla was removed and cut into small pieces. After collagenase digestion and washing, cells were plated on tissue dishes and cultured in DMEM/F-12 supplemented with insulin/transferrin/selenium, 40 ng/ml hydrocortisone, 0.11 μg/ml recombinant human epidermal growth factor, 100 units/ml penicillin, and 100 μg/ml streptomycin at 37 °C in a 5% CO_2 incubator.

2.9. Lentivirus preparation and gene transfer

Recombinant lentiviruses were produced by using a self-inactivating lentiviral transduction system [26], then purified and concentrated by use of Vivapure LentiSELECT 40 (Sartorius Stedim Biotech, Bohemia,

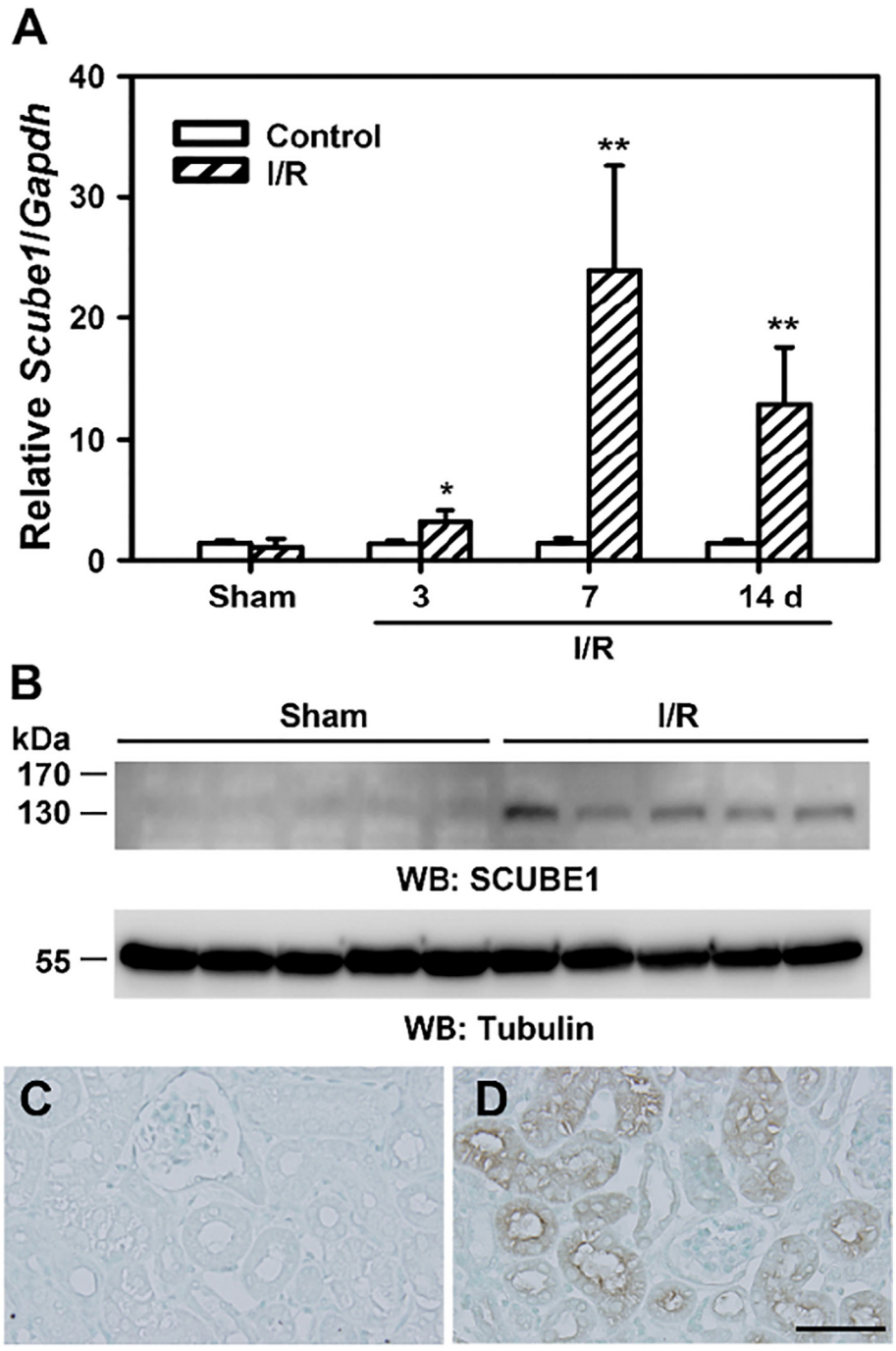

Fig. 1. SCUBE1 mRNA and protein levels were up-regulated after renal ischemia-reperfusion (I/R) injury. (A) Quantitative RT-PCR analysis of *Scube1* mRNA level from renal cDNA derived from mice at 3, 7, 14 days after renal I/R injury as compared with contralateral control kidneys. Expression levels were normalized to that of *Gapdh*. Experiments were performed twice in duplicate with similar results. Data are mean ± SD (n = 5). $^{*}P < 0.05$; $^{**}P < 0.01$. Sham, sham-operated animals. (B) Western blot analysis of SCUBE1 protein expression in kidneys at 7 days after I/R. Protein extracts (20 μg) from control (Sham) and I/R kidneys (n = 5 in each group) were subjected to western blot analysis with the anti-SCUBE1-specific antibody. Anti-tubulin blotting was a loading control. (C and D) Localization of SCUBE1 in I/R kidney. Kidney sections from sham (C) or I/R (D) mice were probed with anti-SCUBE1-specific antibody. Renal SCUBE1 protein expression was present in proximal tubular epithelial cells with I/R injury after 7 days (D) but was barely seen in sham control kidneys (C). Bar = 50 μm.

