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Title Page

Kidney-targeted delivery of PHD2 siRNA with nanoparticles alleviated renal ischemia/reperfusion injury

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Running title page:

Kidney-targeted delivery of PHD2 siRNA and renal ischemia

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Abbreviations：

AKI, acute kidney injury; BUN, blood urea nitrogen; Cr, serum creatinine; CKD, chronic kidney disease; DMSO, dimethyl sulfoxide; FA, folic acid; G5, polyamidoamine dendrimer generation 5; G5-FA, folic acid-decorated polyamidoamine dendrimer generation 5; GLUT1, glucose transporter type 1; HIF, hypoxia-inducible factor; I/R, ischemia/reperfusion injury; KIM-1, kidney injury molecule-1; NGAL, neutrophil gelatinase-associated lipocalin; NIR-G5-FA, near infrared fluorescent dye labeled G5-FA; PHDs, prolyl hydroxylase domain proteins; PAMAM, polyamidoamine; PAS, Periodic-Acid Schiff; siRNA, small interfering RNA;

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Abstract

Background: Inhibition of HIF-prolyl hydroxylase (PHD) has been shown to protect against various kidney diseases. However, there are controversial reports on the effect of PHD inhibition in renoprotection. The present study determined whether delivery of PHD2 siRNA using a siRNA carrier, folic acid (FA)-decorated polyamidoamine dendrimer generation 5 (G5-FA), would mainly target kidneys and protect against renal ischemia/reperfusion injury (I/R). **Methods:** The renal I/R was generated by clipping the renal pedicle for 30 minutes in uninephrectomized mice. Mice were sacrificed 48 hours after I/R. Normal saline or G5-FA complexed with control or PHD2 siRNA was injected via tail vein 24 h before ischemia. **Results:** After the injection of near-infrared fluorescent dye-labeled G5-FA, the fluorescence was mainly detected in kidneys, but not in other organs. The reduction of PHD2 mRNA and protein was only observed in kidneys but not in other organs after injection of PHD2-siRNA- G5-FA complex. The injection of PHD2-siRNA-G5-FA significantly alleviated renal I/R injury, as shown by the inhibition of increases in serum creatinine and BUN, the blockade of increases in KIM-1 and NGAL and the improvement of histological damage compared with mice treated with control siRNA. **Conclusion:** PHD2 siRNA can be delivered specifically into kidneys using G5-FA and that local knockdown of PHD2 gene expression within the kidney alleviates renal I/R injury. Therefore, G5-FA is an efficient siRNA carrier to deliver siRNA into the kidney, and that local inhibition of PHD2 within the kidney may be a potential strategy for the management of acute I/R injury.

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Significance Statement

Folic acid (FA)-decorated polyamidoamine dendrimer generation 5 (G5-FA) was demonstrated to be an effective carrier to deliver siRNA into kidneys. Delivery of PHD2 siRNA with G5-FA effectively protected the kidneys against the acute renal ischemia/reperfusion injury.

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Introduction:

The treatment of acute renal ischemia/reperfusion (I/R) injury remains difficult. Prolyl hydroxylase domain proteins (PHDs) are oxygen sensors to promote the degradation of hypoxia-inducible factor (HIF)-1α. Of three PHD isoforms, PHD2 is the primary PHD in kidneys (Schodel et al., 2009; Ito et al., 2020). Inhibition of PHD2 can increase activity of HIF-1α, which has been shown to protect against ischemia-induced kidney injury (Fang et al., 2016; Rajendran et al., 2020) . Among methods for inhibition of gene expression, small interfering RNA (siRNA) is proven to be an effective way for gene silencing. Despite many studies on siRNA treatment in various diseases, there are barriers that restrict its application, especially in the *in vivo* study. The limitations of siRNA in the *in vivo* studies include the requirement for relatively large amounts of siRNA to knock-down target genes, inadequate cellular intake reducing the efficiency of siRNA, and silencing of genes in non-target tissues. To overcome these limitations, it is necessary to develop an effective siRNA delivery method that can specifically carry siRNA to targeted organs.

Recent studies have displayed that nanoparticles are powerful vectors to deliver siRNA. Polyamidoamine (PAMAM) dendrimers have been shown to be promising vectors. PAMAM dendrimers have a highly branched 3D architecture, an initiator core and a number of active surface terminal groups. The surface groups and branches of dendrimers increase exponentially with the generations. The hydrophobic core and numerous surface groups of dendrimers make dendrimers possess a high drug loading capacity and multifunctionality (Menjoge et al., 2010; Xu et al., 2014a). PAMAM dendrimers have an important function that can facilitate the

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transportation of drugs across biological barriers such as cell membranes, because they can be endocytosed readily by virtue of their cationic primary amine groups on the surface. In contrast, siRNA are negatively charged, making it hard to enter the cell, because cell surfaces also possess negative charges. PAMAM dendrimers can take part in the siRNA-binding process and become an effective siRNA delivery vehicle to overcome the cell membrane barrier (Kesharwani et al., 2012).

Previous studies have demonstrated that folic acid (FA)-decorated PAMAM dendrimers are preferentially taken up by cells having high expression of folate receptors, such as kidney tubule cells and tumor cells (Dolman et al., 2010; Knight et al., 2012; Xu et al., 2017). Because kidney tubule cells express high levels of folate receptor, FA-conjugation has also been used for kidney-targeted drug delivery (Dolman et al., 2010; Knight et al., 2012). Utilizing the advantages of carrying siRNA by PAMAM dendrimers (Dzmitruk et al., 2018) and tumor-targeting delivery of siRNA by FA-conjugation (Dolman et al., 2010; Knight et al., 2012), we recently showed successful and sustained tumor-specific siRNA delivery by FA-conjugated PAMAM dendrimers generation 5 (G5-FA) (Xu et al., 2017). Therefore, we hypothesized that G5-FA might be a useful strategy for siRNA delivery specifically targeting kidneys and tested the effect of PHD2 siRNA on I/R-induced AKI, given the fact that renal tubules express high level of folate receptor. It is worth noting that while the luminal side of the tubular epithelial cells in the kidneys can be reached via the blood stream after glomerular filtration, the epithelial cells in other organs pose a barrier that prevents the possibility of macromolecular drug-carrier conjugates to reach the internalizing receptors at the luminal side, which make FA-conjugated dendrimers ideal drug-

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carriers for kidney-specific drug delivery.

