

# Overexpression of angiotensinogen downregulates aquaporin I expression via modulation of Nrf2–HO-1 pathway in renal proximal tubular cells of transgenic mice

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## Abstract

**Introduction:** We aimed to examine the regulation of aquaporin I expression in an angiotensinogen transgenic mouse model, focusing on underlying mechanisms.

**Methods:** Male transgenic mice overexpressing rat angiotensinogen in their renal proximal tubular cells (RPTCs) and rat immortalised RPTCs stably transfected with rat angiotensinogen cDNA were used.

**Results:** Angiotensinogen-transgenic mice developed hypertension and nephropathy, changes that were either partially or completely attenuated by treatment with losartan or dual renin–angiotensin system blockade (losartan and perindopril), respectively, while hydralazine prevented hypertension but not nephropathy. Decreased expression of aquaporin I and heme oxygenase-1 and increased expression of nuclear factor erythroid 2-related factor 2 (Nrf2) and sodium–hydrogen exchanger 3 were observed in RPTCs of angiotensinogen-transgenic mice and in angiotensinogen-transfected immortalised RPTCs. These parameters were normalised by dual renin–angiotensin system blockade. Both in vivo and in vitro studies identified a novel mechanism in which angiotensinogen overexpression in RPTCs enhances the cytosolic accumulation of Nrf2 via the phosphorylation of pGSK3 $\beta$  Y216. Consequently, lower intranuclear Nrf2 levels are less efficient to trigger heme oxygenase-1 expression as a defence mechanism, which subsequently diminishes aquaporin I expression in RPTCs.

**Conclusions:** Angiotensinogen-mediated downregulation of aquaporin I and Nrf2 signalling may play an important role in intrarenal renin–angiotensin system-induced hypertension and kidney injury.

## Keywords

Aquaporin-I, Nrf2, intrarenal renin–angiotensin system, hypertension, kidney injury

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## Introduction

Aquaporin-1 (AQP1) is the major water channel in the renal proximal tubule and the loop of Henle.<sup>1</sup> These two nephron segments are responsible for reabsorbing 80% of the glomerular filtrate.<sup>1</sup> As renal proximal tubular cells (RPTCs) reabsorb 60–70% of filtered sodium (Na) and fluid, changes in the way in which RPTCs reabsorb water (i.e. AQP1) and Na (via increased Na transporter expression)<sup>2</sup> can have profound effects on renal and body fluid balance. AQP1-deficient mice (*Aqp1*-null) displayed

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normal phenotypes with respect to survival, physical appearance and organ morphology, but these mice became severely dehydrated after water deprivation, indicating that AQP1 is required for the formation of a concentrated urine.<sup>3</sup> *Aqp1*-null mice had a relatively low blood pressure phenotype, which can be explained by several possibilities – e.g. polyuria,<sup>4</sup> impaired nitric oxide signalling<sup>5</sup> and reduced renin cell recruitment.<sup>6</sup>

It has been observed that AQP1 expression is upregulated in the kidneys<sup>7</sup> and brain<sup>8</sup> of spontaneously hypertensive rats.<sup>7,8</sup> In contrast, recent studies reported that renal and cardiac AQP1 expression was downregulated and associated with renal fibrosis<sup>9</sup> and high-salt diet-induced hypertension.<sup>10</sup> Thus, it remains unclear whether AQP1 expression can directly or indirectly affect blood pressure and kidney injury.

The intrarenal renin angiotensin system (RAS) plays a key role in blood pressure regulation and renal haemodynamics, and all RAS components are expressed in RPTCs.<sup>11</sup> To date, intrarenal RAS influences on AQP1 expression in either pathological or physiological conditions are poorly understood. Bouley et al. reported that angiotensin II (Ang II) rather than osmolality may be more important in regulating AQP1 levels in renal proximal tubules (RPTs).<sup>12</sup> Ang II at low concentrations ( $10^{-9}$  and  $10^{-8}$  M) or infusion of Ang II at 80 ng/min/kg increased AQP1 expression in cultured rat immortalised renal proximal tubular cells (IRPTCs) *in vitro* and in rat kidneys *in vivo*, respectively. In contrast, Ang II at high concentration ( $10^{-7}$  M) inhibited AQP1 expression in IRPTCs. Thus, the intrarenal RAS appears to regulate AQP1 expression, influencing water reabsorption and body fluid homeostasis.

Our lab has established that transgenic mice specifically overexpressing angiotensinogen (the sole precursor of all angiotensins) in their RPTCs developed hypertension and nephropathy with elevated intrarenal reactive oxygen species (ROS) production.<sup>13–15</sup> In the present study, we aimed to determine whether intrarenal RAS-induced hypertension and kidney injury in our angiotensinogen-transgenic (Agt-Tg) mice could be mediated, at least in part, via alteration of AQP1 expression and whether RAS blockade in this transgenic model could reverse this effect. We further aimed to define the underlying molecular mechanisms both *in vivo* and *in vitro*.