NY). Lentiviral infection involved adding lentivirus to cells in growth media-containing polybrene.

3. Results

3.1. Induction of SCUBE1 in proximal tubular cells after I/R injury

Because kidneys injured by ischemia can regenerate via a mechanism involving endogenous induction of protective factors, such as BMP7 [8,27], we first investigated whether *Scube1* expression could be induced in the kidney of a mouse model of unilateral I/R injury. As compared with sham or contralateral control kidneys, I/R-injured kidneys showed upregulated *Scube1* mRNA level at 3, 7, and 14 days after I/R injury (Fig. 1A), which was consistent with renal SCUBE1 protein expression (Fig. 1B). We used immunohistochemistry to further confirm the cell-type expression of SCUBE1 and found marked induction of SCUBE1 in proximal tubular cells from the outer medulla (verified by co-staining with *Lotus tetragonolobus* lectin) of mice at 7 days after I/R; SCUBE1 expression was absent or barely detectable in kidneys not subjected to I/R (Fig. 1C and D).

3.2. SCUBE1 can interact with BMP7 or its receptor subunits and enhance BMP7 signaling

Zebrafish Scube1 can act as a co-receptor for BMP2/4 to promote BMP signaling during primitive hematopoiesis [22]. BMP7 [28] and enhancers of BMP7 signaling such as kielin/chordin-like protein [10,11] acting via its anti-inflammatory, anti-apoptotic as well as proliferative effects can lessen the severity of acute tubular injury and accelerate the recovery phase. Both SCUBE1 (Fig. 1) and BMP7 [29,30]

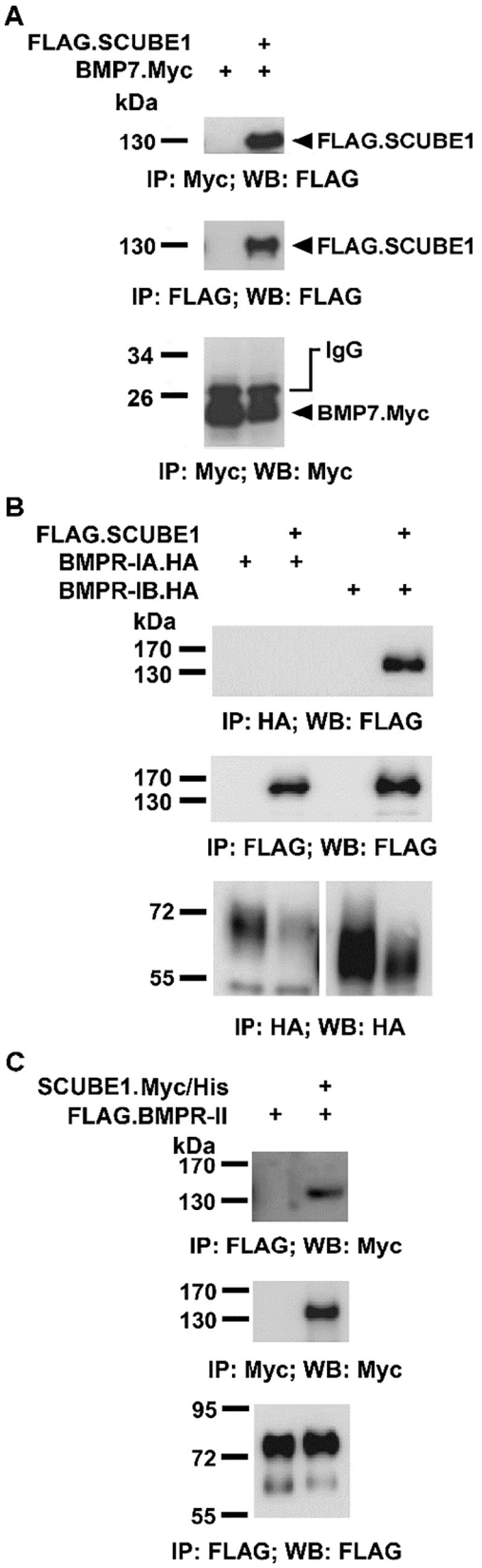

Fig. 2. SCUBE1 binds BMP7 ligand and receptors. The expression plasmid encoding the Myc-tagged BMP7 (A), HA-tagged BMPR-IA or BMPR-IB (B) or Myc-tagged BMPR-II (C) was transfected alone or with FLAG-tagged SCUBE1 in HEK-293T cells. After 2 days, cell lysates underwent immunoprecipitation (IP) then western blot (WB) analysis with indicated antibodies to examine protein–protein interactions.