Although activation of HIF-1α by inhibiting PHD has been shown to protect the kidney against injury, there are controversies about the effect of global vs. local activation of HIF-1α in kidney diseases. Some studies showed that global activation of HIF-1α inhibited kidney damage (Kobayashi et al., 2012), whereas other studies suggested that local activation of HIF-1α within the kidneys caused damage (Higgins et al., 2007; Kimura et al., 2008). Therefore, it is imperative to explore further about the local effect of PHDs in kidney. In addition, the controversial reports may also be due to different effects between pharmacological and genetic interventions. There has been no report on the effect of local genetic knockdown of PHDs within the kidney in an acute I/R model. The present study determined the effectiveness of G5-FA as promising vectors to deliver siRNA into renal tubules and the effect of PHD2 siRNA locally in kidneys on I/R renal injury. These results would be helpful to dissect the effects of systemic vs. local as well as genetic vs. pharmacological interventions for PHD inhibition in the kidneys when compared with the reports in the literature.

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Method and materials

Animals

Adult male mice (20-25 g) were used in this experiment. All mice had free access to laboratory food and water under standard conditions (12 h light-dark cycles, 22 °C ± 0.5 °C).

G5-FA preparation, siRNA delivery and AKI model

G5-FA was prepared as we described previously (Xu et al., 2016; He et al., 2017). Briefly, 19 mg of FA (Sigma-Aldrich, MO, USA) was reacted with 113 mg of 1-(3-Dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride in a mixture of 4 mL dimethyl sulfoxide (DMSO) and 12 mL of DMF for 1 hour. The mixture was then added dropwise to 50 mL of G5 aqueous solution containing 203 mg of G5. The reaction mixture was stirred vigorously in darkness for two days, then dialyzed with 7,000 molecular weight cut-off tubing (Thermo Scientific, IL, USA) against DI water for 2 days and freeze-dried. The obtained G5-FA was characterized by ^{1}H NMR in D_2O and its purity assessed by High Performance Liquid Chromatography. To prepare near infrared fluorescent dye labeled G5-FA (NIR-G5-FA), IRDye 800CW NHS ester (Li-COR Biotechnology, Lincoln, NE) was reacted with G5-FA to form IRDye 800CW-labeled G5.

G5-FA/siRNA polyplexes were prepared as we described before (Xu et al., 2016). Briefly, 325 μg of G5-FA was dissolved in 0.5 mL of RNAse-free normal saline and 130 μg of siRNA in 0.5 mL of Rnase-free saline. The solutions were vortexed for 10 s and equilibrated for 10 min at room temperature. The G5-FA solution and siRNA solution were then mixed by vortexing for 10 s, and allowed to equilibrate for 30 min at room temperature. The resulting siRNA G5-FA

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solution was then injected into mice via a tail vein bolus.

Acute kidney ischemia/reperfusion (I/R) model was produced according to a previous article (Wang et al., 2018b). Briefly, mice were anesthetized by intraperitoneal ketamine (60 mg/kg) and xylazine (6 mg/kg) and placed on a warm pad to keep a stable body temperature at 37 °C during surgery. The kidneys were exposed by dorsal incision, the right kidney removed and the renal pedicle of left kidney was clipped by microvascular clamp for 30 minutes. Control groups were treated the same way as I/R groups except for clipping of the renal pedicle. After surgery, mice were allowed free access to food and water. Mice were euthanized 48 hours after I/R, and blood, kidney, liver and heart collected for further experiments.

Mice were randomly divided into control saline group, control G5-FA group, IR+ PHD2 siRNA saline group, IR+ control siRNA G5-FA group, and IR+ PHD2 siRNA G5-FA group. Mice in IR+ PHD2 siRNA G5-FA group were injected with 7ug siRNA/17.5ug G5-FA per 10g body weight. The rest of groups received the same amount of normal saline, G5-FA, PHD2 siRNA saline, and control siRNA G5-FA, respectively. All solutions were injected 24 hours prior to I/R surgery.

Examination of NIR-G5-FA distribution in different organs by fluorescent imaging

Following the i.v. injection of a single dose of NIR-G5-FA or normal saline, mice were sacrificed 3 days later, and major organs were assessed by the Imaging System IVIS-200 (PerkinElmer).

Immunohistochemistry of G5-FA and PHD2 in kidney tissue

The kidneys were fixed in 4% formaldehyde, paraffin-embedded, and cut into 4-μm

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sections. Immunostaining was done as we described previously (Zhu et al., 2011). The primary antibodies were against folic acid (Genetex, USA) or PHD2 (Novus, USA). Image-Pro Plus was used to calculate the percentage of positive staining area as described previously (Zhu et al., 2011).

Renal function

After mice were sacrificed, blood samples were centrifuged at 1500g for 10 minutes to isolate the serum. Serum creatinine (Cr) and blood urea nitrogen (BUN) were analyzed by commercial kits (Fujifilm, Japan).

Isolation of RNA and Real-Time RT-PCR analysis of PHD2 and glucose transporter type 1 (GLUT1) mRNA levels

TRIzol reagent (Life Technology, Rockville, USA) was used to isolate total RNA. RNA was reverse-transcribed using an iScript cDNA Synthesis kit (Bio-Rad, Hercules, USA). The TaqMan Gene Expression Assays kit (Applied Biosystems) was used to amplify the reverse transcribed cDNA products. The expression level of 18S rRNA was chosen as an endogenous control. Relative PHD2 and GLUT1 mRNA levels were calculated according to ΔΔCt method.

Western blot analysis of PHD2 and renal injury markers, the kidney injury molecule molecule-1 (KIM-1) and neutrophil gelatinase-associated lipocalin (NGAL)

Protein preparation and Western blot were performed as we described previously (Zhang et al., 2019). The primary antibody against KIM-1 was from Invitrogen (rabbit polyclonal, 1:1000) and NGAL from Abcam (rabbit polyclonal, 1:1000). GAPDH (rabbit polyclonal, 1:3000, Proteintech) was used as a loading control. Image J was used to analyze the densitometry in all

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of the blots. All ratios of band intensities were normalized to the mean value of averaged intensities in the control group. Specificities of antibodies used were verified by the previous publications listed in company websites and by the results of using siRNA in the present study.

Histological analysis

Periodic-Acid Schiff (PAS) staining (Sigma-Aldrich) was used to examine the structures of tubular histology as published previously (Wang et al., 2015). The minimum of 20 cortical fields were independently scored by 2 examiners blinded to the different groups. The severities of tubular injuries were classified in 5 grades: 0 = normal; 1 = <10%; 2 = 10–25%; 3 = 26–75%; 4 = >75%.