## Materials and methods

### *Animal models and ethics statement*

Agt-Tg mice overexpressing renal rat angiotensinogen were generated by employing the kidney-specific, androgen-regulated protein promoter (KAP2) linked to rat angiotensinogen cDNA as reported previously.<sup>16</sup> There is no need to administer exogenous androgen since the

circulating level of testosterone in adult male Agt-Tg mice (from 12 weeks of age, as is the case here) is sufficiently high to drive KAP2 promoter to express the transgenes.<sup>13,14,16</sup> Thus, male Agt-Tg mice were employed and studied starting at 10 weeks of age and treated with or without hydralazine (15 mg/kg/day, in drinking water), losartan (losartan 30 mg/kg/day, in drinking water) and/or dual RAS blockers (losartan 30 mg/kg/day plus perindopril 4 mg/kg/day, in drinking water) from week 13 until week 20<sup>13–15</sup> (eight to 15 mice per group). Non-transgenic littermates served as controls. All animals had free access to standard mouse chow (Diet #2918, Harlan Teklad, Montreal, Canada) and water.

The animal study was carried out under strict conditions according to the recommendations in the guide for the care and use of laboratory animals of the National Institutes of Health. Animal care and procedures were approved by the animal care committee from the Centre de Recherche du Centre Hospitalier de l'Université de Montréal (CRCHUM). Mice were killed by sodium pentobarbital overdose (75 mg/kg of body weight (BW)) and efforts were made to minimise suffering.

### *Physiological studies*

Mean systolic blood pressure (SBP) was monitored by the tail-cuff method with the Visitech BP-2000 blood pressure analysis system for mice (Visitech System Inc., Apex, NC, USA), as reported elsewhere.<sup>13–15</sup> Animals in each group were acclimated to longitudinal SBP measurement (2-week period of pre-training starting at 11 weeks of age, followed by actual measurement of SBP thrice weekly from 13 weeks until 20 weeks of age) to minimise stress to the animals. While the technique of tail-cuff measurement is generally considered less sensitive than telemetry, our SBP data include a 2-week pre-study training period and substantial numbers of animals ( $N=8–15$  mice per group) and longitudinal measurement (8 weeks excluding the 2-week pre-study training period).

Twenty-four hours before the mice were killed, BW was recorded and mice were individually housed for 24 hours in metabolic cages. Blood was collected individually via intracardiac exsanguination before death and then centrifuged to obtain serum. Urine was collected and assayed for albumin and creatinine ratio ( $\mu\text{g}/\text{mg}$ ) (enzyme-linked immunosorbent assay (ELISA), Albuwell and Creatinine Companion, Exocell, Inc., Philadelphia, PA, USA), angiotensinogen and Ang II measurement (i.e. C18 Sep-Pak columns (Waters, Mississauga, ON, Canada); extraction kits (Bachem Americas, Torrance, CA, USA); ELISAs (Bachem Americas)) as reported previously.<sup>13–15</sup> Kidney weights were rapidly recorded. The left kidney was utilised for renal histology and the right kidney was reserved for RPTs isolation by the Percoll gradient method for protein expression experiments as previously reported.<sup>13–15</sup>

## Renal morphology, immunohistochemistry and immunofluorescence

The renal morphology and immunostaining (immunohistochemistry and immunofluorescence) were performed as described previously.<sup>14,16</sup> Briefly, the kidney morphology was studied with periodic-acid Schiff (PAS) and Masson's trichrome staining. The antibodies were used for immunohistochemistry and immunofluorescence including: anti-AQP1 (Santa Cruz Biotechnology, Santa Cruz, CA, USA); anti-nuclear factor-erythroid 2p45 (NF-E2) related factor-2 (Nrf2) and anti-kelch-like ECH-associated protein 1 (Keap1) antibodies (Abcam, Cambridge, MA, USA); anti-heme oxygenase-1 (HO-1) (Assay Designs, Ann Arbor, MI, USA); anti-catalase (Cat) and anti- $\beta$ -actin antibodies (Sigma-Aldrich, Oakville, ON, Canada); anti-collagen type IV (Chemicon International, Temecula, CA, USA); anti-glycogen synthase kinase 3 $\beta$  (GSK3 $\beta$ ) (27C10) and anti-phospho-GSK3 $\beta$  (Ser 9) (pGSK S9, an inactive form) (5B3) as well as anti-histone H3 (3H1) antibodies (Cell Signaling, Boston, MA, USA); anti-TGF $\beta$ 1 and anti- $\beta$ -catenin (total) antibodies (R&D Systems, Inc., Burlington, ON, Canada); anti-GSK3 $\beta$  (pY216, an active form) (BD Transduction Laboratories, Mississauga, ON, Canada); anti-phospho- $\beta$ -catenin (Ser33/37/Thr41) (Cell Signaling, ON, Canada); anti-phospho- $\beta$ -catenin (Ser552) (Thermo Fisher Scientific, Rockford, IL, USA). The sodium-hydrogen exchanger 3 (NHE3) antibody was a gift from Dr Orson Moe (University of Texas Southwestern Medical Center, Dallas, TX, USA). A rabbit polyclonal antibody against rAgt was generated in our laboratory<sup>17</sup> and is specific for intact rat and mouse angiotensinogen (55–62 kDa) and does not cross-react with pituitary hormone preparations or other rat or mouse plasma proteins, as described elsewhere.<sup>13–15</sup>

## Rat immortalised renal proximal tubular cells

The IRPTC cell line<sup>11,18</sup> and an IRPTC stable clone that has been stably transfected with the control plasmid pRC/RSV (designated as 'pRSV-IRPTC') or with a plasmid pRC/RSV containing the rat angiotensinogen cDNA (designated as 'pRSV/rAgt-IRPTC')<sup>13,19</sup> were employed for our *in vitro* studies. We have previously reported that as compared to naive IRPTC and pRSV-IRPTC, pRSV/rAgt-IRPTC express significantly high amounts of rat angiotensinogen mRNA and protein as well as significantly higher amounts of Ang II secreted into the culture medium.<sup>13,19</sup> All *in vitro* studies were performed in the normal 150 nM NaCl final concentration with an osmolarity of 415 mOsm/kg in normal glucose (5 mM D-glucose) DMEM as reported by Bouley et al.<sup>12</sup>

Nuclear protein and cytosolic protein extracts were prepared using the NE-PER nuclear and cytoplasmic extraction kit (Thermo Scientific, Burlington, ON, Canada).<sup>18</sup>

Cobalt protoporphyrin (CoPP, an activator of HO-1 expression) was purchased from Sigma-Aldrich Canada (Oakville, ON, Canada).