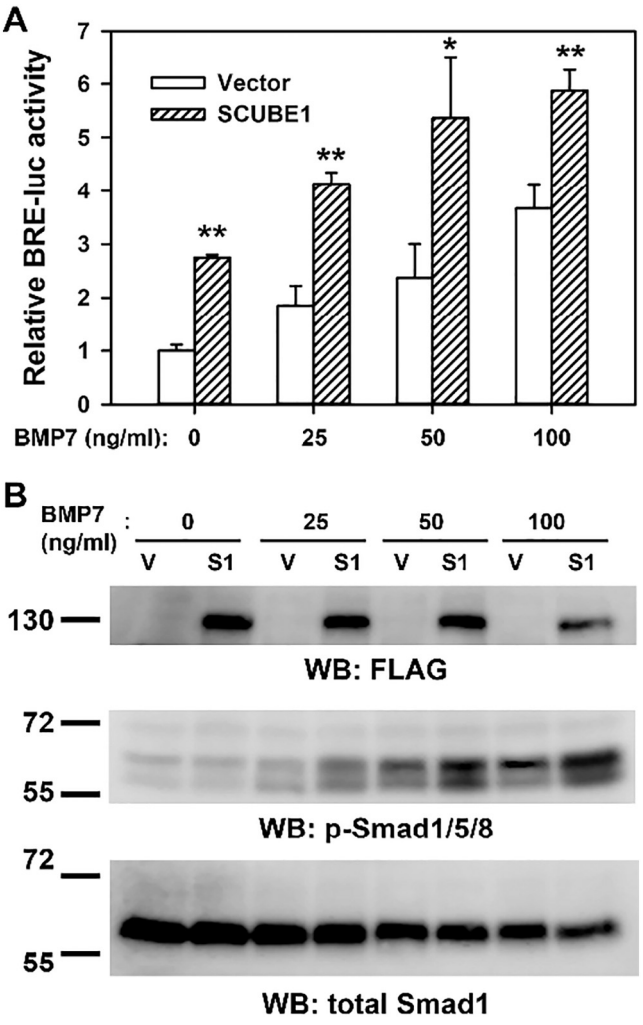

Fig. 3. SCUBE1 augments BMP7 signaling activity. (A) SCUBE1 enhances BMP7 signaling. HepG2 cells were transfected with the BMP-responsive luciferase reporter (BRE-luc) and pRL-TK alone or with the expression plasmid encoding SCUBE1. After 24 h, transfected cells were incubated for another 24 h with and without BMP7 (25, 50, and 100 ng/ml), then luciferase activity was measured. Relative luciferase activity represents firefly luciferase values normalized to Renilla activity. Data are mean ± SD. $^{*}P < 0.05$; $^{**}P < 0.01$. (B) HEK-293T cells were transfected with control vector or plasmid encoding the FLAG-tagged SCUBE1. Cells were maintained in normal growth medium for 24 h after transfection, then medium was replaced with serum-free medium and cultured for 24 h. Cells were treated with BMP7 (0, 25, 50, or 100 ng/ml) for 30 min. Cell lysates underwent western blot analysis with antibody specific for phosphorylated (p) Smad1/5/8 or total Smad1 protein. Experiments were performed twice in duplicate with similar results.

protein expression is induced and co-expressed in proximal tubular cells after I/R. Thus, we examined whether SCUBE1 could bind to the BMP7 ligand or its receptors, and if so, whether SCUBE1 could modulate BMP7-mediated downstream signaling.

HEK-293T cells were transfected with the expression plasmid encoding Myc-tagged BMP7 alone or with FLAG-tagged SCUBE1. At 2 days after transfection, cell lysates were immunoprecipitated with the anti-Myc antibody, and precipitates were analyzed by immunoblotting with anti-FLAG antibody to determine protein–protein interaction. Immunoprecipitation with anti-Myc antibody resulted in co-immunoprecipitation of SCUBE1 protein (Fig. 2A, top panel). Reciprocal immunoprecipitation also confirmed that SCUBE1 could bind BMP7 and form a complex with BMP7 (not shown).

Because BMP7 signals through heterotetrameric receptor complexes containing BMPR-I and -II that phosphorylate Smad1/5/8 transcription

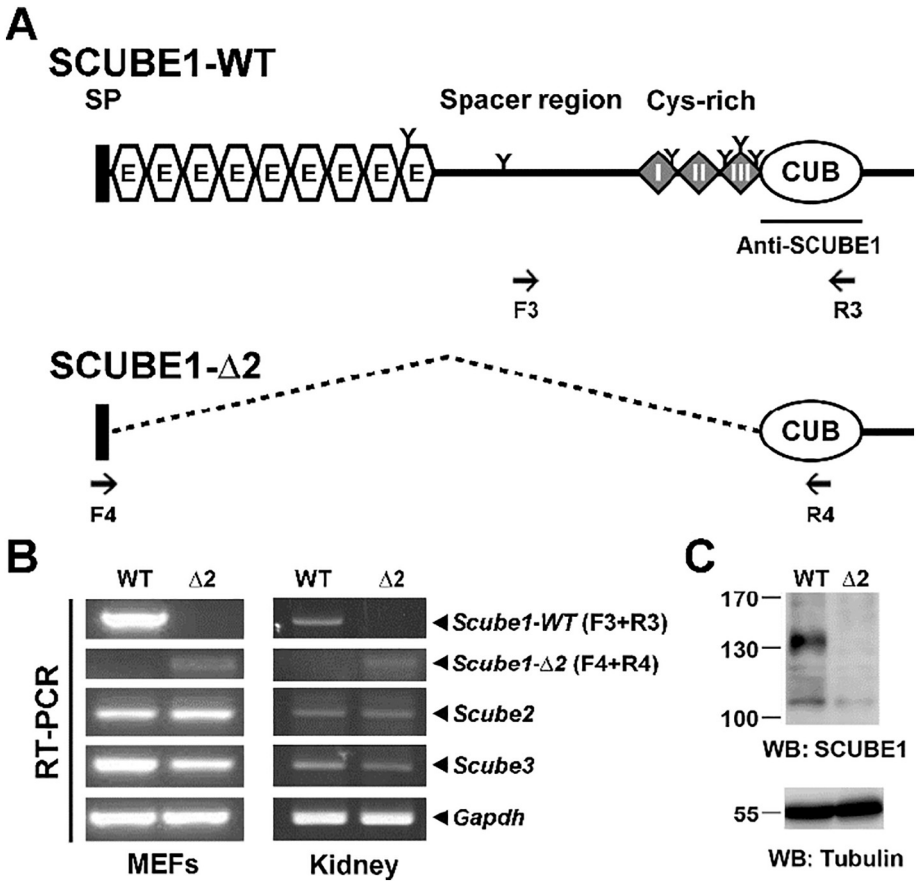

Fig. 4. Targeted deletion of *Scube1* was confirmed at both mRNA and protein levels. (A) Graphic illustration of protein structure encoded by *Scube1* WT or deletion (Δ2) transcript. Locations of oligonucleotide primers used for RT-PCR and protein antigen used for anti-SCUBE1–specific antibody for western blot analysis are marked. (B) Mouse embryonic fibroblasts (MEF) or kidney tissue cDNA derived from WT and Δ2 mice underwent RT-PCR amplification with the WT or Δ2-specific oligonucleotide primers F3 + R3 or F4 + R4, respectively. (C) Western blot analysis of SCUBE1 expression in renal lysates from WT or Δ2 mice at 7 days after renal I/R surgery. Anti-tubulin was a loading control. WB, western blot analysis.