Detection of hypoxia by pimonidazole staining

A Hypoxyprobe™ -1 Kit (HPI, Inc. Burlington, MA) was used to detect the renal tissue hypoxia using the manufacturer's protocol as described previously (Zhu et al., 2011). Briefly, pimonidazole hydrochloride (60 mg/kg ip) was injected 30 minutes before I/R surgery, and immunostaining of pimonidazole hydrochloride then performed in kidney tissue slides from different groups described above. Image-Pro Plus was used to calculate the percentage of positive staining area as described previously (Zhu et al., 2011) .

Statistics

Data are shown as the mean ± standard error of the mean (SEM). Comparisons among groups were performed with one-way analysis of variance (ANOVA) followed by Tukey's test. Student's *t*-test was used to assess statistically significant differences between two groups.

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Statistical significance was defined as $P<0.05$.

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Results

High kidney uptake of NIR-G5-FA

The fluorescent imaging showed that kidneys had substantially higher fluorescent intensity, whereas other organs, including heart, liver, lung and spleen, had undetectable or minimal fluorescence, indicating that G5-FA was mainly taken up by the kidneys (Fig. 1). The immunostaining of folic acid showed that the kidney from the G5-FA group had much more extensive staining than the control group and that FA was mainly located in proximal tubules (Fig. 2). These results indicated that G5-FA may be used as a carrier for kidney-specific delivery.

PHD2 mainly expressed in tubules

The expression of PHD2 was assessed in normal kidney tissue, and the result showed that PHD2 is mainly expressed in renal tubules (Supplement Fig. 1), which was consistent with previous reports (Schodel et al., 2009). These results suggest that G5-FA-mediated siRNA delivery is able to silence majority of PHD2 expression in the kidney by targeting the tubules.

I/R surgery caused equal hypoxia in surgery groups

The area of positive staining by the hypoxia probe, pimonidazole, was significantly increased in all mouse groups subjected to the I/R surgery, and there was no difference among different I/R groups. This confirms that the I/R surgery produced equal ischemia in the kidney in different groups (Supplement Fig. 2).

PHD2 mRNA and protein were knocked down in kidney but not in heart and liver

The levels of PHD2 mRNA and protein in kidney were significantly lower in the IR+PHD2

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siRNA G5-FA group than other groups (42 and 51% of the Ctrl groups, respectively), suggesting that G5-FA successfully carried PHD2 siRNA into kidneys and effectively silenced PHD2 locally (Fig. 3). The effects on the expression of PHD2 assessed by immunostaining showed similar results (Fig. 4); notably, in siPHD2-G5-FA-treated kidney the PHD2 staining was hardly visible in proximal tubules, whereas the positive PHD2 staining was not affected in other tubules, such as distal tubules and thick acceding limbs of Henle's loop, as identified by the morphology of the tubules. These results further demonstrated that siRNA delivered by G5-FA mainly targeted the proximal tubules, which is consistent with other reports showing that FA-conjugated drugs unable to target tubules other than proximal tubular cells in the kidneys (Shillingford et al., 2012; Samodelov et al., 2019; Shi et al., 2019).

However, the levels of PHD2 mRNA and protein were not changed in heart and liver (Fig. 5), suggesting that G5-FA did not carry PHD2 siRNA into other organs except kidneys. The apparent limited degree of observed knockdown of PHD2 by the G5-FA-mediated delivery of PHD2 siRNA may be due to selective targeting to the proximal tubules which express FA receptors at higher levels relative to other nephron segments. Therefore, the FA-G5-mediated siRNA delivery only targeted certain nephron segments, leading to a partial reduction of the overall expression of targeted gene in kidneys. The PHD2 knockdown mainly in proximal tubules was also supported by the immunostaining showing that some other tubules were still PHD2-positive in the IR+siPHD2 G5-FA group (Fig. 4).

Silencing of PHD2 increased the mRNA expression of HIF-1α target gene glucose transporter 1 (GLUT1) in kidneys.

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In order to further assess whether knockdown of PHD2 stimulated HIF-1α-mediated gene activation, the level of GLUT1, a target gene of HIF-1α (Makanji et al., 2014; Pan et al., 2021) and abundantly expressed in proximal tubules (Thorens et al., 1990; Dominguez et al., 1992), was measured by RT-PCR. The results showed that the GLUT1 level was significantly higher in IR+PHD2 siRNA G5-FA group compared with other groups, indicating the activation of HIF-1α-mediated gene regulation by G5-FA-mediated delivery of PHD2 siRNA in kidney (Fig. 3C).

Silencing of PHD2 improved renal function in I/R mice

The results of serum creatinine and BUN analyses showed no significant difference between the control saline and control G5-FA groups, indicating no adverse effect of G5-FA alone on the kidneys. Levels of serum creatinine (Cr) and BUN were significantly lower in the IR+PHD2 siRNA G5-FA group compared to the IR+PHD2 siRNA saline and IR+ control siRNA G5-FA groups, suggesting that silencing of PHD2 improves kidney function in I/R-induced injury (Fig. 6). The impressive degree of protection against the effects of I/R on Cr and BUN by the siPHD2 G5-FA treatment contrasts to the lower magnitude effects observed in reductions of PHD2 mRNA and protein levels (Fig. 3). We suggest that this seeming discrepancy is accounted for by the critical role of proximal tubules in renal I/R injury (Bajwa et al., 2010; Bonventre and Yang, 2011; Xu et al., 2014b; Ying et al., 2014), together with the selective targeting of PHD2 siRNA to proximal tubules by the FA-directed delivery system. Although PHD2 expression in proximal tubules represent partial levels of overall PHD2 in the kidneys, knockdown of PHD2 in this tubular segment leads to a bigger reservation in renal function after I/R injury because of the

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major role of proximal tubules in renal I/R injury.

Silencing of PHD2 blocked the increases of AKI biomarkers in I/R mice

The levels of NGAL，KIM-1 and IL-1β showed no significant difference between the control saline and control G5-FA group, indicating no adverse effect of G5-FA on the kidneys. The levels of NGAL，KIM-1 and IL-1β were significantly increased in I/R mice, whereas the increases in these markers were much lower in IR+PHD2 siRNA G5-FA group than IR+PHD2 siRNA saline and IR+ control siRNA groups. These results further demonstrated that the silencing of PHD2 improved AKI induced by I/R (Fig. 7).

Silencing of PHD2 attenuated morphological damage in I/R injury model

Morphological analysis assessed by PAS staining showed no histological damage in control G5-FA groups compared with control saline groups and that kidney tubular damage in I/R groups, as indicated by changes in tubular dilation, loss of brush border, vacuolar degeneration, tubular necrosis, and cast formation, were significantly attenuated in IR+PHD2 siRNA G5-FA group compared with other I/R groups, suggesting that silencing of PHD2 in the kidney improved renal I/R injury, (Fig. 8).