## Statistical analysis

Statistical significance between the experimental groups was analysed by one-way analysis of variance, followed by the Bonferroni test using Graphpad Software, Prism 5.0 (La Jolla, CA, USA, <http://www.graphpad.com/prism/Prism.htm>). A probability level of  $P \leq 0.05$  was considered to be statistically significant and was followed by a Bonferroni analysis with adjustment for multiple comparisons.<sup>13–15</sup>

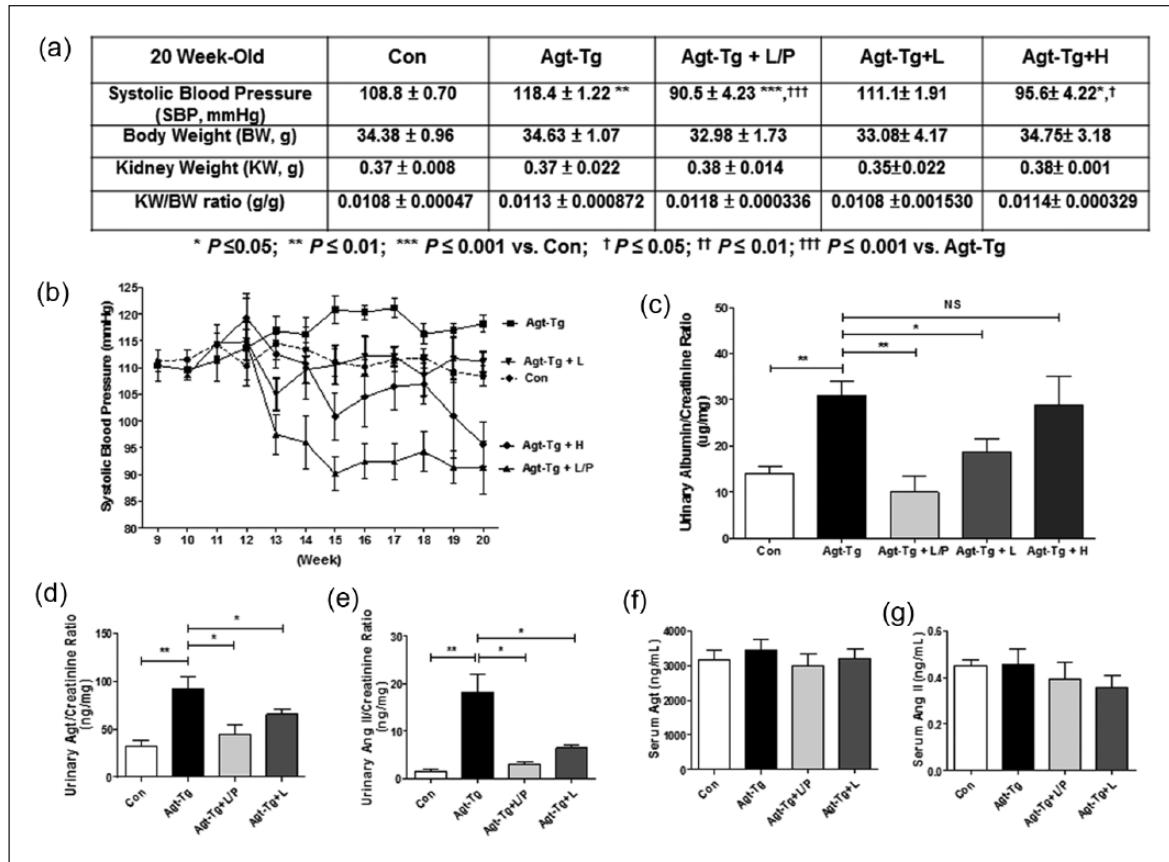
## Results

### Physiological parameters

We measured biological parameters in five subgroups of animals at the age of 20 weeks – non-transgenic littermates as controls (Con,  $N=12$ ); Agt-Tg ( $N=14$ ); Agt-Tg + RAS blockade (losartan and perindopril treatment, Agt-Tg + L/P,  $N=15$ ); Agt-Tg + losartan (Agt-Tg + L,  $N=14$ ) and Agt-Tg + hydralazine (Agt-Tg + H,  $N=8$ ) as shown in Figure 1. There were no significant differences in BW, kidney weight and the kidney weight/BW ratio (Figure 1(a)) among the five groups. However, as compared to the control group, SBP (Figure 1(a), a cross-sectional measurement at week 20; Figure 1(b), a longitudinal measurement (weeks 13–20)), urinary albumin/creatinine ratio (ACR,  $\mu\text{g}/\text{mg}$ ) (Figure 1(c)), angiotensinogen/creatinine ratio (ng/mg) (Figure 1(d)) and Ang II/creatinine ratio (ng/mg) (Figure 1(e)) were relatively increased in Agt-Tg mice; these changes were prevented by the treatment of losartan alone and/or dual RAS blockade in Agt-Tg mice. It appears that dual RAS blockade was more effective than losartan alone in decreasing the urinary ACR (Figure 1(c)). In contrast, although hydralazine treatment was able to decrease SBP in Agt-Tg mice over the follow-up period (Figure 1(a) and (b)), the urinary ACR was unchanged (Figure 1(c)). The serum level of angiotensinogen (Figure 1(e)) or Ang II (Figure 1(f)) did not differ between the groups.