factors [4,5], we next investigated whether SCUBE1 could interact with BMPR-I or -II and whether such SCUBE1-mediated interaction could modulate BMP7 signaling. Immunoprecipitation experiments further verified that SCUBE1 specifically interacted with BMPR-IB (but not IA) and -II receptors (Fig. 2B and C). This interaction appeared to be specific because SCUBE1 could not bind Toll-like receptor 4 (not shown) or even BMPR-IA (Fig. 2B, second lane).

Furthermore, BMP7 treatment dose-dependently increased BMP-responsive luciferase reporter (BRE-Luc) [31] activity with SCUBE1 overexpression (Fig. 3A). Of note, SCUBE1 alone was sufficient to elicit BRE-driven reporter activity even in the absence of exogenous BMP7 treatment (Fig. 3A), possibly because of the SCUBE1-facilitated cross-linking of BMPR-I and -II receptors. In agreement with BRE-Luc reporter activity assays, SCUBE1 significantly facilitated the BMP7-stimulated downstream signaling activity as measured by markedly increased phosphorylation of Smad1/5/8 (p-Smad1/5/8) (Fig. 3B). Together, enhanced BMP7 signaling may result from increased receptor–ligand interactions mediated by the co-receptor function of SCUBE1.

3.3. Genetic ablation of Scube1 exacerbates acute renal injury

Because SCUBE1 is upregulated and co-expressed with the renoprotective molecule BMP7 in proximal tubular cells after renal I/R injury, we hypothesized that SCUBE1 might play an important role in regeneration and repair after I/R damage. We used a genetically altered mouse strain deleting the *Scube1* gene to evaluate its in vivo involvement in regulating tubular cell proliferation and re-epithelialization during resolution of kidney injury. Because the COOH terminal CUB domain is required for brain development [20], we recently generated a mutant mouse strain (Δ2) deleting exons 2 to 18 that code for NH_2-terminal EGF-like repeats, the spacer and cysteine-rich motifs, but keeping the exons that code for the CUB domain at the COOH terminus (Fig. 4A, bottom panel). Homozygous Δ2 mutants were viable and fertile and had no gross phenotypic abnormalities. In addition, the mRNA expression of *Scube2* and *Scube3* remained unchanged between WT and Δ2 mouse embryonic fibroblasts (MEFs) and adult kidney tissue (Fig. 4B); direct sequencing of the RT-PCR product confirmed the complete deletion of coding sequences for the 9 EGF-like domains, spacer, and 3 cysteine-rich repeats (Fig. 4A). In agreement with Δ2 mRNA level, SCUBE1 protein expression was eliminated in Δ2 renal lysates after I/R (Fig. 4C). Therefore, we succeeded in producing Δ2 mice deficient in the expression of SCUBE1 at both mRNA and protein levels. Mice with the Δ2 allele were backcrossed with the WT C57BL/6 strain for at least 6 generations before phenotyping analyses.

To study the role of *Scube1* in I/R injury, we subjected adult (8 to 10 weeks old) WT and Δ2 male mice to unilateral I/R and compared morphological changes in kidneys at 3 and 14 days post-I/R injury. Compared to sham control WT or Δ2 kidneys (Fig. 5A and B), WT I/R kidneys showed extensive damage, such as tubular necrosis, protein casts, tubular dilation, and loss of brush border (Fig. 5C), with more severe morphological changes in Δ2 kidneys, such as further widespread tubular necrosis and dilation, as shown by denuded basement membranes (Fig. 5D). At 14 days after I/R, endogenous repair was evident, with reduced numbers of protein casts or reabsorbing casts and tubules showing progressive re-epithelization in WT kidneys (Fig. 5E). However, Δ2 renal tubular structures remained severely dilated and denuded (Fig. 5F) with prominent neutrophil infiltrates (Supplementary Fig. S2A and B). In agreement with severe immune cell infiltration, the expression of chemokine and pro-inflammatory cytokines including monocyte chemoattractant protein-1 (MCP-1), tumor necrosis factor-α (TNF-α), and interleukin-6 (IL-6) was significantly elevated in Δ2 kidneys after I/R as compared with the WT (Supplemental Fig. S2C). Consistent with these findings, the mRNA and/or protein expression of 2 biomarkers of acute kidney injury, neutrophil gelatinase-associated

