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Meprin β activity modulates cellular proliferation via trans-signaling IL-6-mediated AKT/ERK pathway in IR-induced kidney injury

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Abstract

Background Inflammation plays a central role in the progression of kidney injury induced by ischemia/reperfusion (IR). Meprin metalloproteinases have been implicated in the pathophysiology of IR-induced kidney injury. Existing data from in vitro and in vivo studies show that meprins modulate interleukin-6 (IL-6)-mediated inflammation via proteolytic processing of IL-6 and its receptor. IL-6 trans-signaling induces proliferation through either Mitogen-activated protein kinase /extracellular signal-regulated kinase (MAPK/ERK) or Phosphatidylinositol 3-Kinase/ protein kinase B (PI3K/AKT) pathway or in crosstalk with AKT/ERK. We previously showed that meprin β modulates cellular survival B-Cell Lymphoma/Leukemia 2 (BCL-2) through IL-6/Janus kinase/ Signal Transducer and Activator of Transcription (IL-6/JAK/STAT) signaling pathway in IR-induced kidney injury. However, it's not known how meprin β modulation of the IL-6 signaling pathway impacts the cellular proliferation in IR-induced acute kidney injury. The goal of the current study was to determine how meprin β modulation of the IL-6 signaling pathway impacts downstream cellular proliferation in IR-induced kidney injury.

Methods We induced Ischemia/Reperfusion injury with unilateral IR as a model of renal inflammation in wild-type (WT) and meprin β knockout (βKO) mice, with the contralateral kidneys serving as controls. The mice were sacrificed at 96 h post-IR, and kidney tissue processed for evaluation by RT-PCR and immunohistochemistry. Statistical analysis utilized two-way ANOVA.

Results RT-PCR data showed a significant increase in mRNA levels for IL-6 and proliferating cell nuclear antigen (PCNA) in WT and βKO mice at 96 h-post IR when compared to WT control kidneys. However, the baseline mRNA levels for PCNA were significantly higher in βKO when compared to WT kidneys. Immunohistochemical data showed significant increases in IL-6, PCNA, p-AKT and p-ERK in select tubules in both genotypes at 96 h post-IR when compared to control kidneys for each genotype. Data from immunofluorescence counterstaining of kidney tissues revealed that at 96 hours post-IR, IL-6, PCNA, p-AKT, and p-ERK were primarily expressed in meprin β-expressing proximal tubules (PTs), where meprins are abundantly present. However, high levels of IL-6 were also present in the lumen of PTs and DTs from WT and βKO kidneys at 96 h post-IR, suggesting increased release/shedding into filtrate and subsequently into urine.

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Conclusion In conclusion, this study highlights the role of meprin β activity in regulating cellular proliferation through PCNA regulation, driven by the IL-6-mediated AKT/ERK signaling pathway during the recovery phase following IR-induced kidney injury.

Clinical trial number Not applicable.

Keywords Interleukin-6, Proliferation, Ischemia-reperfusion, Meprin metalloprotease β

Introduction

Ischemia/Reperfusion (IR) is one of the leading causes of acute kidney injury (AKI) and is associated with high morbidity and mortality rates [1, 2]. Inflammation contributes to the pathology of AKI [3, 4], particularly, IR-induced kidney injury [5–7]. At the onset of inflammation, monocytes are recruited to the injury site and differentiate into M1 macrophages, key players in the inflammatory response [8]. These macrophages release and respond to IL-6, which signals through two pathways: the classic pathway via membrane-bound IL-6 R (mbIL-6 R), predominantly on immune cells, and the trans-signaling pathway via soluble IL-6 R (sIL-6 R), enabling IL-6 activity in cells lacking mbIL-6 R, such as renal proximal tubule epithelial cells [9, 10]. IL-6 trans-signaling plays a protective role in repair processes, particularly in ischemia-reperfusion-induced AKI models. Meprins metalloproteases, abundant in brush-border membranes of proximal kidney tubules [11], modulate inflammation by inactivating IL-6 and cleaving IL-6 R, generating sIL-6 R and influencing the balance between IL-6 pathways [11–14]. Proliferating cell nuclear antigen (PCNA) is a well-known cellular proliferation marker that is induced through activation of IL-6 trans-signaling [15–17]. When IL-6 binds to its receptor, it forms the IL-6/IL6R complex, which binds to the membrane-bound gp130 dimer, leading to activation phosphorylation of Janus Kinase- Signal Transducer and Activator of Transcription (JAK/STAT), Mitogen-activated protein kinase (MAPK) and phosphatidylinositol 3-kinase/Akt kinase (PI3K/AKT) pathways [18]. Activation of these pathways modulates the expression of different inflammation related downstream pathways, such as cellular apoptosis, survival and proliferation [19]. We previously showed that meprin β modulates cellular apoptosis and survival through IL-6/JAK/STAT signaling pathway in IR-induced kidney injury [20]. However, proliferation was reported to be induced via trans-signaling IL-6 through MAPK/ERK signaling pathway [21, 22], PI3K/AKT pathway [23–25] or their crosstalk (AKT/ERK) [19, 26–28]. It's not known which of these mechanisms is involved in inducing the cellular proliferation when meprin β regulates the trans-signaling IL-6. Therefore, the goal of the current study was to determine how meprin β activity impact IL-6 mediated ERK/AKT pathway and downstream cellular proliferation in IR-induced kidney injury.

Materials and methods

Experimental animals

This study utilized twelve-week-old male Wildtype (WT) and meprin β knockout (βKO) mice, both maintained on a C57BL/6 background. While WT mice express both meprin A and meprin B, βKO mice lack meprin B (β-β) as well as the heterodimeric form of meprin A (α-β). The βKO strain was originally developed in laboratory of Dr. Judith Bond at Pennsylvania State University College of Medicine by targeted disruption of the Mep1b gene, in which exons 3–5 were replaced by a neomycin resistance cassette, resulting in a null allele [29]. The absence of meprin β expression in the experimental mice was validated by RT-PCR and Western blot analysis, confirming the loss of mRNA and protein in kidney tissues using previously described methods [29, 30]. The βKO mice were backcrossed onto the C57BL/6 J background for at least six generations to ensure genetic consistency. The experimental mice were maintained at North Carolina A&T State University's (NC A&T) Laboratory Animal Resource Unit (LARU). Age-matched WT controls mice were sourced from Charles River Laboratories (Wilmington, MA). The animals were housed in standard cages, with a maximum of five mice per standard cage, under a controlled 12-hour light-dark cycle. They had unrestricted access to water and a standard rat chow diet (Purina, St. Louis, MO). All procedures involving animals were conducted in accordance with the ethical research standards set by the NC A&T Institutional Animal Care and Use Committee (IACUC) under protocol # LA-20-012.

Surgical induction of ischemia/reperfusion

To induce ischemia/reperfusion injury (IR), we carried out surgical procedures on twelve-week-old male mice following our established protocol [20] and previously described methods [31, 32]. Mice weighing 25–35 grams were anesthetized via intraperitoneal injection of a Ketamine-Xylazine mixture at a dosage of 4.3 µl/g body weight [33, 34]. The procedure involved clamping the renal pedicle for 27 minutes, followed by a 96-hour reperfusion period, while the contralateral kidney remained unclamped and served as an internal control for each mouse. After removing the clamp and suturing the incisions, a subcutaneous injection of pre-warmed (37 °C) saline at 0.05 ml/g body weight was administered

as previously described [20, 31, 32]. To support post-surgery recovery, the mice were placed in temperature-controlled cages for recovery. Between 8–12 h post-surgery, mice received Buprenorphine (3.5 µl/g body weight) for pain management [35–38]. At 96 h post-IR, the mice were then euthanized by CO2 asphyxiations and kidney tissues harvested for gene and protein expression analysis.