### Renal morphology and extracellular matrix protein

Both PAS staining (Figure 2(a)) and Masson's trichrome staining (Figure 2(b)) of kidney sections revealed enhanced extracellular matrix (ECM) protein accumulation in the glomerulo-tubular areas in hypertensive Agt-Tg mice, a finding that was confirmed by collagen type IV (Figure 2(c)) and TGF $\beta$ 1-immunohistochemistry staining (Figure 2(d)). The degree of oxidative stress was confirmed by lower catalase expression in kidneys of hypertensive



**Figure 1.** Physiological measurements. (a) Biological parameters in five groups of mice (Con, Agt-Tg, Agt-Tg + L/P, Agt-Tg + L, and Agt-Tg + H) at 20 weeks old. \* $P \leq 0.05$ , \*\* $P \leq 0.01$ , \*\*\* $P \leq 0.001$  vs. Con; † $P \leq 0.05$ , †† $P \leq 0.01$ , ††† $P \leq 0.001$  vs. Agt-Tg. (b) Longitudinal SBP (mmHg) measurement in five groups of mice from age 9 to 20 weeks. (c) Urinary albumin/creatinine ratio (ACR,  $\mu\text{g}/\text{mg}$ ) in five groups of mice. (d) Urinary angiotensinogen/creatinine ratio (ng/mg). (e) Urinary Ang II/creatinine ratio (ng/mg). (f) Serum level of angiotensinogen (ng/ml). (g) Serum level of Ang II (ng/ml). \* $P \leq 0.05$ , \*\* $P \leq 0.01$ , \*\*\* $P \leq 0.001$  vs. Con; NS: non-significant.

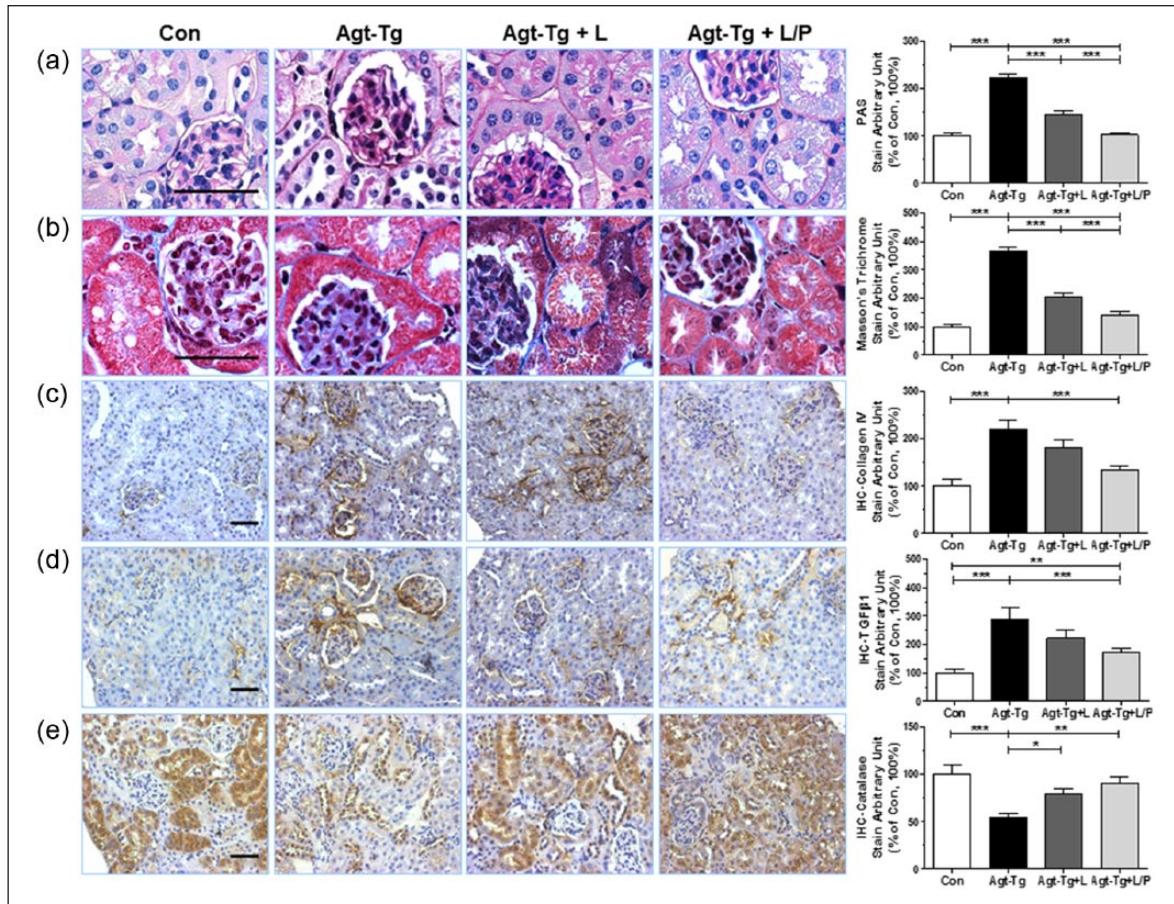
angiotensinogen mice (Figure 2(e)). Semi-quantitative analysis revealed that dual RAS blockade was more effective in preventing ECM accumulation and collagen type IV/TGF- $\beta$ 1 expression as well as in normalising catalase expression in Agt-Tg mice as compared to losartan treatment alone. Given the greater effectiveness of dual blockade, the remainder of our mechanistic experiments were done with Agt-Tg mice treated with dual RAS blockade.