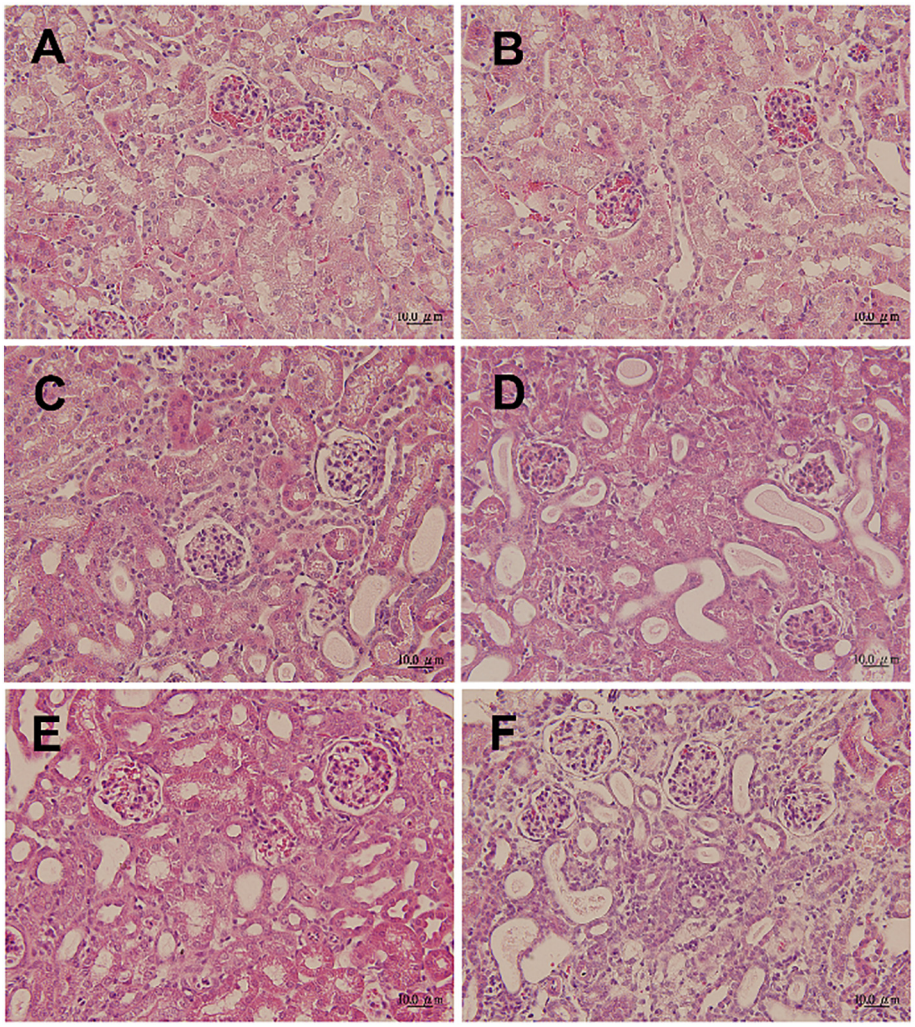

Fig. 5. Δ2 mice showed severe histological features after renal I/R injury. WT (A, C, E) and Δ2 (B, D, F) mice underwent sham operation or I/R injury and were killed at 3 or 14 days. Representative histological sections of sham (A and B) and I/R kidneys at 3 days (C and D) and 14 days (E and F).

lipocalin (NGAL) and kidney injury molecule-1 (KIM-1) [32,33] was significantly increased by I/R injury in Δ2 mice as compared with WT animals (Supplementary Fig. S3). Together, these data suggest that SCUBE1 plays a protective role in regulating tubular cell proliferation and re-epithelization during the resolution of kidney I/R injury.

3.4. Reduced BMP7 signaling in Δ2 MEFs or kidneys

Besides exogenous gain-of-function approaches, we further evaluated the loss-of-function effect of SCUBE1 deficiency on the response to intrinsic BMP7 signaling in MEFs and kidneys from WT or Δ2 mice. MEFs were treated with increasing doses of BMP7 for 20 min, and whole cell lysates were examined for downstream signaling as reflected by the phosphorylation level of Smad1/5/8 (p-Smad1/5/8). BMP7-stimulated p-Samd1/5/8 level was much reduced in Δ2 versus WT MEFs (Fig. 6A and B). As well, endogenous BMP signaling detected by p-Smad1/5/8 level was also markedly lower in Δ2 than WT kidneys after I/R injury (Fig. 6C and D). These data suggest that I/R-induced SCUBE1 might enhance BMP7 signaling via Smad1 in kidneys after I/R.

3.5. Δ2 kidneys showed increased tubular epithelial cell apoptosis and decreased cell proliferation after I/R injury

In addition to its well-known anti-inflammatory function [27,34,35], BMP7 was recently found to promote proliferation of nephron progenitors and maintain a renal progenitor pool in an undifferentiated state via its anti-apoptotic effect [36,37]. We first investigated the role of *Scube1* in tubular epithelial cell apoptosis after renal I/R by terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL). TUNEL-positive cells were virtually undetectable in kidneys of sham-operated WT and Δ2 mice (Fig. 7A and C). After I/R injury, the number of TUNEL-positive cells was increased in WT mice (Fig. 7B and E). In line with previous reports, TUNEL-positive cells were mainly identified in renal proximal and distal tubules [38,39]. Of note, substantially more TUNEL-positive cells were seen in Δ2 kidneys after I/R insult (Fig. 7D and E).

Caspases, a family of cysteine proteases acting as molecular executioners for programmed cell death, are induced and activated under I/R [39,40]. We further examined caspase-3 activation in kidneys of WT and Δ2 mice by western blot analysis with an antibody that specifically reacts with the active, cleaved form of caspase-3. Consistent with TUNEL assay results, Δ2 kidneys under I/R injury exhibited more caspase-3 activation, as reflected by the total amount of cleaved caspase-3 (17/19 kDa) products, as compared with WT kidneys (Fig. 7F). Moreover, immunostaining for Ki-67 (a proliferation marker) indicated a reduced number of proliferating tubular cells in Δ2 than WT kidneys after I/R injury (Fig. 8). Together, these results suggest that the SCUBE1-enhanced BMP7 signaling may participate in suppressing tubular cell apoptosis and promoting cell proliferation after I/R injury.