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Discussion

In the present study, the main findings were as followings: G5-FA delivered PHD2 siRNA into kidneys and produced knockdown of the target gene in kidney without influencing other major organs; delivery of PHD2 siRNA with G5-FA significantly improved kidney functions, kidney injury markers and morphological damage in I/R-induced AKI.

In addition to pharmacological methods, siRNA has proven to be a more accurate approach to inhibit functions of target genes (Lares et al., 2010; Chakraborty et al., 2017). Compared with systemic administration of siRNA, the delivery of siRNA with a vector to specifically target tissues has many advantages similar to that of organ-specific drug delivery (Draz et al., 2014). These advantages include increasing drug bioavailability, decreasing the dosage of drug, and reducing systemic toxicity. Although efforts have been made for kidney-targeted drug delivery (Dolman et al., 2010), there is nearly no report for specific kidney-targeted delivery of siRNA. The present study demonstrated the specific detection of NIR-G5-FA fluorescence and selective PHD2 gene knockdown in kidney but not other organs after IV injection, suggesting a successful kidney-targeted delivery of siRNA that utilizes the combined properties of carrying siRNA by PAMAM dendrimers (Kesharwani et al., 2012) and directing its kidney-uptake by FA-conjugation (Dolman et al., 2010; Knight et al., 2012).

PHD2 is an important oxygen sensor to regulate HIF-1α (Appelhoff et al., 2004). Many studies have found that PHD2 plays a vital role in ischemic diseases. It has been demonstrated that inhibition of PHD2 protects against I/R injury in different organs, such as brain, heart and limb (Takeda et al., 2011; Olenchock et al., 2016; Shi et al., 2016). A recent study showed that

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inhibition of PHD2 in endothelial cells protected kidneys from post-ischemic injury (Rajendran et al., 2020). Consistent with these results, the present study demonstrated that inhibition of PHD2 in kidney tubules improved kidney function and histological damage in I/R-induced AKI. Interestingly, we found that systemic delivery of an equal amount of PHD2 siRNA without G5-FA did not alleviate I/R-induced injury, whereas delivery of PHD2 siRNA with G5-FA significantly improved I/R-induced injury. Therefore, G5-FA can serve as a useful vector to deliver siRNA for local gene knockdown in kidney tissue.

In term of mechanisms by which PHD2 inhibition protects the kidneys from I/R-induced injury, the activation of HIF-1α-mediated gene regulation would be accountable for the protection. HIFs play a central role in mediating cellular protection in I/R injury (Howell and Tennant, 2014). PHD inhibition has been shown to induce HIF-mediated ischemic pre-conditioning, thereby, attenuating I/R injuries in various organs, including kidneys (Heyman et al., 2016). Different downstream pathways that contribute to the renal protection after HIF-1α activation by PHD inhibition have been elucidated, such as inhibiting inflammation, suppressing ROS production, attenuating apoptosis, blocking neutrophil and macrophage recruitment, and enhancing autophagy (Kapitsinou and Haase, 2015; Fang et al., 2016; Yang et al., 2018; Xie et al., 2019; Li et al., 2020; Rajendran et al., 2020; Miao et al., 2021). Consistent with the literature, the present study showed that in kidneys from mice treated with siPHD2-G5-FA the increased levels of inflammatory factor IL-1β were reduced, which validated the inhibition of downstream injurious pathway in I/R-induced AKI by PHD2 inhibition in the present study.

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Notably, results from the present study represent both a technical and scientific advance. Technically, FA-conjugated dendrimers as a carrier for kidney-targeted drug and DNA delivery is an innovative technique. Some other studies also used FA-conjugated drug to target tubular cells for the treatment of kidney diseases, such as polycystic kidneys and AKI (Knight et al., 2012; Shillingford et al., 2012). However, these studies directly conjugated FA with the drugs. Our approach to conjugate FA to dendrimers as drug carriers for kidney-targeted delivery bears several advantages compared with the FA-drug conjugation. Firstly, conjugation of a chemical to FA may change properties of the drug, thereby reducing the therapeutic efficacy of the drug. Secondly, conjugation of FA to a carrier has the potential to deliver different drugs, which will be significantly convenient and broaden the application of this technology. Thirdly, our FA-dendrimer drug carriers have been shown to efficiently deliver siRNA or DNA that cannot be directly conjugated with FA, and then provide a novel kidney-targeted genetic intervention, which is more convenient than generating conditional gene KO animals, and more importantly, bears clinical implication in gene therapy. Scientifically, results from the present study, for the first time, reveal renoprotection against I/R by genetic inhibition of PHD to activate HIF-1α-mediated gene regulation locally in the kidneys. These findings clarify some controversial reports and advance our understanding in the mechanisms associated PHD/HIF pathway in kidney damages.

Finally, results from the present study suggest interesting clinical implications of the findings. Nanoparticles as drug carriers have been used successfully in humans. Some examples include cytarabine and daunorubicin contained in nanoparticles to treat acute myeloid leukemia

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in phase I clinical study and optimize dosing for further phase II trials (Feldman et al., 2011); hafnium oxide, a radioenhancer, contained in nanoparticles, added to radiotherapy for improved therapy of advanced soft-tissue sarcoma (Bonvalot et al., 2019); and microRNA-loaded nanoparticles, TargomiRs, for treatment of malignant pleural mesothelioma (van Zandwijk et al., 2017). Based on these human studies, findings from our present study indicate that FA-dendrimers could be used as a potential approach clinically for kidney-targeted delivery of drugs or siRNAs in the management of I/R-induced AKI or other kidney diseases. Scientifically, there are human studies targeting PHD, mostly using PHD inhibitor to treat anemia in CKD patients. Multiple trials have demonstrated that inhibition of PHD effectively improve anemia in those patients with less adverse effects (Akizawa et al., 2020a; Akizawa et al., 2020b; Fishbane et al., 2021). There is no clinical research about effect of PHD inhibition on AKI. However, many pre-clinical studies have proved that inhibition of PHD to increase the expression of HIF-1α protects against cisplatin- or I/R-induced AKI (Bernhardt et al., 2006; Yang et al., 2018). Similarly, administration of recombinant HIF-1α protein protects rats against I/R-induced AKI (Wang et al., 2018a). Combined with the findings from our present study and literature, it is a promising potential therapy to deliver PHD inhibitor or siRNA for patients with AKI.