Processing of kidney tissues

Harvested kidneys were de-capsulated, and sections were processed accordingly for RNA extraction and paraffin embedding for immunohistochemical analysis. For immunohistochemistry, 2 mm mid-section tissue samples were initially fixed in Carnoy's fixative (60% ethanol, 30% chloroform, 10% acetic acid) overnight at 4 °C, followed by transfer to 70% ethanol at 4 °C until further processing. Tissue embedding and sectioning onto slides were performed at the Wake Forest University Pathology Laboratory. For RNA extraction, kidney tissues were stabilized in RNALater® solution (Invitrogen, Carlsbad, CA) for 24 hours at 4 °C, after which the solution was removed, and the samples were stored at – 80 °C until used for RT-PCR analysis.

Assessment of kidney injury

To assess kidney damage, we performed quantified immunohistochemical staining for kidney injury molecule-1 (KIM-1), a well-established biomarker of renal injury, as an alternative to biochemical assessment using blood samples, which was not feasible since the contralateral kidney remained uninjured. Optic densitometry was used to quantify KIM-1 expression levels, while immunofluorescence analysis was performed to determine its localization. The results demonstrated a significant increase in KIM-1 expression in select tubules at 96 hours post-IR in both genotypes ($p < 0.001$), confirming IR-induced kidney injury (Fig. 1). Previous studies from out lab have shown that IR coupled with unilateral nephrectomy results significant increases in the standard biomarkers of kidney injury, including NGAL, Cystatin C and serum creatinine [39]. For this reason, we are confident that the surgical procedures we used cause kidney injury and that the quantitation of the KIM-1 intensity in IHC kidney sections is appropriate for this model of kidney injury as it focuses on the transition from acute to chronic kidney injury.

RNA extraction and cDNA synthesis

Total RNA was extracted using the Qiagen RNeasy Mini Kit (Qiagen, Germantown, MD) following the manufacturer's instructions as previously reported [20]. RNA concentration and purity were determined using a NanoDrop 2000 spectrophotometer (Thermo Scientific, Wilmington, DE) at 260/280 and 260/230 absorbance ratios. Reverse transcription was performed in a 20 µl reaction volume using the High-Capacity cDNA Reverse Transcription Kit with RNase Inhibitor (Thermo Fisher, Waltham, MA) under the following conditions: 37 °C for 90 minutes, 85 °C for 3 minutes, followed by rapid cooling at 4 °C. The synthesized cDNA was stored at – 20 °C until further analysis.

Real-time PCR analysis

Gene expression was assessed using quantitative real-time PCR (qPCR) with QuantiFast SYBR® Green PCR Reagents (Qiagen, Germantown, MD) and Bio-Rad Multiplate™ 96-Well PCR Plates, following our previously developed protocol [20]. The qPCR cycling conditions consisted of 50 °C for 2 minutes, 95 °C for 10 minutes, followed by 40 cycles of 95 °C for 15 seconds and 58 °C for 1 minute. To ensure specificity, melting curve analysis was performed. Oligonucleotides for the target genes in this study were designed as mouse-specific primer pairs and obtained from Integrated DNA Technologies (IDTDNA) (Coralville, IA).Gene-specific primers included: IL-6 (Forward: GTT CTC TGG GAA ATC GTG GA, Reverse: TGT ACT CCA GGT AGC TAT GG), PCNA (Forward: GAC GCG GCG GCA TTA AAC, Reverse: GTT CAC GCC CAT GGC CAG), and GAPDH (Forward: AGG TCG GTG TGA ACG GAT TTG, Reverse: GGG GTC GTT GAT GGC AAC A). Gene expression was determined using the $2 - \Delta\Delta Ct$ method, normalizing target gene expression to GAPDH [40] and presented as fold change compared to control WT kidney samples.

Immunohistochemical analysis

We conducted immunohistochemical staining to evaluate the expression of target proteins in paraffin-embedded kidney sections following our methodology outlined previously [20, 32, 39, 41]. Briefly, the sections were deparaffinized and subjected to antigen retrieval in 10 mM sodium citrate buffer (pH 6.0) at boiling temperature. To block endogenous peroxidase activity, the sections were treated with methanol containing 25% hydrogen peroxide, followed by blocking with PBS containing 1% normal goat serum to prevent non-specific binding. Tissue sections were then incubated with primary antibodies overnight at 4 °C or for 1 hour at room temperature, followed by incubation with fluorophore-conjugated secondary antibodies. The primary antibodies used for immunohistochemistry included Meprin β (goat polyclonal, R&D Systems, Cat# AF3300, 1:200), Villin (mouse monoclonal, Santa Cruz, Cat# sc-58,897, 1:200), KIM-1 (rabbit polyclonal, Abcam, Cat# ab47635, 1:100), IL-6 (rabbit monoclonal, Thermo Fisher, Cat# 416–7061-82, 1:400 with meprin β), PCNA (rabbit monoclonal, Cell