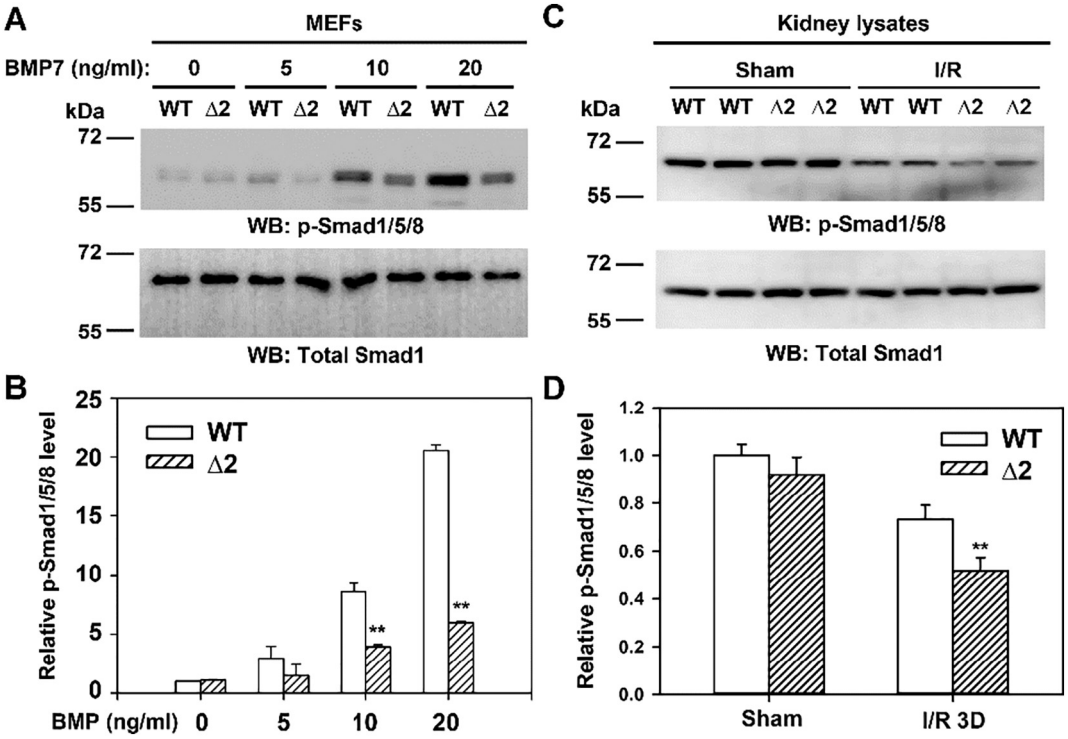

Fig. 6. Reduced BMP7 signaling in Δ2 MEFs and I/R kidneys. (A and B) Deletion of *Scube1* decreases BMP7-induced signal. Before BMP7 stimulation, WT and Δ2 MEFs isolated from E13.5 embryos were first cultured in serum-free medium for 24 h. MEFs were then incubated with different concentrations of BMP7 (0 to 20 ng/ml) for 20 min. Cell lysates were probed with anti-phosphorylated (p) Smad1/5/8 or total Smad1 antibodies, respectively (A). (B) Quantitative analysis of relative p-Smad1/5/8 expression. Data are mean ± SD from 3 independent experiments. $^{**}P < 0.01$, Δ2 versus WT. (C and D) I/R Δ2 kidneys show reduced BMP signaling compared to WT kidneys. Renal lysates from WT and Δ2 were subjected to sham operation or I/R injury. P-Smad1/5/8 and total Smad1 protein levels were examined by western blot analysis (C) and quantified (D). Experiments were performed twice with similar results (n = 5 in each group). $^{**}P < 0.01$, Δ2 versus WT.

3.6. Restoration of the protective role of SCUBE1 in Δ2 proximal tubular epithelial cells (PTECs) by lentivirus-mediated gene transfer

To further verify the protective function of SCUBE1, we used lentivirus-mediated gene transfer of SCUBE1 in freshly isolated PTECs from Δ2 mice. Δ2 PTECs were transduced with recombinant lentivirus carrying FLAG-tagged SCUBE1 (pSIN-SCUBE1) or empty vector (pSIN) as a control followed by normoxia or hypoxia–reoxygenation (H/R) treatment. BMP signaling, measured by p-Smad1/5/8 level, was significantly increased, and the apoptotic response, determined by cleaved caspase-3 activation induced by H/R, was suppressed in Δ2 PTECs overexpressing SCUBE1 as compared with the control (Supplementary Fig. S4). Again, these data validate that SCUBE1 plays a protective role after renal I/R injury, at least by enhancing BMP7 signaling and anti-apoptotic actions.

4. Discussion

In addition to its endothelial origin and platelet expression [12,19,21,23], here, we found SCUBE1 upregulated and localized to PTECs in damaged kidneys after I/R injury in mice. SCUBE1 expression peaked at 7 days after I/R, when tubules have a remarkable capacity to regenerate and replace lost cells. Besides its function in peritubular capillary endothelial cells, which has reparative effects on adjacent renal tubules in a paracrine fashion [23], we describe a new endogenous cell-autonomous function for I/R-induced SCUBE1 protein in proximal tubules. Indeed, membrane-associated SCUBE1 acts as a BMP co-receptor to facilitate protective BMP7 signaling for epithelial repair and regeneration via proliferating, anti-inflammatory, and anti-apoptotic effects.