It should be noted that both folic acid and PAMAM dendrimers have been shown toxic effects. Folic acid-induced kidney damage has been commonly used as an AKI animal model (Samodelov et al., 2019). However, the dose of folic acid used to induce kidney damage is 250mg/kg in mice (Stallons et al., 2014; Kumar et al., 2015; Fan et al., 2017; Fu et al., 2018). The dose of folic acid used in the present study was much lower and unlikely to induce kidney

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damage by the folic acid per se. Indeed, our data showed that there were no renal injuries in G5-FA-treated mice, suggesting a safe dose of folic acid in the present study. In addition, although PAMAM dendrimers have been widely used for drug delivery, dendrimers-induced cell toxicity has also been demonstrated (Neerman et al., 2004; Araújo et al., 2018; Chis et al., 2020). The PAMAM dendrimers-induced toxicity is also dose-dependent, but no toxic effects are observed in studies using doses of 5, 10 and 95 mg/kg in mice (Roberts et al., 1996; Malik et al., 1999; Yu et al., 2017). Thus, the dose used in the present study was within the safe range, and consistent with the literature, no kidney damages were observed in G5-FA-treated mice in the present study.

There are several limitations in the present study. We chose I/R-induced AKI because its pathogenesis is closely related the PHD2/HIF pathways and that the ischemia is most commonly associated with AKI in hospital (Liaño and Pascual, 1996). However, HIF-1α signaling has also been shown to be involved in the pathogeneses of other forms of AKI, such as cisplatin-, radiocontrast-, sepsis-, and gentamicin-induced AKI (Shu et al., 2019). Although the mechanisms in the pathogeneses of those forms of AKI are different, whether G5-FA-mediated delivery of PHD2 siRNA to target the proximal tubules can also protect the kidneys in those forms of AKI is worth future investigation. In addition, the disease course of the current study is short. The duration of G5-FA-siPHD2-produced PHD2 inhibition remains unclear. It is important to clarify the duration of PHD2 inhibition, for example, whether there will be a prolonged inhibition of PHD2 continuing after the recovery of AKI. To clarify the duration of PHD2 inhibition, or more generally, gene silencing by G5-FA-mediated siRNA delivery into the kidneys is also important for the potential application of this technique in a chronic and longer time disease model, such as

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CKD, for example, whether repeated injections are needed and in what interval between injections. Furthermore, the present study was carried out under a pathological condition and attempted to prime the PHD2/HIF-1α pathway for the protection against I/R-induced AKI. However, whether long-term inhibition of PHD2 has harmful effect and what the consequence of prolonged PHD2 inhibition in a chronic and longer time disease model remain unknown. All these important questions need to be elucidated in future investigations.

In conclusion, the results of our study demonstrated that G5-FA was an effective carrier to deliver siRNA into kidneys and that local knock down of PHD2 in renal tubules alleviated I/R induced AKI. It is suggested that G5-FA-mediated local delivery of PHD2 siRNA may be used as a potential therapeutic strategy for the prevention or treatment of acute renal I/R injury.

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Authorship Contributions

Participated in research design: Li, Xie, Yang

Conducted experiments: Xie, Wang, Hu, Chen, Qu

Performed data analysis: Xie, Wang, Yang, Li, Qu

Wrote or contributed to the writing of the manuscript: Xie, Wang, Yang, Ritter, Li

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Figure legend

Fig 1: Fluorescent imaging of important organs by the IVIS-200 imaging system. Top panel: Representative ex vivo fluorescent images of different organs. Bottom panel: Summarized fluorescent intensities of different organs. *P < 0.05 vs. all other groups, n=3.

Fig. 2: Immunohistochemical analysis of folic acid in the kidney tissue slides. Top panel: Representative microphotographs of kidney sections from a control (G5-Ctrl) and G5-FA injected mouse, stained using a folic acid primary antibody. Bottom panel: Percentage of positively stained area normalized to the value in control. *P < 0.05 vs. the other group, n=3.

Fig. 3: PHD2 mRNA and protein levels in kidneys. A, Real-Time RT-PCR analysis of PHD2 mRNA levels. B, Western blot analysis of PHD2 protein levels: upper panel, representative gel documents depicting the protein levels of PHD2; lower panel, summarized band intensity ratios to GAPDH normalized to the value in control. *P < 0.05 vs. all other groups. n=3-5

Fig. 4: Immunohistochemical analysis of PHD2 in kidney tissue slides from mice with different treatments. Top panel: Representative microphotographs showing IHC of PHD2. Bottom panel: Percentage of positively stained area normalized to the value in control. *P < 0.05 vs. all other groups, n=3.

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Fig. 5: PHD2 mRNA and protein levels in heart and liver. A & B: Real-Time RT-PCR analysis of PHD2 mRNA levels in heart and liver, respectively. C&D: Western blot analysis of PHD2 protein levels in heart and liver: upper panel, representative gel documents depicting the protein levels of PHD2; lower panel, summarized band intensity ratios to GAPDH normalized to the value in control. *P < 0.05 vs. all other groups. n=3-5

Fig. 6: Levels of serum creatinine and blood urea nitrogen (BUN). *P < 0.05 vs. the indicated groups. NS: no significant difference. n=3-5

Fig 7: Levels of KIM-1, NGAL and IL-1β in kidney tissues by Western blot. Upper panel: representative band of KIM-1, NGAL and IL-1β; Lower panel: summarized band intensity ratios to GAPDH normalized to the value in control. *P < 0.05 vs. other I/R groups. n=3-5

Fig. 8: Morphological examination by PAS staining in kidney tissue slides. Upper panel: representative microphotographs showing tubular structures; Lower panel: semiquantitation of tubular damages. *P < 0.05 vs. all other groups. n=3-5

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Footnotes.

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Fig. 1

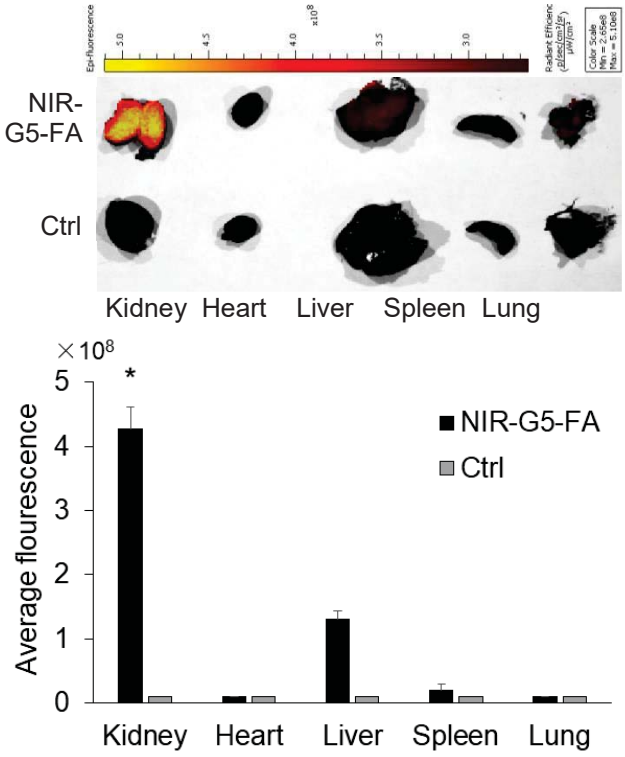

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Fig 2.