### Renal angiotensinogen, AQP1 and HO-1 protein expression

We assessed angiotensinogen, AQP1 and HO-1 protein expression in the renal cortex by immunohistochemistry (Figure 3(a)) and in isolated RPTs by western blot (WB) (Figure 3(b)). AQP1 shows a two-band WB pattern (glycosylated (38 kDa) and non-glycosylated AQP1 fractions (28 kDa)), matching its original described character as an N-proteoglycan.<sup>20</sup> The functional significance of AQP1 glycosylation is unknown but it could play a role in AQP1 oligomerisation,<sup>21</sup> removal of sugars from the

AQP1 molecule seems not to influence AQP1 water transport function.<sup>22</sup> Thus, in the current study, we evaluated the change of total AQP1 including both glycosylated and non-glycosylated AQP1. Compared to non-transgenic control littermates, increased angiotensinogen, but decreased AQP1 and HO-1 protein expression were observed in RPTs of Agt-Tg mice and these changes were normalised with dual RAS blockade. These data indicate an inverse relationship between angiotensinogen expression and AQP1 and HO-1 expression in RPTs of Agt-Tg mice.

To establish a functional relationship among angiotensinogen, AQP1 and HO-1 expression, we performed in vitro studies by using IRPTCs.<sup>11,18</sup> In the presence of CoPP, an activator of HO-1, both HO-1 and AQP1 protein expressions were increased (Figure 3(c)) while the angiotensinogen protein expression was reduced (Figure 3(d)) in a dose-dependent manner. Furthermore, these effects seem selective because CoPP did not affect Nrf2 expression (Figure 3(e)). We further confirmed those results in naive IRPTCs and IRPTCs transiently



**Figure 2.** Renal morphology and immunohistochemistry. (a) PAS staining (magnification, 600 $\times$ ); (b) Masson's trichrome staining (magnification, 600 $\times$ ); (c) IHC-collagen type IV (magnification, 200 $\times$ ); (d) IHC-TGF $\beta$ 1 (magnification, 200 $\times$ ); and (e) IHC-catalase (magnification, 200 $\times$ ), in four groups of mice (Con, Agt-Tg, Agt-Tg + L and Agt-Tg + L/P) at 20 weeks old. Scale bar 50  $\mu$ m. \* $P=0.05$ , \*\* $P\leq 0.01$ , \*\*\* $P\leq 0.001$  vs. Con; IHC: immunohistochemistry; NS: non-significant.

transfected with rat angiotensinogen cDNA followed by the stimulation of 2  $\mu$ M CoPP (Figure 3(f)).

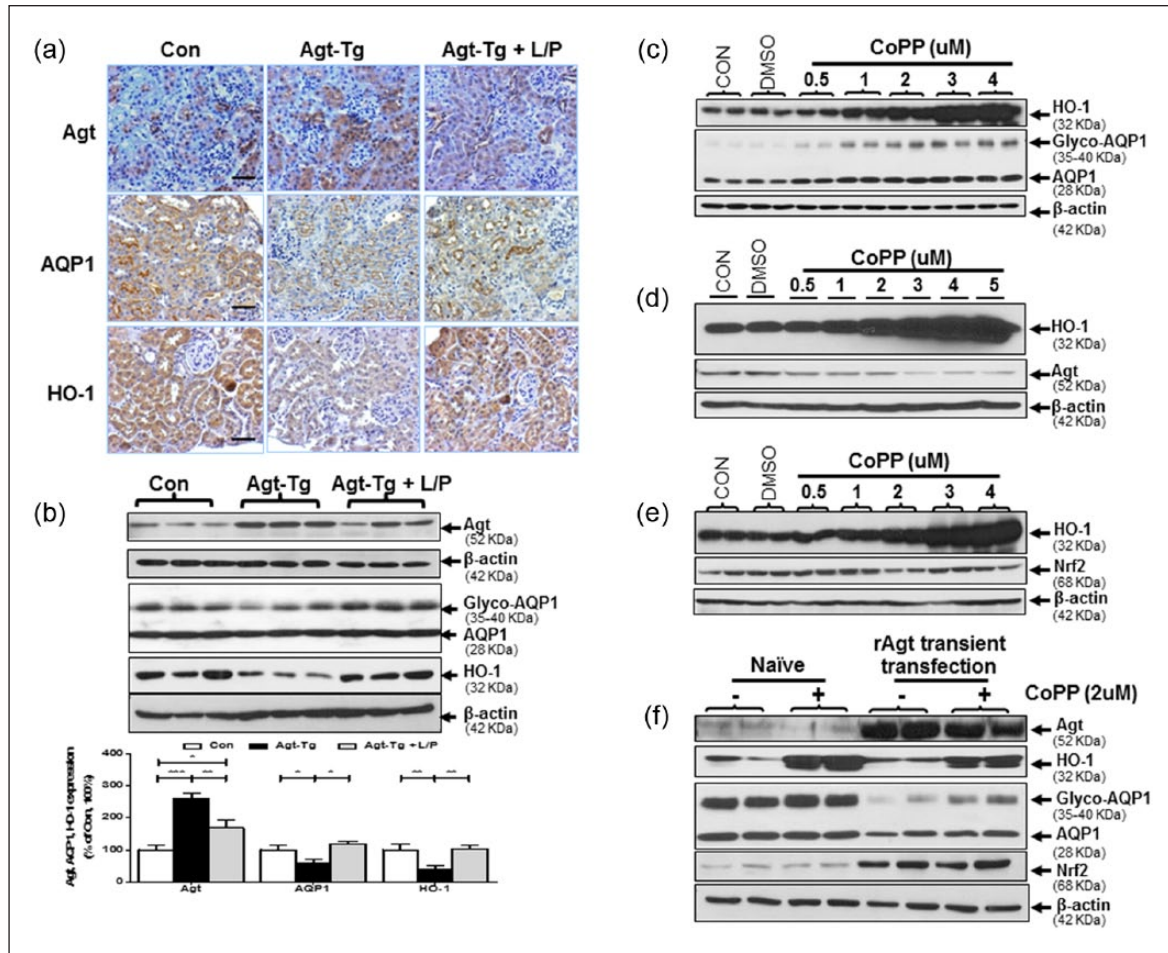
### Renal angiotensinogen, AQP1 and Nrf2-Keap1 expression