Tissue repair and regeneration in adult organs is well known to involve the re-expression of a number of developmental genes and developmental pathways that are normally switched off at birth [41]. In the context of kidneys, the re-expression of proteins involved in nephrogenesis and proliferation of resident tubular epithelial cells plays major roles in kidney regeneration after ischemic injury [42,43]. For example, BMP7, which plays pivotal roles in nephron formation during kidney development, is induced and contributes to the proliferation and repair of tubular cells after ischemia [8,9,28,44]. Likewise, mouse *Scube1* is expressed in the urogenital system and during branching morphogenesis, which suggests an important role in the differentiation of the metanephric mesenchyme during kidney development [45,46]. Consistently, our studies reveal a cooperative mechanism between these 2 genes, which recapitulates a development program, but also a role for SCUBE1-promoted BMP7 signaling that may help stimulate the regeneration of epithelial cells after acute kidney injury. Moreover, BMP7 has an anti-fibrotic effect in chronic renal diseases [47,48]. Therefore, additional investigation is required to further clarify whether SCUBE1-augmented BMP7 activity has a beneficial effect in chronic renal diseases.

One interesting finding from this study is that tubular epithelial SCUBE1 appears to be a stress-responsive gene under I/R. Although the precise mechanism underlying its I/R-induced upregulation remains unknown, we found at least 5 hypoxia-inducible factor (HIF)-binding sites (A/GCTGA) within the 1.5-kb proximal promoter region of SCUBE1 that may be involved in HIF-mediated regulation in tubular cells. However, further studies are needed to confirm this hypothesis. In addition, whether such stress-induced expression of epithelial SCUBE1 plays roles in other (patho)physiological conditions besides kidneys remains for further investigation.

From its secretory nature, plasma SCUBE1 level in platelets and/or endothelial cells is significantly elevated and is a potential biomarker of platelet activation in acute coronary syndrome and acute ischemic stroke [21] as well as in hemodialysis or hypertension conditions

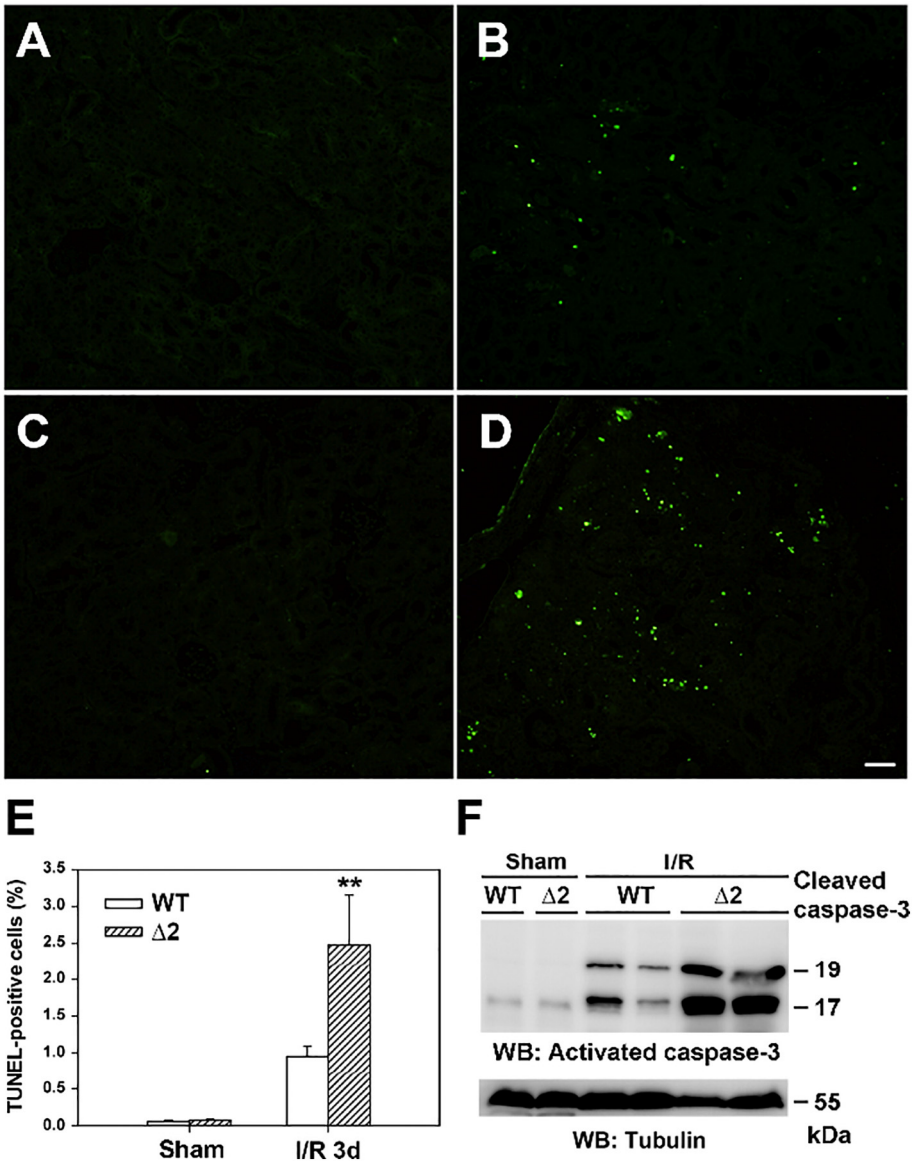

Fig. 7. Δ2 mice showed renal tubular cell apoptosis after I/R injury. WT (A and B) and Δ2 (C and D) mice underwent a sham operation (A and C) or renal I/R for 3 days (B and D). TUNEL staining of representative kidney sections from each experimental group is shown. Bar = 50 μm. (E) Quantitative analysis of TUNEL-positive renal epithelial nuclei per total nuclei in WT and Δ2 mice after sham operation or I/R injury. Data are mean ± SD (n = 5). $^{**}P < 0.01$ (Δ2 versus WT). (F) Active caspase-3 protein expression. Renal lysates derived from WT or Δ2 mice subjected to sham operation or renal I/R were probed with specific antibody against the cleaved, active form of caspase-3. Experiments were performed twice with similar results (n = 5 in each group).