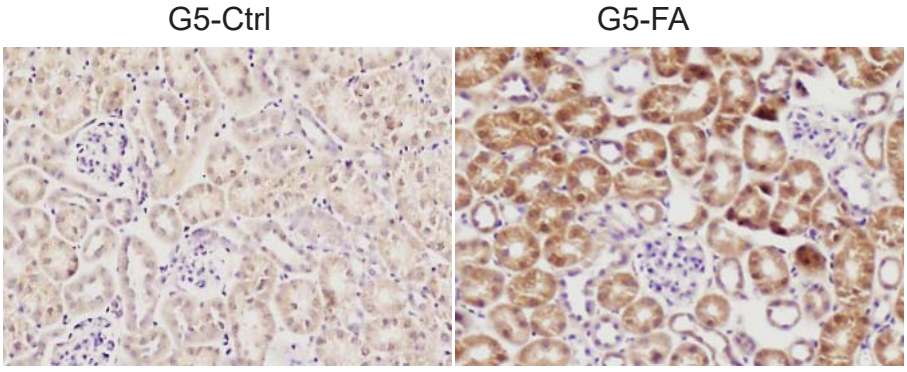

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Fig. 3

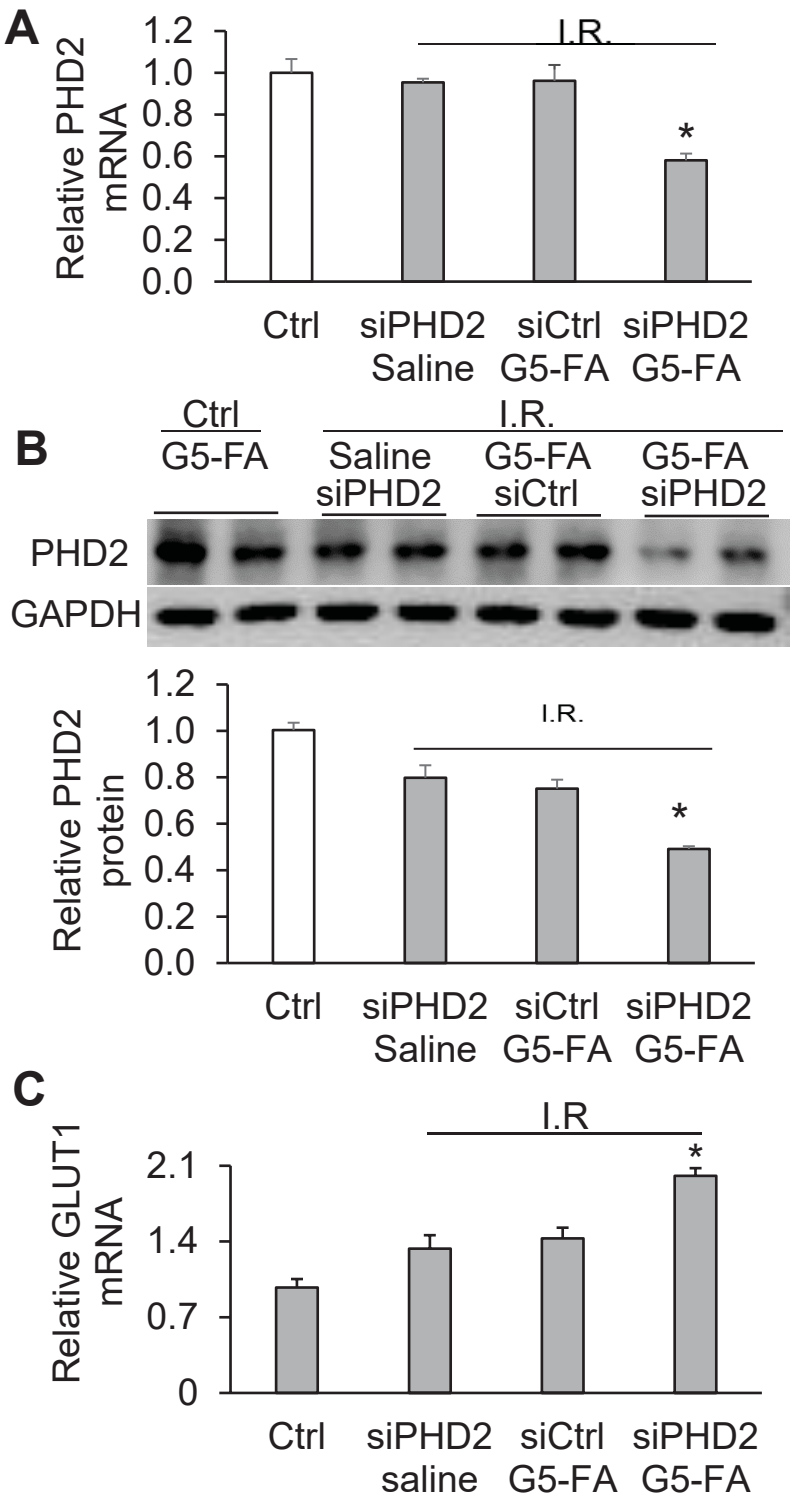

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Fig 4.