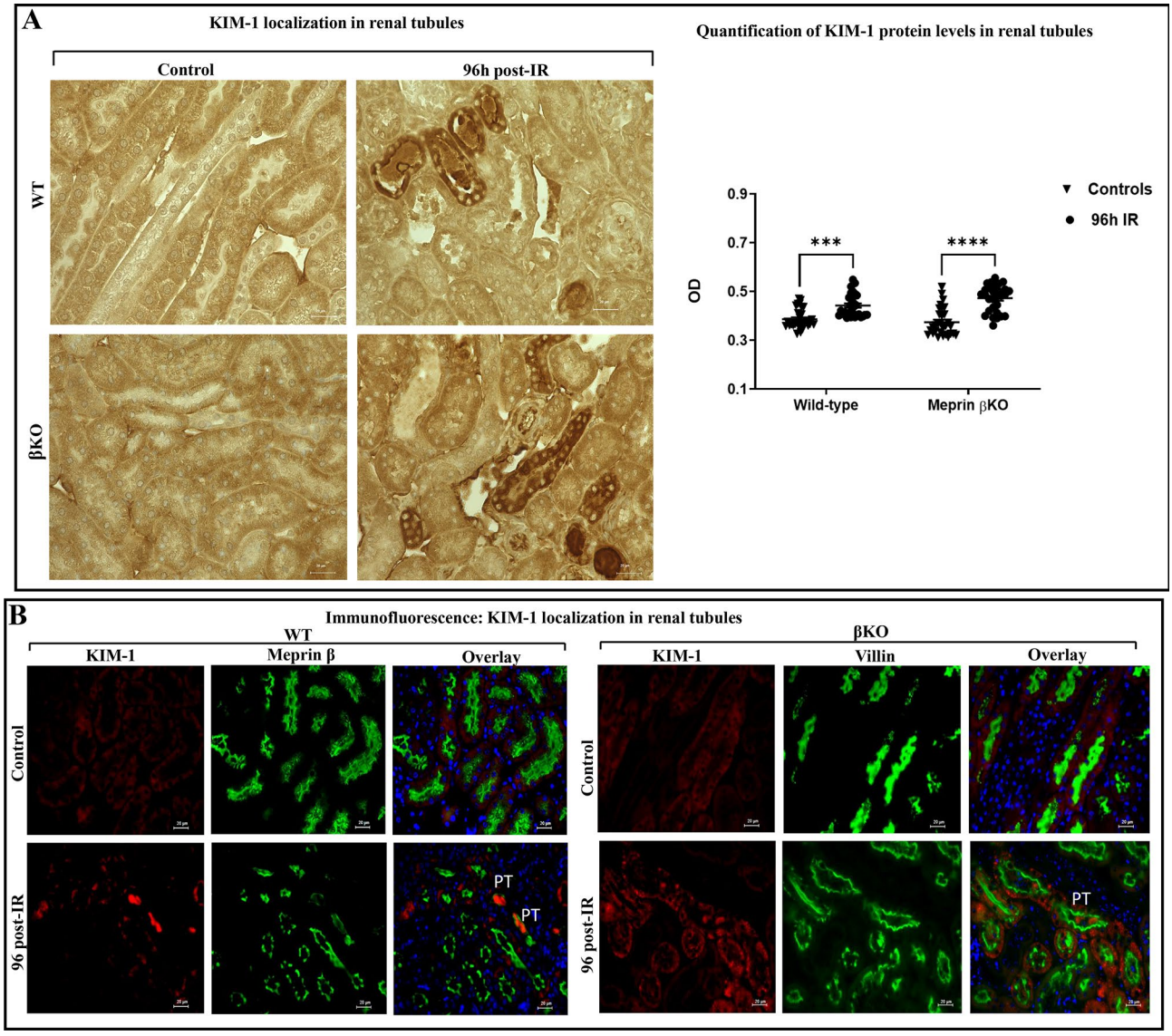

Fig. 1 Kidney injury assessment in kidney tissue from wild type (WT) and meprin β deficient mice (βKO) at 96 h post-IR. Immunohistochemical staining for kidney injury marker 1 (KIM-1) in wild-type (WT) and meprin β knockout (βKO) kidneys at 96 h post-IR. OD data were quantified using Image J analysis Software (ImageJ/Fiji 1.46) and analyzed by two-way ANOVA for 10 non-overlapping fields from renal tubular sections from each kidney (**A**). Images at 60 × magnification and the scale bar representing 20 µm. Immunofluorescence counterstaining of KIM-1 (red) and meprin β (green) in wild-type (WT) and villin (green) in meprin β knockout (βKO) kidneys to determine KIM-1 protein localization as an indicator of kidney injury in kidney tissues of both genotypes (**B**). DAPI (blue) was used to stain the nuclei. Images at 60 × magnification with a scale bar representing 20 µm. There was significant increase in KIM-1 in select tubules at 96 h post-IR in both genotypes confirming kidney injury. Data is expressed as mean ± SEM with P values as indicated, P ≤ 0.05 are considered statistically significant

Signaling, Cat# 13,110, 1:400), p-AKT (rabbit polyclonal, Cell Signaling, Cat# 9271, 1:100), and p-ERK1/2 (rabbit polyclonal, Cell Signaling, Cat# 4370, 1:200). Quantitative analysis of staining intensity was conducted to determine protein expressions of IL-6, PCNA, p-AKT, and p-ERK1/2 using a KEYENCE light microscope (Elmwood, NJ) and BZ-X700 analysis software. Ten non-overlapping fields from tubular and renal corpuscle sections were imaged at 60 × magnification with a 20 µm scale bar. Optical density (OD) values were quantified from calibrated 8-bit images using ImageJ/Fiji 1.46 software.

Immunofluorescence staining

To further confirm protein localization, we conducted immunofluorescence staining according to our established protocol [20, 39]. Following deparaffinization and antigen retrieval, tissue sections were blocked with PBS containing 5% normal goat serum, 5% normal chicken

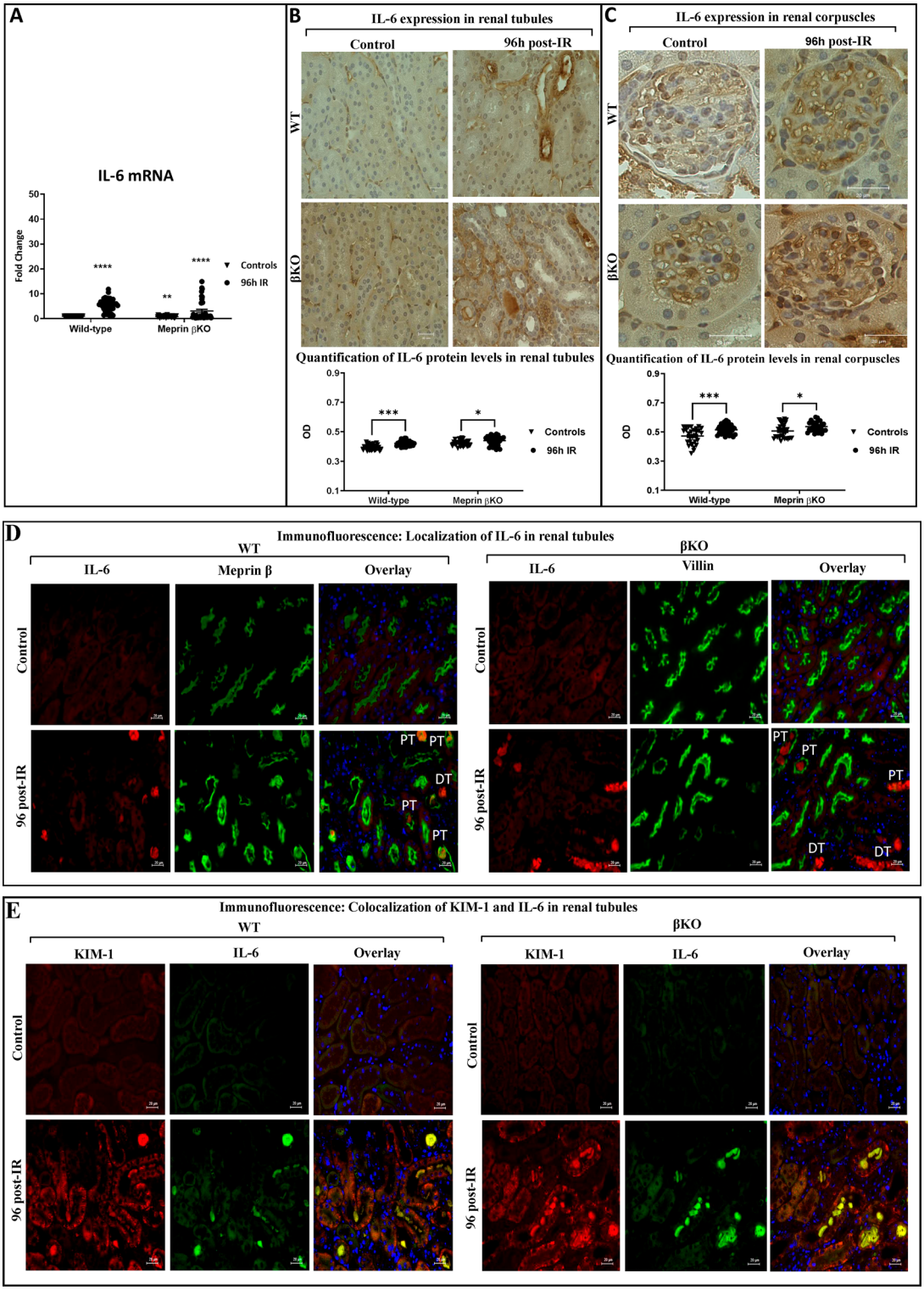

Fig. 2 (See legend on next page.)

serum, or 5% normal donkey serum, depending on the host species of the secondary antibody.

Sections were incubated with the same primary antibodies used for immunohistochemistry, with the addition of IL-6 (mouse monoclonal, Abcam, Cat# ab9324, 1:1000 with KIM-1). This was followed by a 1-hour incubation with fluorophore-conjugated secondary antibodies at room temperature.

(See figure on previous page.)