As compared to controls, there was augmented Nrf2 protein expression in kidneys of both Agt-Tg and Agt-Tg mice treated with dual RAS blockade (Figure 4(a)), and that expression pattern was further confirmed in the fresh isolated RPTs by WB (Figure 4(b)). However, higher magnification of immunohistochemistry staining revealed that the augmented Nrf2 was mostly localised to the cytosolic portion in RPTCs of Agt-Tg mice with some Nrf2 staining in the nuclei of RPTCs (Figure 4(a)). In contrast, in the kidneys of Agt-Tg mice treated with dual RAS blockade, the majority of positive immunohistochemistry-Nrf2 was localised in the nuclei of RPTCs (Figure 4(a)). Keap1, a protein involved in Nrf2 degradation showed no change in expression in the kidneys among three groups by either immunohistochemistry staining (Figure 4(a)) or WB (Figure 4(b)).

Next, we validated the renal Nrf2 translocation pattern in our pRSV/rAgt-IRPTC stable transformants.<sup>13,19</sup> As compared with naive IRPTC and pRSV-IRPTC control transformants, the pRSV/rAgt-IRPTC stable transformants expressed high amounts of rat angiotensinogen and Nrf2 protein without any change in Keap1 protein expression (Figure 5(a)). Also, AQP1 expression was dramatically suppressed in the pRSV/rAgt-IRPTC stable clone (Figure 5(a)). Similar to the in vivo observation, the higher and lower Nrf2 expression was observed in the cytosolic fraction and nuclear fraction of pRSV/rAgt-IRPTC stable transformants, respectively, as compared to pRSV-IRPTC controls (Figure 5(b)). Moreover, the lower AQP1 expression pattern in the pRSV/rAgt-IRPTC stable clone was further confirmed by immunofluorescence-AQP1 staining (Figure 5(c)).

### Renal angiotensinogen and phosphorylation of GSK3 $\beta$ and $\beta$ -catenin

Since studies have reported that phosphorylated (p)GSK S9 increases Nrf2 nuclear translocation whereas pGSK



**Figure 3.** Angiotensinogen, HO-1 and AQP1 expression in vivo and in vitro. (a, b) Angiotensinogen, AQP1 and HO-1 protein expression in three groups of mice (Con, Agt-Tg and Agt-Tg + L/P) at 20 weeks old. (a) IHC staining (magnification 200×). Scale bar 50 μm. (b) Western blot (WB) in the isolated RPTCs. The relative densities of angiotensinogen, AQP1 and HO-1 were compared with their own β-actin. Control values were considered as 100%. Each point represents the mean ± SEM of three independent experiments. \*\*P ≤ 0.01 vs. Con. (c–f) Cobalt protoporphyrin (CoPP) effect analysed by WB in vitro; (c) CoPP dose-dependent effect on HO-1 and AQP1 protein expression in naive IRPTCs; (d) CoPP dose-dependent effect on HO-1 and angiotensinogen protein expression in naive IRPTCs; (e) CoPP dose-dependent effect on HO-1 and total Nrf2 protein expression in naive IRPTCs; (f) CoPP (2 μM) effect on naive IRPTCs and IRPTCs transient transfection of rat angiotensinogen cDNA.