[49,50]. Thus, membrane-associated SCUBE1 may be secreted into urine from injured or regenerating tubular cells, which may have diagnostic or prognostic value for acute kidney injury. However, further clinical studies are needed to verify the biomarker potential of urinary SCUBE1 level in renal diseases.

Because recombinant SCUBE1 is a large glycoprotein with limited secretion ability [12], we have not been able to purify sufficient quantities of soluble, recombinant SCUBE1 protein to test its efficacy as an anti-ischemic agent directly in a mouse I/R model. Given the susceptibility of Δ2 mutants to I/R injury and the novel BMP7-enhancing effects of SCUBE1, its potential as a therapeutic agent for renal diseases warrants further exploration.

Conflict of interest

The authors declare no conflict of interest.

Transparency document

The Transparency document associated with this article can be found, in online version.

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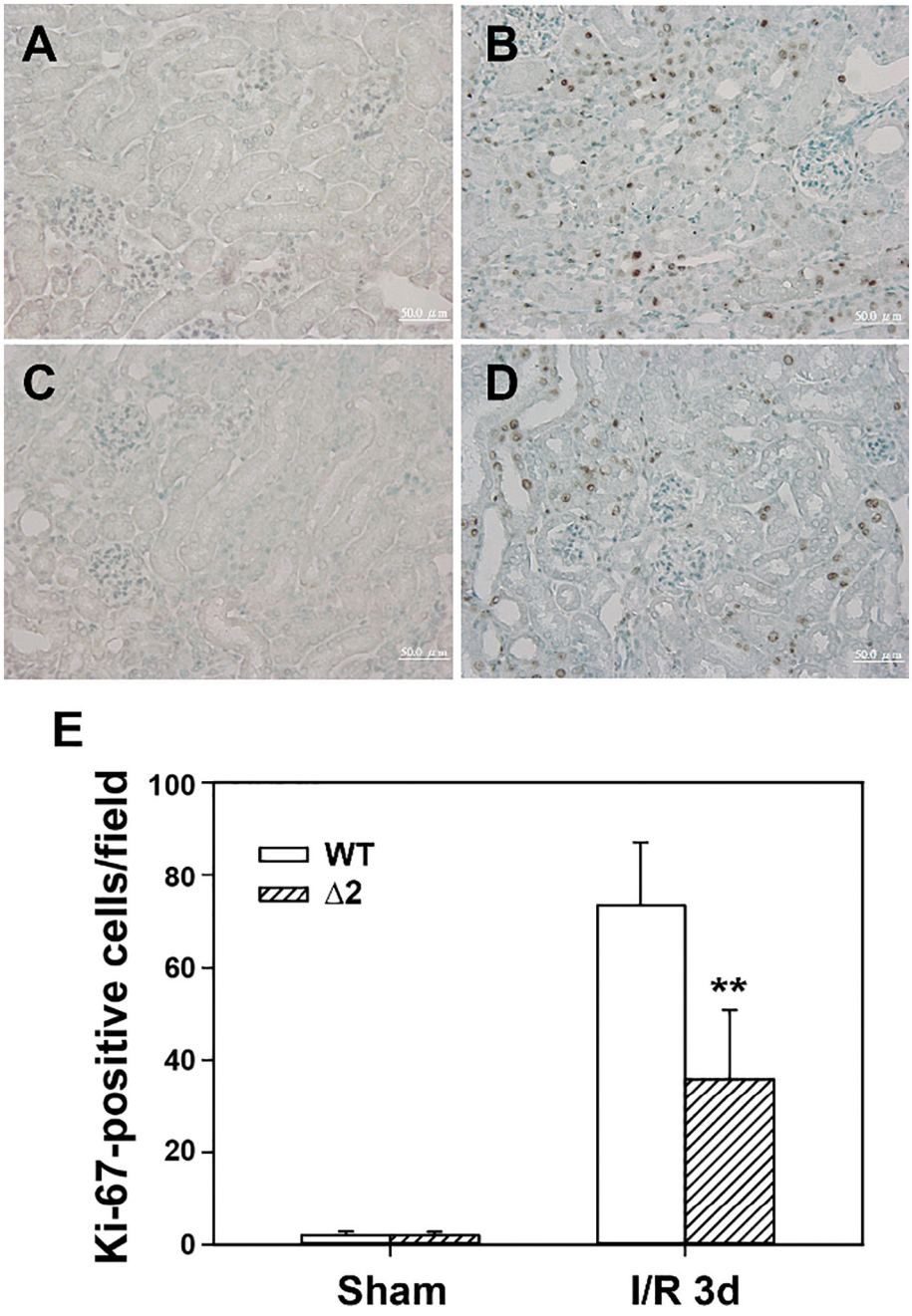

Fig. 8. Δ2 mice showed decreased renal tubular cell proliferation after I/R injury. WT (A and B) and Δ2 (C and D) mice underwent a sham operation (A and C) or 45 min of renal ischemia followed by 3 days of reperfusion (B and D). Staining for Ki-67 (a proliferation marker) in representative kidney sections from each experimental group is shown. Bar = 50 μm. (E) Quantification of Ki-67–positive renal epithelial nuclei per total nuclei in WT and Δ2 mice subjected to sham operation or I/R injury. Data are mean ± SD (n = 5 in each group). $^{**}P < 0.01$ (Δ2 vs WT).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbadis.2018.10.038.

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