Fig. 2 IL-6 mRNA and protein expression in kidney tissue from wild type (WT) and meprin β deficient mice (βKO) at 96 h post-IR. The real-time PCR data showed that mRNA expression levels of IL-6 increased in both genotypes subjected to IR (**A**). Values for IL-6 mRNA levels were presented as fold change relative to control WT kidneys and normalized to GAPDH mRNA. Each value represents the mean ± SEM of triplicate combinations from 4 mice per group. Immunohistochemical staining for IL-6 in kidney tubules (**B**) and in renal corpuscles (**C**). OD data were quantified using Image J analysis Software (ImageJ/Fiji 1.46) and analyzed by two-way ANOVA. Ten non-overlapping fields for tubular and 10 non-overlapping fields of renal corpuscle sections from each kidney were imaged at 60 × magnification and the scale bar representing 20 µm. There was a significant increase in IL-6 mRNA and an increase in IL-6 protein expression in select tubules and in renal corpuscle at 96 h post- IR in both genotypes. Immunofluorescence counterstaining of IL-6 (red) in kidney tubules with the proximal tubule markers, meprin β (green) in wild-type (WT) and villin (green) in meprin β knockout (βKO) kidneys in renal tubules (**D**). DAPI was used to stain the nuclei (blue). IL-6 expression was notably observed in the lumen of PTs and DTs in both genotype kidney sections at 96 h post-IR, suggesting IL-6 excretion and clearance into the urine at 96 h post-IR. Immunofluorescence counterstaining of IL-6 (green) and KIM-1 (red) in wild-type (WT) and meprin β knockout (βKO) kidneys (**E**). DAPI was used to stain the nuclei (blue). Images at 60 × magnification with a scale bar representing 20 µm. IL-6 expression was positively associated with KIM-1 within the same tubules, particularly in the lumen, indicating an association between IL-6 and KIM-1 in both genotypes subjected to IR. Data is expressed as mean ± SEM with P values as indicated, $P \leq 0.05$ are considered statistically significant

The secondary antibodies used for immunofluorescence included Alexa Fluor® 647 (Abcam, Cat# ab150075) for rabbit antibodies (KIM-1, IL-6, PCNA, p-AKT, p-ERK1/2), Chicken anti-mouse Alexa Fluor® 488 (Invitrogen, Cat# A-21,200) for IL-6 (when counterstained with KIM-1), Goat anti-mouse IgG1 Alexa Fluor™ 488 (Thermo Fisher, Cat# A-21,121) for villin, Chicken anti-goat Alexa Fluor® 488 (Invitrogen, Cat# A-21,467) for meprin β, DAPI (Vector Laboratories, Cat# SK-4100) for nuclear staining. Tissue sections were qualitatively analyzed to differentiate proximal from distal tubules. Protein expression and localization were assessed using a BZ-X700 fluorescence microscope (KEYENCE, Elmwood, NJ) with BZ-X700 analysis software, captured at 60 × magnification with a 20 µm scale bar. Exposure settings for all microscopic images were kept consistent across all groups to ensure identical acquisition parameters and minimize variability.

Statistical analysis

All data were analyzed by two-way ANOVA with Tukey's pair-wise comparisons using GraphPad 7.0 Prism Software (GraphPad, La Jolla, CA). For mRNA expression data analysis, an unpaired t test was utilized to perform gene expression analysis for each group with WT control serving as the baseline control (n = 4 mice/group). Data for light microscopy utilized the calibrated ODs. Data of equal number of animals across all groups are presented as mean ± SEM. The p values ≤ 0.05 were considered statistically significant.

Results

At 96 hours post-IR, elevated levels of the kidney injury biomarker KIM-1 were observed in select tubules for kidneys of both genotypes subjected to IR, but not in the counterpart control kidneys (Fig. 1), thus confirming the induction of kidney injury. Immunofluorescence counterstaining with proximal tubule markers (meprin β in WT and villin in βKO kidneys) showed that increased KIM-1 expression was primarily in proximal tubules (PTs) and not distal tubules (DTs) (Fig. 1). Interestingly, KIM-1 shedding into the PT lumen was detected in kidneys for both genotype, suggesting its excretion and clearance into the urine after kidney injury, as reported in previous AKI studies [20, 42, 43].

Ischemia/reperfusion associated with increased IL-6 protein levels in kidney tissue at 96 h post-IR

To determine the impact of meprin β expression/activity on IL-6 levels in vivo, mRNA and protein expression of IL-6 were evaluated in kidney tissue at 96 h-post-IR. RT-PCR data showed significant increases in mRNA levels of IL-6 in both WT ($P \leq 0.0001$) and βKO mice ($P \leq 0.001$) kidneys subjected to IR when compared to control kidneys at 96 h-post IR (Fig. 2). Additionally, quantification of IL-6 staining intensity in kidney sections showed that significant increases in IL-6 expression in select renal tubules (Fig. 2) for both WT ($P \leq 0.0001$) and βKO mice ($P \leq 0.05$) subjected to IR when compared to their respective control kidneys at 96 h-post IR. Similarly, IL-6 protein expression increased significantly in the renal corpuscles of kidney sections subjected to IR compared to their counterpart controls WT ($P \leq 0.0001$) and βKO mice ($P \leq 0.05$) (Fig. 2). To identify the localization of increased IL-6 protein expression in kidney tubules, we used immunofluorescence counterstaining with proximal tubule biomarkers, meprin β in WT and villin in βKO kidneys. IL-6 expression was notably observed in PTs, which express meprin with some expression also detected in DTs, which lack meprins (Fig. 2). We also observed increased IL-6 levels in the lumen of PTs and DTs in both genotype kidney sections at 96 h post-IR, suggesting IL-6 excretion and clearance into the urine at 96 h post-IR. We also used the immunofluorescence counterstaining to identify the co-localization of increased KIM-1 and IL-6 expression in kidney tissues, The data showed co-expression of KIM-1 and IL-6 within the same tubules, particularly in the lumen, indicating a positive association between IL-6 and KIM-1 (Fig. 2). This suggests a tight connection between inflammation and tubular injury, with IL-6 signaling potentially driving kidney damage in tubules after 96 h of ischemia-reperfusion (IR) induction across both genotypes. The IL-6 protein staining intensity in renal corpuscles also increased

significantly in both WT ($P \leq 0.0001$) and βKO ($P \leq 0.01$) at 96 h post-IR (Fig. 2).

Ischemia/reperfusion and meprin β expression associated with increased renal p-AKT levels at 96 h post-IR

To determine whether meprin-β expression affects downstream modulators of the IL-6 signaling pathway, levels of phosphorylated Serine/Threonine Protein Kinase B, (PKB/AKT) on Serine 473 (p-AKT^{Ser473}) protein were evaluated using quantitative immunohistochemical staining approaches. Light microscopy and analysis of the immunostaining kidney sections showed that p-AKT protein levels significantly increased in select tubules of both genotypes' kidney ($P \leq 0.0001$) at 96 h following IR when compared to counterpart control kidneys (Fig. 3). The staining intensity for p-AKT in renal corpuscles showed a similar significant increase pattern as in renal tubules of WT ($P \leq 0.001$) and βKO ($P \leq 0.0001$) at 96 h post-IR (Fig. 3). To determine whether meprin expression correlates with p-AKT expression in mice kidney sections, we used immunofluorescent counterstaining with proximal tubule (PT) markers (meprin β for WT and villin for βKO, respectively). Our data showed that at 96 hours post-IR, p-AKT expressions were primarily observed in meprin β-expressing proximal tubules (PTs), compared to distal kidney tubules (DTs), which do not express meprin β (Fig. 3). Additionally, immunofluorescence staining showed p-AKT expression in the lumen of PTs of both genotypes' kidney sections at 96 h post-IR, suggesting release p-AKT into filtration and subsequently into urine.