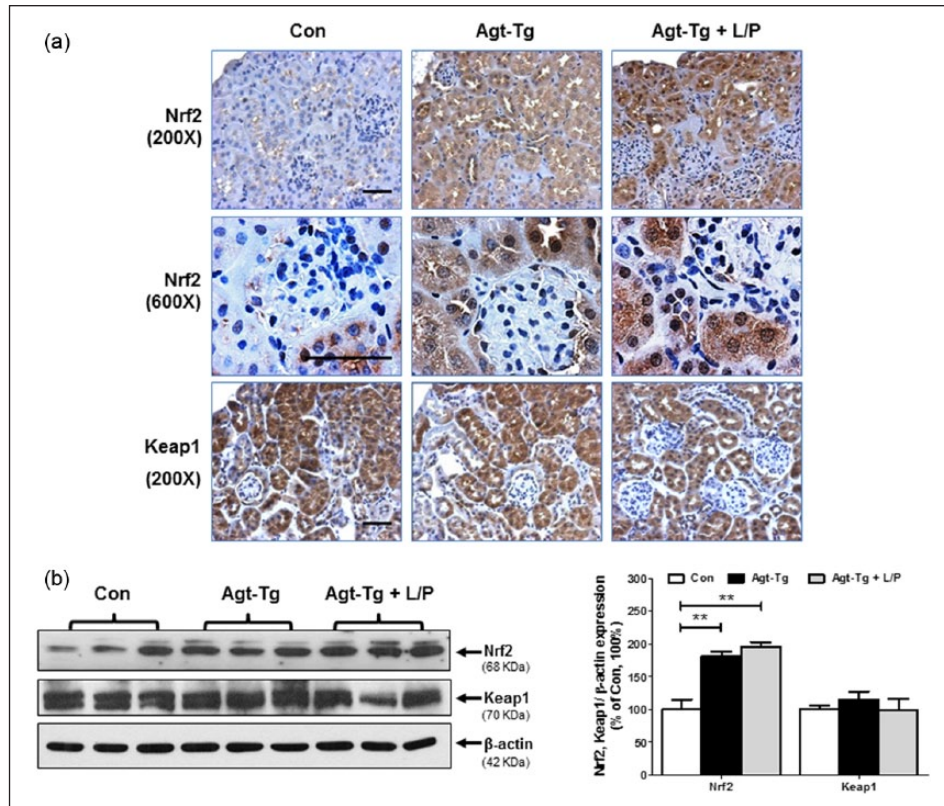
Y216 enhances Nrf2 nuclear export, we investigated the expression of pGSK S9 and pGSK Y216 in vivo and in vitro. As compared to control animals, the expression of pGSK S9 was decreased whereas pGSK Y216 expression was increased in the kidneys of Agt-Tg mice, and dual RAS blockade treatment reversed these changes in Agt-Tg mice (Figure 5(d)). The expression pattern of pGSK S9 and pGSK Y216 was further confirmed by WB in the fresh isolated RPTs (Figure 5(e)). The similar expression pattern of pGSK S9 and pGSK Y216 was also observed in our pRSV-IRPTCs and pRSV/rAgt-IRPTC stable transformants (Figure 6(a)).

Studies indicate that both Ang II and AQP1 can interact with the GSK3β and β-catenin pathways to trigger renal injury. Thus, we studied these interactions in vitro. Our data indicated that the phosphorylation of β-catenin

(Ser33/37/Thr41 and Ser552) was significantly inhibited in pRSV/rAgt-IRPTC stable transformants as compared to naive IRPTC and/or pRSV-IRPTC control transformants (Figure 6(a)).

### Renal angiotensinogen and NHE3 expression

Co-immunofluorescence staining of AQP1 and NHE3 revealed that Agt-Tg mice expressed less AQP1 protein and augmented NHE3 protein in their RPTCs as compared to control littermates and that the treatment with dual RAS blockade reversed these changes (Figure 6(b)). These observations were further confirmed by WB for AQP1 and NHE3 in isolated RPTs of these mice (Figure 6(c)). The increased NHE3 expression was also



**Figure 4.** Renal Nrf2-Keap1 expression in vivo. (a) Immunohistochemistry staining (magnification 200 $\times$  and 600 $\times$ ). Scale bar 50  $\mu$ m. (b) Western blot in the isolated RPTCs. Con (white bar); Agt-Tg (black bar); Agt-Tg+L/P (shadow bar); the relative densities of Nrf2 and Keap1 were compared with their own  $\beta$ -actin. Control values were considered as 100%. Each point represents the mean  $\pm$  SEM of three independent experiments. \*\*,\*\*\* $P$  $\leq$ 0.01 vs. Con.

confirmed in pRSV/rAgt-IRPTC stable transformants (Figure 6(d)).