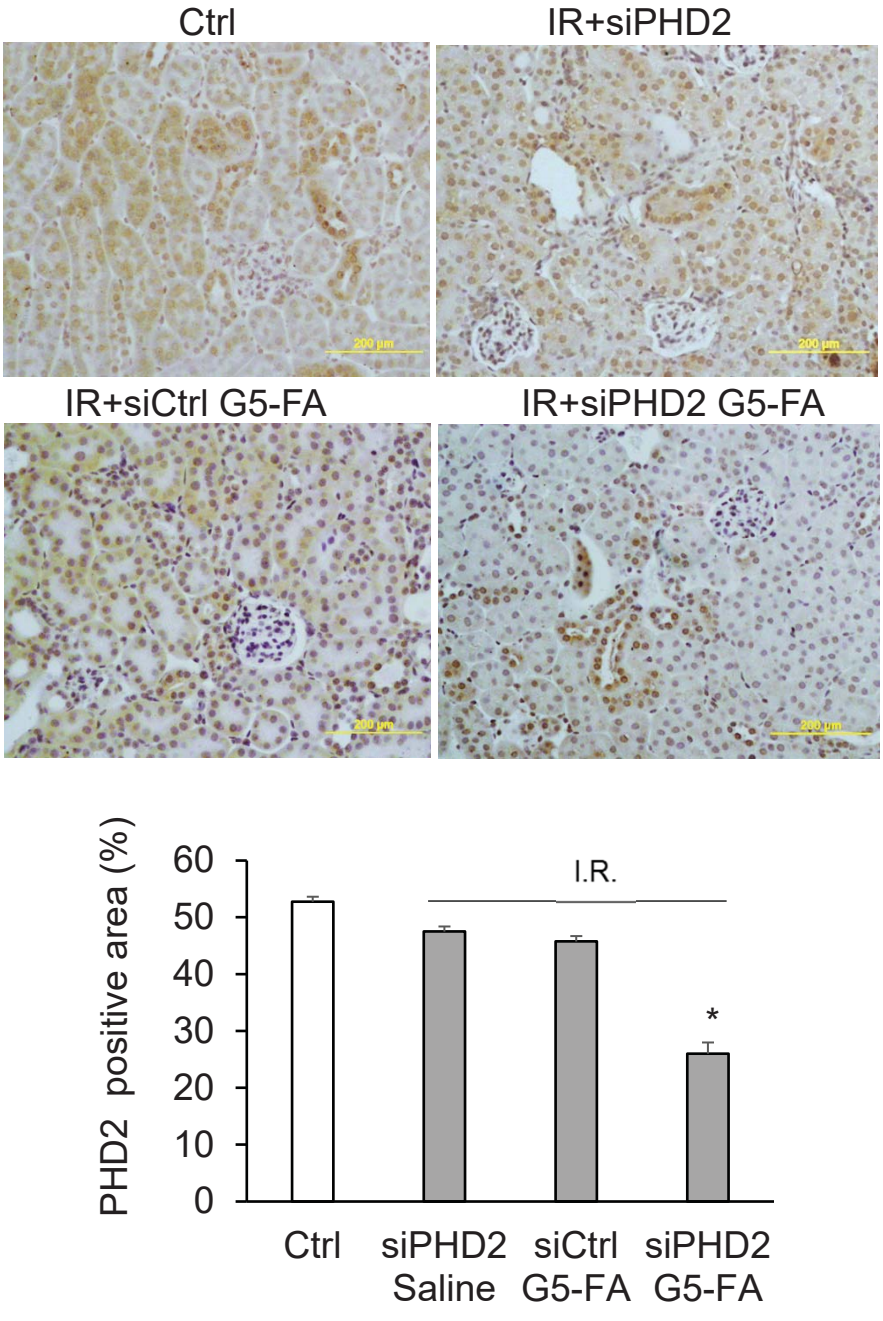

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Fig. 5

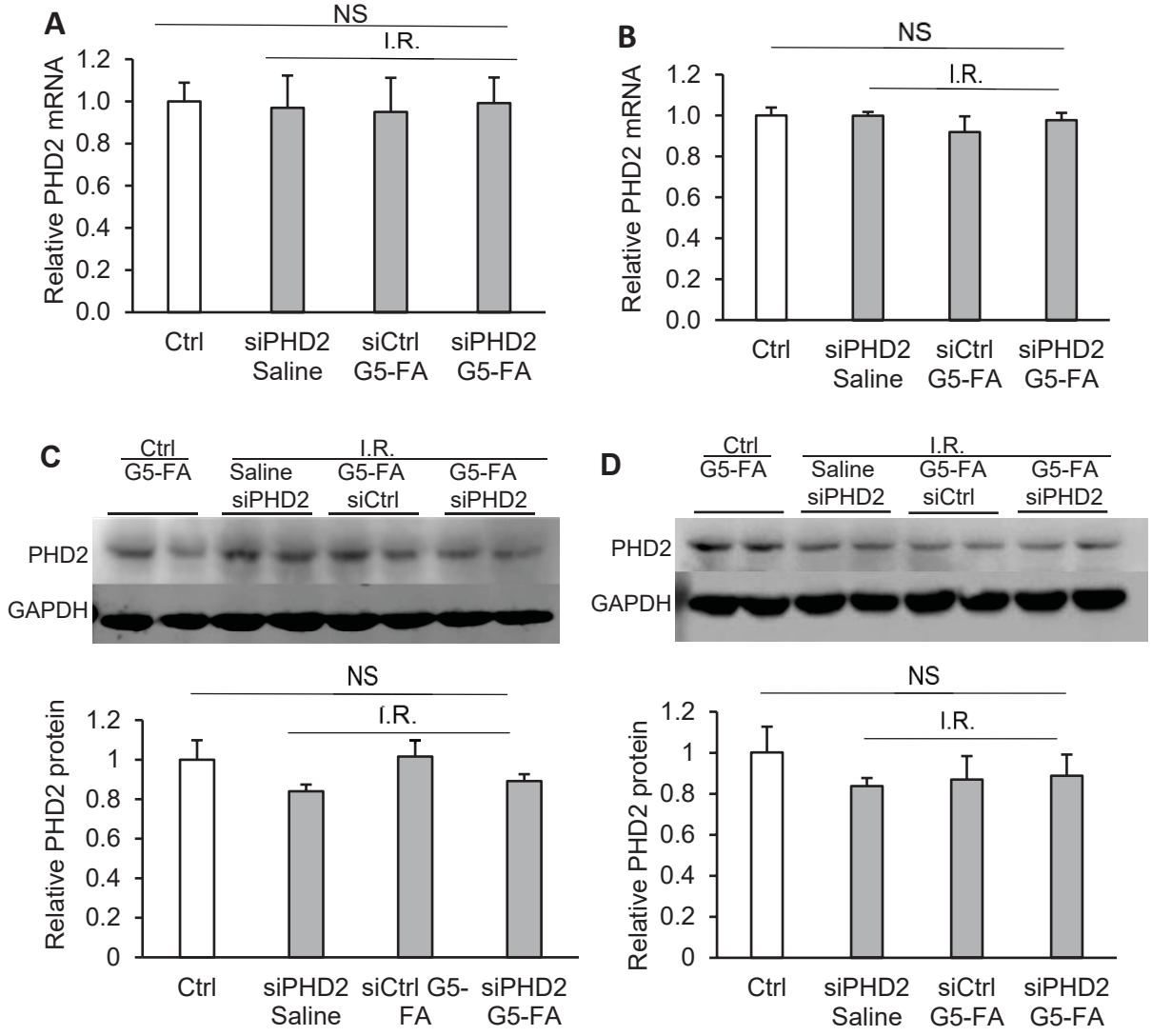

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Fig 6.