Meprin β deficiency associated with increase p-ERK1/2 protein levels in kidney tissue at 96 h post-IR

To assess whether meprin β expression influences additional downstream modulators of the IL-6 signaling pathway, we evaluated phosphorylated Mitogen-Activated Protein Kinases (MAPKs) Phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204). The antibodies used to detect the endogenous levels of p44 and p42 MAP Kinase (Erk1 and Erk2) which are either dually phosphorylated at Thr202 and Tyr204 of Erk1 (Thr185 and Tyr187 of Erk2) or singly phosphorylated at Thr202. Immunohistochemical staining coupled with evaluation by light microscopy were used to evaluate the levels of p-ERK. Quantification of the immunostaining intensity showed that the levels of p-ERK proteins were significantly increased in select tubules in WT ($P \leq 0.01$) and βKO ($P \leq 0.0001$) kidneys after 96 h post-IR when compared to their control counterparts (Fig. 4). Similar significant increases of the staining intensity for p-ERK were demonstrated in renal corpuscles in both genotypes subjected to IR (Fig. 4). Immunofluorescence counterstaining method was used to determine the localization of the p-ERK1/2 expression in the kidney tubules (Fig. 4). Like p-AKT, p-ERK1/2 expression was mainly observed in PTs of the kidney tissues of WT and βKO compared to DTs at 96 h post-IR. Also, accumulation of p-ERK1/2 protein was observed in the lumen of the PTs only of both genotype kidney sections subjected to IR, implying that p-ERK1/2 is discharged into the filtration process and subsequently excreted through urine.

Meprin β deficiency associated with mediators of cellular proliferation in renal corpuscles at 96 h post-IR

To determine whether meprin β expression impacts downstream cellular proliferation of the IL-6 signaling pathway at 96 h post-IR (considered the repair phase level), mRNA and protein levels of cellular proliferation marker (proliferating nuclear antigen cell, PCNA) were evaluated in kidney tissue. The data showed that mRNA levels of PCNA significantly increased in kidneys subjected to IR for both genotypes when compared to control mice ($P < 0.01$) at 96 h-post IR (Fig. 5). Increased PCNA protein levels were confirmed using light microscopy, with a quantitative immunostaining intensity method for PCNA. Immunohistochemistry data showed that PCNA significantly increased ($P < 0.05$) in specific tubules of both genotypes for kidneys at 96 h post-IR when compared to control kidneys (Fig. 5). When compared to βKO kidneys, the PCNA baseline levels were lower in WT kidneys ($P < 0.01$). Interestingly, the immunohistochemical analysis showed that the staining intensity for PCNA in renal corpuscles significantly increased in βKO only ($P \leq 0.0001$) and not in WT at 96 h post-IR (Fig. 5). Immunofluorescence counterstaining of PCNA with proximal tubule markers (meprin β in WT and villin in βKO) showed expression of PCNA mainly in PTs of both genotypes' kidneys subjected to IR (Fig. 5). Additionally, PCNA levels were observed in the lumen of PTs in both genotypes at 96 h post-IR, indicating discharge PCNA into the filtration process and subsequently passing into the urine.

Discussion

Ischemia-reperfusion (IR) is a major contributor to acute kidney injury (AKI), a condition associated with high morbidity and mortality rates [1, 2]. Several studies have shown that inflammation is a key mechanism in the progression of AKI induced by IR [3, 4, 7]. At the initial stages of inflammation, circulating monocytes are recruited to the injury site, where they differentiate into classically activated M1 macrophages [8]. These macrophages release pro-inflammatory cytokines, including Interleukin-6 (IL-6), as part of a process that promotes cell proliferation and survival during immune responses to cellular stress [44, 45]. When IL-6 is bound to its soluble receptor (sIL-6 R), it activates the IL-6 trans-signaling

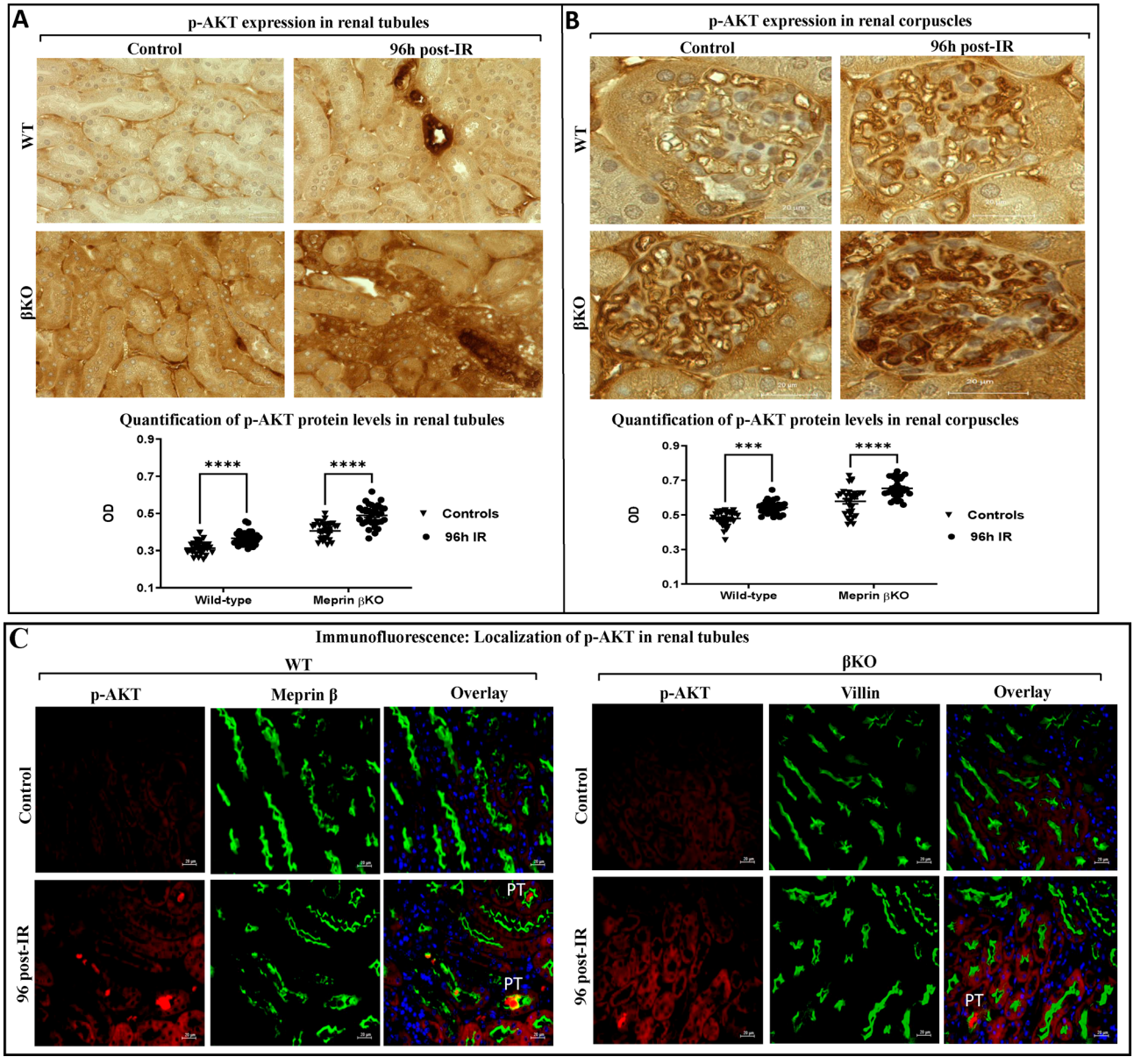

Fig. 3 Protein expression of p-AKT in kidney tissue from wild type (WT) and meprin β deficient mice (βKO) at 96 h post-IR. Immunohistochemical staining for phosphorylated AKT protein in renal tubules in wild-type (WT) and meprin β knockout (βKO) kidneys (**A**) and renal corpuscles (**B**). OD data were quantified using Image J analysis Software (ImageJ/Fiji 1.46) and analyzed by two-way ANOVA for 10 non-overlapping fields of tubular and 10 non-overlapping fields of renal corpuscle sections from each kidney and were imaged at 60 × magnification with a scale bar representing 20 µm. There were significant increases in p-AKT levels in select tubules and in renal corpuscles of both genotypes' kidneys subjected to IR. Immunofluorescence counterstaining of p-AKT (red) in kidney tubules in both genotypes (**C**). Meprin β (green) and villin (green) were used as the proximal tubule biomarkers in WT and βKO, respectively. DAPI was used to stain the nuclei (blue). Images at 60 × magnification with a scale bar representing 20 µm. Immunolocalization of p-AKT expression observed primarily in the lumen of PTs in both genotypes at 96 h post-IR. Data is expressed as mean ± SEM with P values as indicated, $P \leq 0.05$ are considered statistically significant

pathway, an anti-inflammatory process in IR-induced AKI [9, 46–51], promoting repair processes. Meprin metalloproteinases are implicated in the pathophysiology of kidney injury and play a critical role in inflammation, in part by processing and inactivating IL-6 [14, 52]. Meprins are abundantly expressed in the brush-border membrane (BBM) of kidney proximal tubules. Interestingly, meprins undergo redistribution from BBMs to the cytoplasm and basolateral compartments of proximal tubule cells in IR-induced AKI [31]. This redistribution brings meprin β in direct contact with extracellular matrix (ECM) in the basal lamina and enhances their accessibility for proteolytic cleavage [53]. Meprin β modulates IL-6 activity by inactivating it through proteolytic

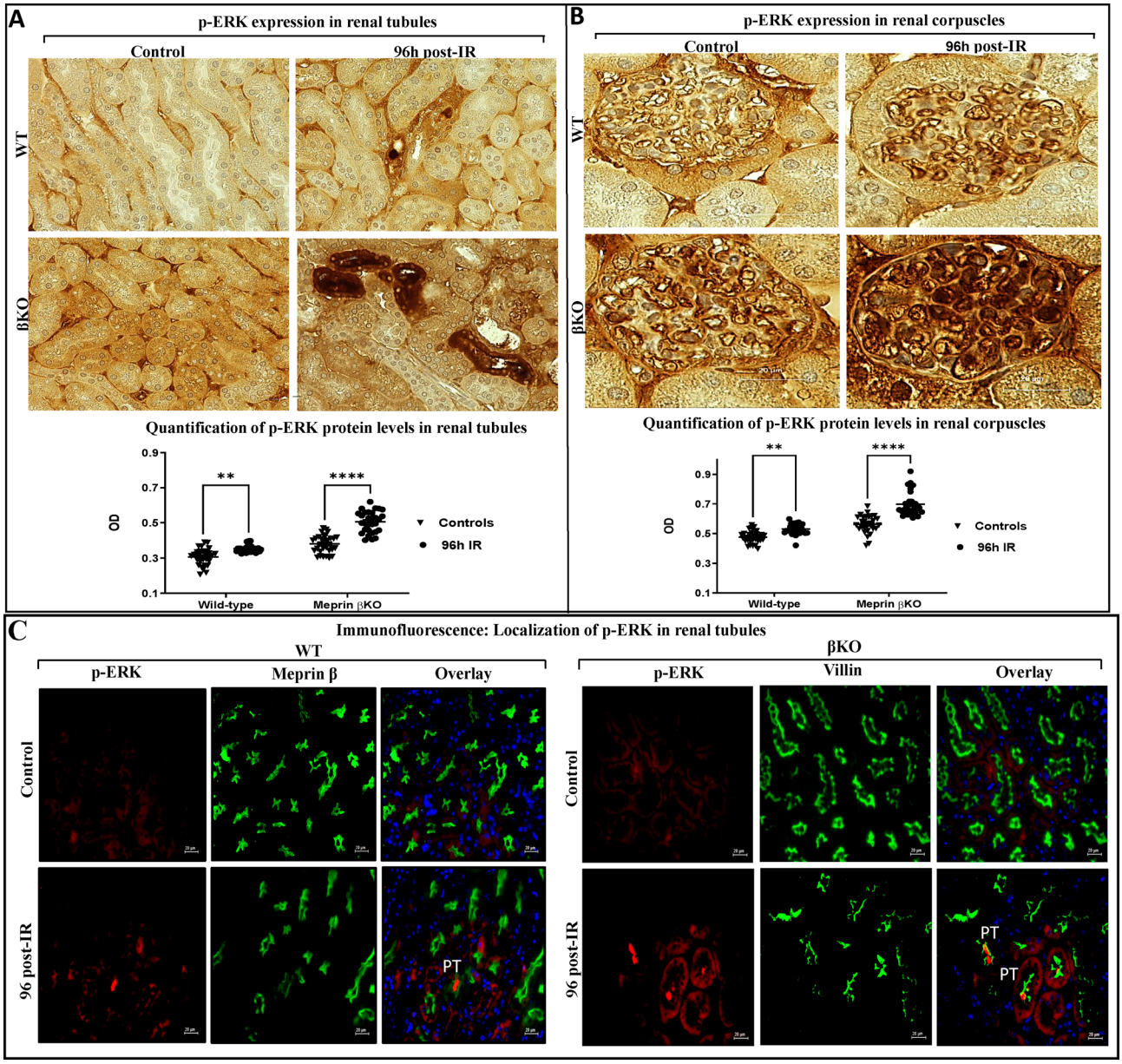

Fig. 4 Protein expression of p-ERK in kidney tissue from wild type (WT) and meprin β deficient mice (βKO) at 96 h post-IR. Immunohistochemical staining for phosphorylated ERK in WT and meprin βKO kidney tubules (**A**). Immunohistochemical staining for p-ERK in renal corpuscles of WT and meprin βKO mice kidneys (**B**). OD data were quantified using Image J analysis Software (ImageJ/Fiji 1.46) and analyzed by two-way ANOVA for 10 non-overlapping fields from renal tubular and renal corpuscle sections from each kidney. Images at 60 × magnification and the scale bar representing 20 µm. There were significant increases in p-ERK levels in select tubules and in renal corpuscles of both genotypes 96 h post-IR. Immunofluorescence counterstaining of p-ERK (red) in kidney tubules with the proximal tubule markers, meprin β (green) in wild-type (WT) and villin (green) in meprin β knockout (βKO) kidneys in renal tubules (**C**). DAPI was used to stain the nuclei (blue). Images at 60 × magnification with a scale bar representing 20 µm. Protein expression of p-EKT was observed in the lumen of PTs only in both genotypes' kidneys subjected to IR. Data is expressed as mean ± SEM with P values as indicated, P ≤ 0.05 are considered statistically significant

processing [14]. It also cleaves the membrane-bound IL-6 receptor (mbIL-6 R), enabling classical IL-6 signaling in cells expressing mbIL-6 R [54]. In contrast, the release of the soluble IL-6 receptor (sIL-6 R) activates trans-signaling in cells lacking mbIL-6 R, such as proximal tubule epithelial cells and macrophages [12, 55]. Data from the current study revealed an increase in IL-6 mRNA expression in both genotypes at 96 h post-IR. Additionally, immunohistochemical staining showed that IL-6 protein expression increased in select renal tubules and in renal corpuscles of both genotypes. These data align with our previous findings at 24 hours post-IR [20], which is the early phase of kidney injury. The high levels of IL-6 at 96 h post-IR, considered a reparative phase of