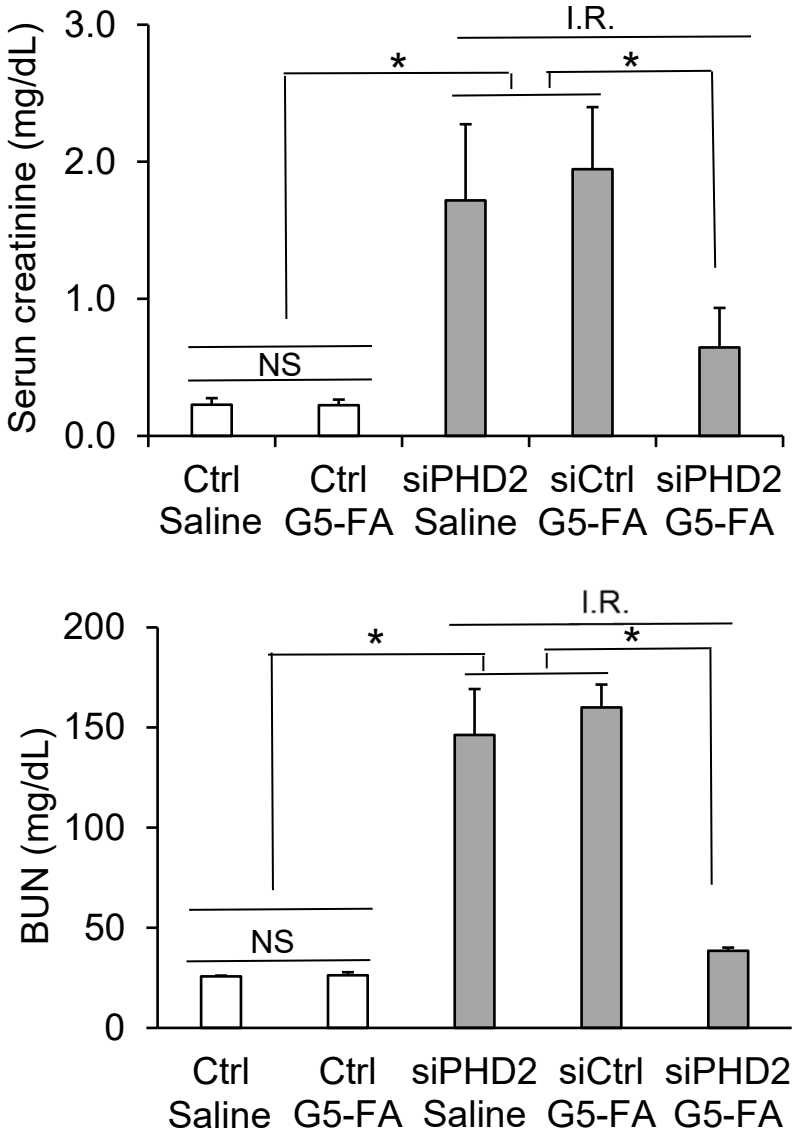

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Fig. 7

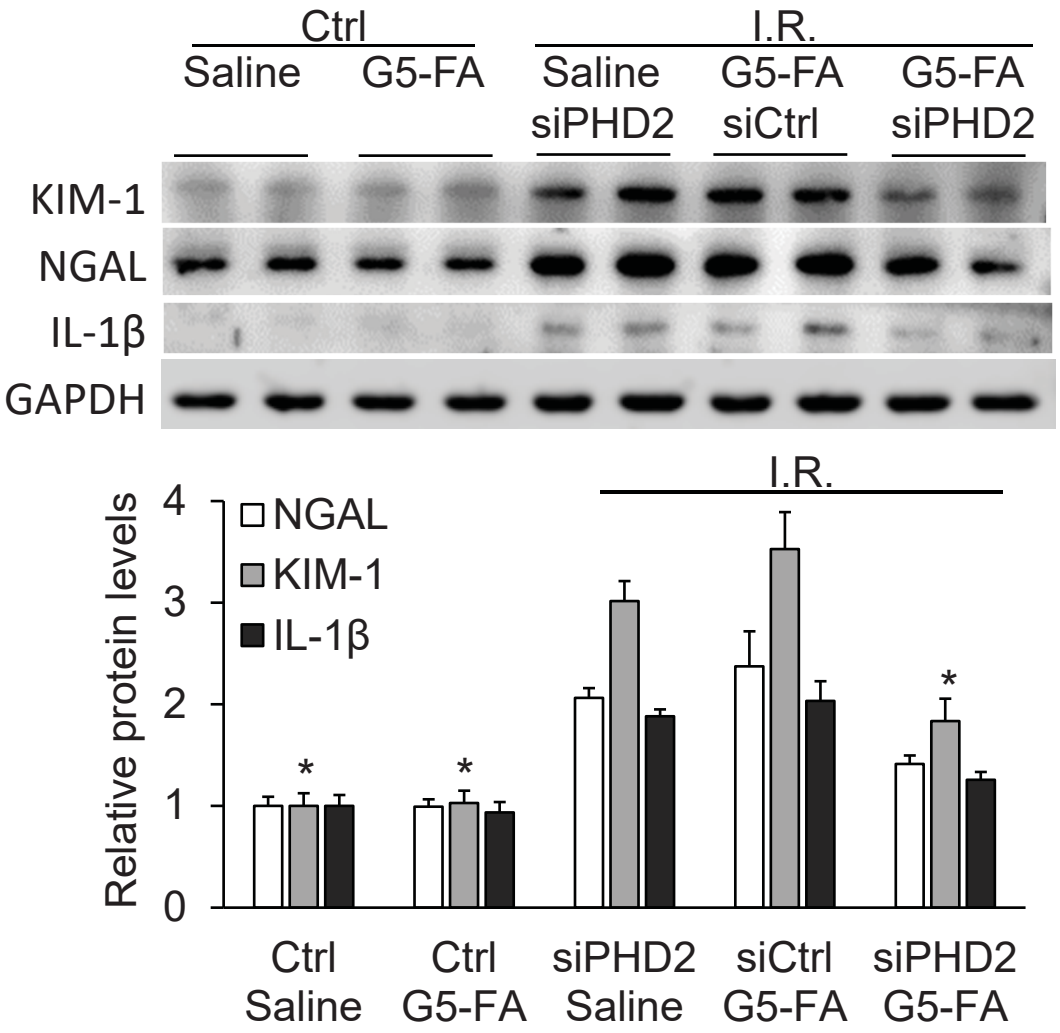

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Fig.8