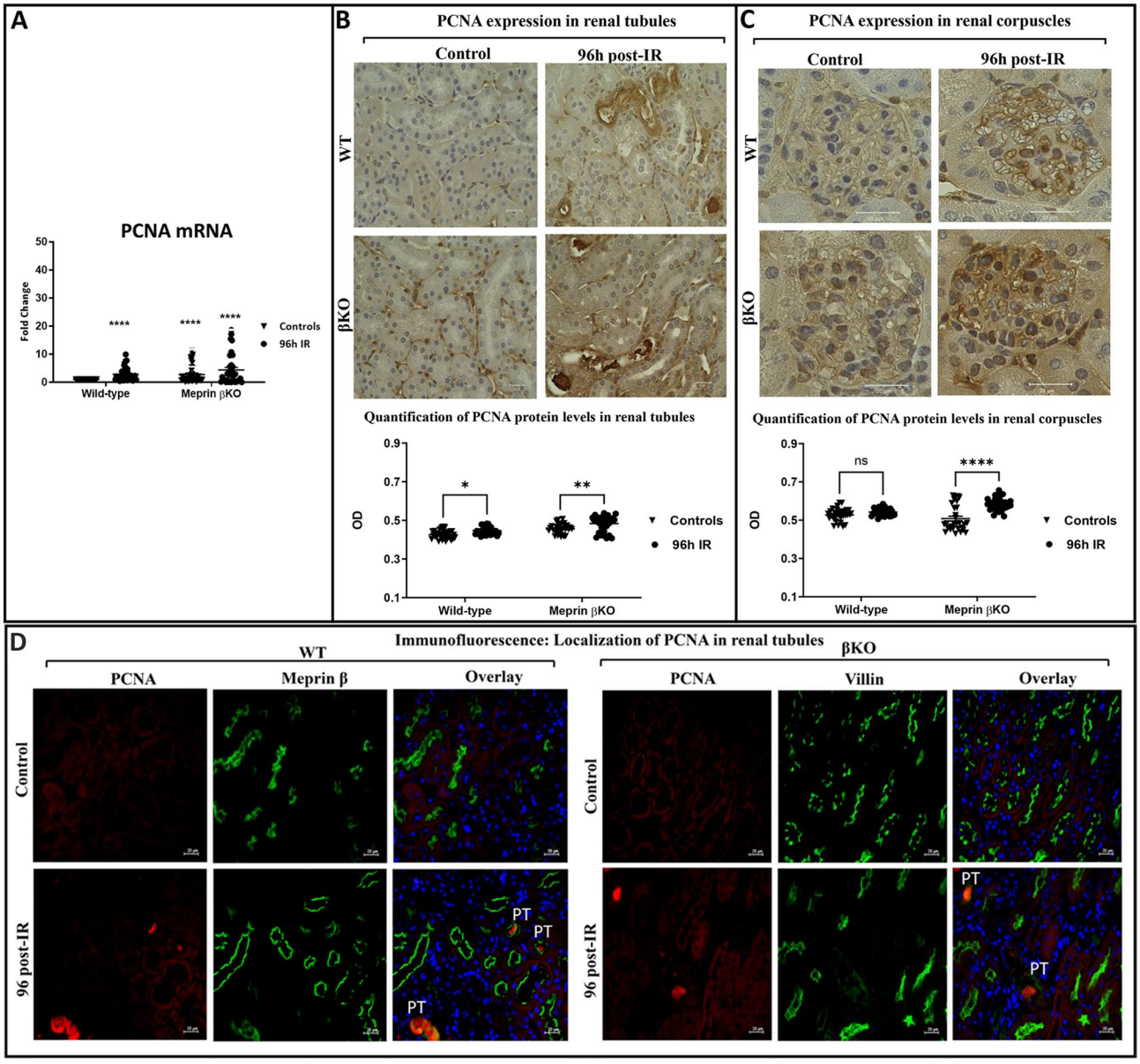

Fig. 5 PCNA protein expression in kidney tissue from wild type (WT) and meprin β deficient mice (βKO) at 96 h post-IR. The real-time PCR data showed that mRNA expression levels of PCNA increased in both genotypes subjected to IR (**A**). Values for PCNA mRNA levels were presented as fold change relative to control WT kidneys and normalized to GAPDH mRNA. Each value represents the mean ± SEM of triplicate combinations from 4 mice per group. Immunohistochemical staining for PCNA in kidney tubules in wild-type (WT) and meprin β knockout (βKO) kidneys (**B**). Immunostaining for PCNA in renal corpuscles in both genotypes (**C**). OD data were quantified using Image J analysis Software (ImageJ/Fiji 1.46) and analyzed by two-way ANOVA for 10 non-overlapping fields from renal tubular and renal corpuscle sections from each kidney. Images at 60 × magnification and the scale bar representing 20 µm. There was a significant increase in PCNA protein expression in select tubules in both genotypes, but only in βKO in renal corpuscle of kidneys subjected to IR. Immunofluorescence counterstaining of PCNA (red) in kidney tubules with the proximal tubule markers, meprin β (green) in wild-type (WT) and villin (green) in meprin β knockout (βKO) kidneys in renal tubules (**D**). DAPI was used to stain the nuclei (blue). Images at 60 × magnification with a scale bar representing 20 µm. PCNA was primarily expressed in the lumen of PTs in both genotype kidney sections at 96 h post-IR. Data is expressed as mean ± SEM with P values as indicated, P ≤ 0.05 are considered statistically significant

AKI, suggests that IL-6 plays a role not only in the injury phase but also in the recovery phase of the inflammatory response. Immunofluorescence counterstaining with proximal tubule biomarkers showed that increased IL-6 expression occurred in both proximal tubules (PTs), which express meprin β, with some expression detected in distal tubules (DTs), which lack meprin β. We also observed increases in IL-6 levels in the lumen of both PTs and DTs in WT and βKO kidneys at 96 h post-IR. This suggests a release of IL-6 into filtrate and subsequently into urine as supported by previous studies [31, 56]. Additionally, counterstaining of IL-6 and kidney

injury marker (KIM-1) in the present study showed that IL-6 expression associated with kidney injury in proximal tubules in both genotypes up to 96 h post-IR, which was previously seen at the acute phase [20]. These findings suggest a dual role for IL-6 in both inflammation and tissue repair despite the ongoing processing by meprin β. When IL-6 binds to its receptor, forming the IL-6/IL6R complex and subsequently interacts with the membrane-bound gp130 dimer, it triggers the activation and phosphorylation of Janus Kinase-Signal Transducer and Activator of Transcription (JAK/STAT), Mitogen-Activated Protein Kinase (MAPK), and Phosphatidylinositol 3-Kinase/Akt Kinase (PI3K/AKT) pathways [57]. These activated pathways are known to regulate various downstream processes including cellular apoptosis, survival, and proliferation [27]. Activation of the trans-signaling IL-6 has been demonstrated to enhance cellular proliferation [15, 17]. This activation of the cellular proliferation involves MAPK/ERK signaling pathway [21, 22], the PI3K/AKT pathway [23–25], or their crosstalk AKT/ERK [26–28]. To date, the mechanism by which meprin β regulates IL-6-induced cellular proliferation remains unclear. It is not yet known whether this regulation occurs through the MAPK/ERK pathway, the PI3K/AKT pathway, or their crosstalk (AKT/ERK). Therefore, the current study investigated the effect of meprin β expression on the expression levels of the two main downstream modulators of proliferation involving the IL-6 signaling pathway, AKT and ERK. Our data demonstrated a significant increase in phosphorylated forms of both meditators (p-AKT and p-ERK) in select tubules and in renal corpuscles at 96 hours post-IR, suggesting activation of the AKT/ERK pathway. Previous studies showed that mitochondrial AKT activation plays a role in preventing the progression of chronic kidney disease via attenuating oxidative stress, improving mitochondrial function, and reducing tubular injury [58, 59]. AKT was also shown to play a pivotal role in the pathogenesis of renal cell proliferation [60]. These findings suggest a critical role for the IL-6 in activating the AKT pathway during kidney recovery, potentially driving essential repair mechanisms and contributing to cellular proliferation. Additionally, the observed increase in p-ERK levels could be due to the role of ERK activation in ischemia-reperfusion injury, frequently mediated by reactive oxygen species (ROS) [61, 62]. These ROS-dependent pathways are shown to be essential for ERK signaling driving cell survival and repair processes. ERK1/2 is a conserved family of serine/threonine kinases involved in cell proliferation and differentiation [63, 64]. This pathway is activated through various extracellular stimuli, including mitogens, growth factors, and cytokines including IL-6 [65– 67]. The activation of ERK pathway plays a critical role in the proliferation and repair of renal tubular epithelial cells which helps facilitate the restoration of damaged tubules and preventing fibrosis progression [61]. Previous study findings from our group are aligned with the current data, indicating substantial increases in p-ERK levels in both meprin β knockout (βKO) and wild-type (WT) at 6 hours post-IR [39]. These findings suggest that the increased p-ERK levels at 96 h post-IR may be due to its sustained activity, supporting tissue repair and functional recovery in IR-induced kidney injury. The PI3K/AKT/IL-6 pathway plays a pivotal role in cancer cell proliferation and survival [68]. On the other hand, the IL-6-/ERK pathway was shown to be one of the key activators of cell proliferation, cancer cell growth, and tumor progression [69, 70]. Interestingly, both AKT and ERK signaling pathways are activated in renal tubules and renal corpuscles for up to 96 hours following IR-induced acute kidney injury (AKI). These intriguing observations, coupled with the role of AKT and ERK as crucial intracellular signaling pathways for cellular proliferation, prompted us to investigate whether the IL-6/AKT/ERK axis influences cell proliferation in relation to meprin activity during the repair phase of IR injury at 96 hours post-IR. To explore this, we examined Proliferating Cell Nuclear Antigen (PCNA), a well-established marker of cellular proliferation, which is known to be induced by the activation of IL-6 trans-signaling. PCNA is prominently expressed in the S3 segment of the proximal tubule following ischemic injury indicating its role in kidney repair [71, 72]. However, the mechanisms linking meprin β regulation of IL-6 trans-signaling to this proliferation remain unclear. Our RT-PCR data revealed significant increase of PCNA mRNA expression levels in both genotypes at 96 h post-IR. This was further supported by quantitative immunohistochemical analysis, which demonstrated a substantial increase in PCNA protein expression in specific tubules, suggesting the activation of mitogenic responses essential for tubular repair and regeneration phase [73, 74]. Interestingly, our results demonstrated a significant increase in PCNA protein expression in renal corpuscles of βKO mice, but not in WT, at 96 h post-IR, suggesting a unique proliferative response in the absence of meprin β expression and potential role of meprin β acts as a negative regulator of cell proliferation in the renal corpuscles. Interestingly, PCNA was detected in the lumen exclusively within proximal tubules, suggesting shedding into the urinary tract. Consistent with our findings, PCNA excretion in urine was previously reported [75], emphasizing its potential as a valuable non-invasive biomarker for clinical assessments. Furthermore, as proximal tubules are known to be more susceptible to damage due to their high metabolic activity and critical role in filtration and reabsorption [76], the exclusive detection of PCNA, along with p-AKT and p-ERK, in the lumen of PTs likely reflects proliferative and repair responses to

injury-induced stress mediated by the AKT/ERK signaling pathway during the recovery phase (96 hours post-IR). Collectively, our findings suggest that meprin may regulate cellular proliferation through IL-6-mediated activation of the AKT/ERK signaling pathway, promoting the expression of proliferation proteins such as PCNA and facilitating tissue repair during the kidney recovery phase.

Conclusion

Taken together, data from the current study suggest a potential modulatory role for meprin β in the IL-6-mediated AKT/ERK signaling pathway in IR-induced kidney injury. Furthermore, these findings suggest meprin β interaction with IL-6 modulates the AKT/ERK-mediated cellular proliferation, emphasizing their complex, tissue-specific functions in kidney injury and repair. Their involvement in conditions characterized by dysregulated proliferation, such as IR injury and proliferative kidney diseases, underscores their therapeutic potential. While our results suggest that meprin β regulates cellular proliferation through the IL-6-mediated AKT/ERK signaling pathway during the repair phase of IR-induced kidney injury, further studies are needed to elucidate this interplay and its role in recovery. Targeting PCNA and meprin β could offer therapeutic strategies to limit tumor growth and inflammation-driven progression in kidney injury and cancer.

Abbreviations

2–ΔΔCt method	2 (-Delta Delta C(T)) Method
AKI	Acute Kidney Injury
AKT	Protein kinase B
BBM	Brush-Border Membrane
Bcl-2	B-Cell Lymphoma/Leukemia 2
cDNA	Complementary Deoxyribonucleic Acid
DAPI	4,6-Diamidino-2-Phenylindole
DTs	Distal Tubules
ECM	Extracellular Matrix
ERK	Extracellular signal-Regulated Kinase
FC	Fold Change
GAPDH	Glyceraldehyde-3-Phosphate Dehydrogenase
IACUC	Institutional Animal Care and Use Committee
IL-6	Interleukin 6
IR	Ischemia/Reperfusion
JAK	Janus Kinase
KIM-1	Kidney Injury Molecule-1
LARU	Laboratory Animal Resource Unit of North Carolina
MAPK	Mitogen-activated protein kinase
mbIL-6R	Membrane-bound Interleukin 6 Receptor
Meprin A	Meprin A is a Homooligomer of α Subunits (α-α) or a Heterooligomer of α and β Subunits (α-β)
Meprin B	Meprin B is a Homooligomer of β Subunits (β-β)
mRNA	Messenger Ribonucleic Acid
PBS	Phosphate Buffer Saline
PCNA	Proliferating Cell Nuclear Antigen
PCR	Polymerase Chain Reaction
p-ERK1/2	Phosphorylated Extracellular Signal-Regulated Kinase 1/2
PI3K	Phosphatidylinositol 3-Kinase/Akt kinase
PKB/AKT	Phosphorylated Serine/Threonine Protein Kinase B
PTs	Proximal Tubules
RNA	Ribonucleic Acid
ROS	Reactive Oxygen Species
sIL-6R	Soluble Interleukin 6 Receptor
STAT	Signal Transducer and Activator of Transcription
WT	Wild-Type
βKO	Meprin-β Knockout Mice, Deficient in Meprin B (β-β) and the Heterodimeric Form of Meprin A (α-β)

Author contributions

S.A. is the first author contributed to the conception, design, data acquisition, analysis, interpretation of data, and drafting of the manuscript and is the primary author. F.A. made substantial contributions to data acquisition, analysis, interpretation, drafting of the manuscript, and substantive revision. A.A. contributed to data acquisition, analysis, and interpretation, as well as the creation of new software used in the work. A. A. was involved in data analysis C.A. was involved in data interpretation, drafting of the manuscript, and substantive revision. E.O. contributed to the design of the work, data acquisition, analysis, interpretation, drafting of the manuscript, and substantive revision.

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Data availability

The datasets used and analyzed during the current study are available from the corresponding author upon reasonable request.

Declarations

Ethics approval and consent to participate

The work in this study was reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) at North Carolina A&T State University.

Consent for publication

Not applicable.

Competing interests

The authors declare no competing interests.

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