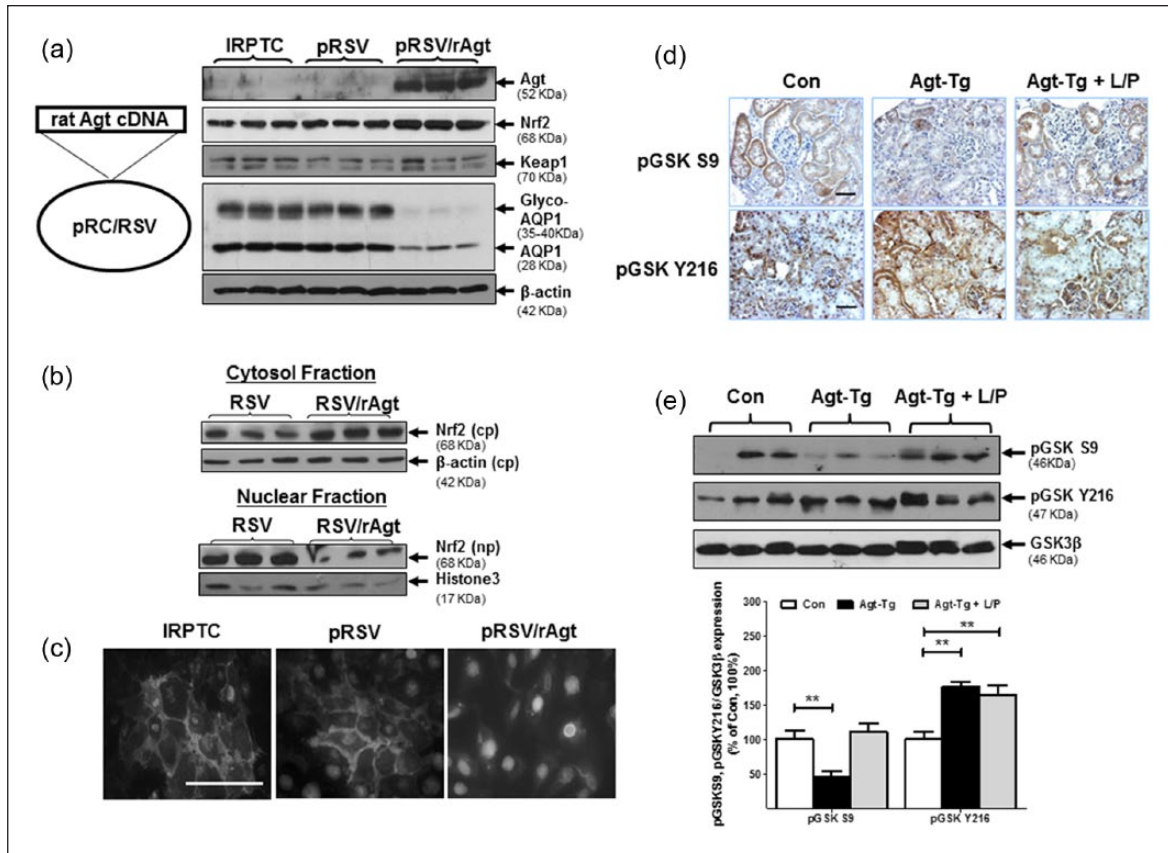
## Discussion

The present report identifies novel mechanism(s) by which angiotensinogen overexpression inhibits AQP1 expression in RPTCs, resulting in renal injury and hypertension (see our concept of a molecular model in Figure 7). In brief, angiotensinogen overexpression in RPTCs enhances cytosolic accumulation of Nrf2 via the phosphorylation of pGSK3 $\beta$  Y216. Consequently, less intranuclear Nrf2 is available to trigger HO-1 expression as a defence mechanism. As a result, AQP1 expression in RPTCs is subsequently diminished. The depleted AQP1 expression through  $\beta$ -catenin-dependent signalling further contributes to hypertension that involves the intrarenal RAS (via NHE3) and nephropathy.

In this study, we are focusing on the functional interaction between angiotensinogen and AQP1 in RPTCs, given the fact that *Aqp1*-null mice appear not to develop homeostasis disturbances although they have slight dehydration.<sup>3</sup> In concert with our previous findings,<sup>13–15</sup> we observed that Agt-Tg mice specifically overexpressing rat

angiotensinogen in their RPTCs developed hypertension and nephropathy. As we only detected a significantly increased urinary Agt/Cre ratio and Ang II/Cre ratio in Agt-Tg mice, while serum levels of angiotensinogen and Ang II remained unchanged, it suggests that angiotensinogen derived predominantly from RPTCs rather than other sources<sup>23,24</sup> plays the key role in this phenomenon.

The use of combination treatment with an ACE inhibitor and angiotensin-receptor blocker to ameliorate the progression of kidney disease has been controversial because of concern about an increased risk of hyperkalaemia or acute kidney injury.<sup>25,26</sup> However, a recent meta-analysis published in the *Lancet*<sup>27</sup> reported a benefit of dual RAS blockade in the prevention of chronic kidney disease with or without diabetes. Our current data lend support to these observations. We found that as compared to the treatment with losartan alone, dual RAS blockade (losartan and perindopril) was more effective in preventing hypertension induced by activation of the intrarenal RAS and nephropathy progression in Agt-Tg mice. Moreover, although hydralazine decreased systemic hypertension in Agt-Tg mice over the follow-up period, it had no impact on ACR (a marker of renal function), suggesting that intrarenal RAS activation contributed to the development of



**Figure 5.** The phosphorylation of GSK3β expression in vivo and in vitro. (a) Angiotensinogen, AQP1 and Nrf2/Keap1 protein expression in IRPTC stable transformants analysed by western blot (WB); (b) Nrf2 translocation in the isolated cytosol and nuclear fraction analysed by WB; (c) immunofluorescence-AQP1 (magnification 200×); (d, e) phosphorylation of GSK3β in the kidneys of three groups of mice (Con, Agt-Tg and Agt-Tg + L/P) at 20 weeks old; (d) immunohistochemistry staining (magnification 200×), scale bar 50 μm; (e) WB in the isolated RPTCs. The relative densities of pGSK3β S9 and pGSK3β Y216 were compared with total GSK3β. Control values were considered as 100%. Each point represents the mean ± SEM of three independent experiments. \*\* $P \leq 0.01$  vs. Con.

nephropathy independent of systemic hypertension (and possibly associated with elevated ROS production in RPTCs in Agt-Tg mice, as reported previously).<sup>14</sup>

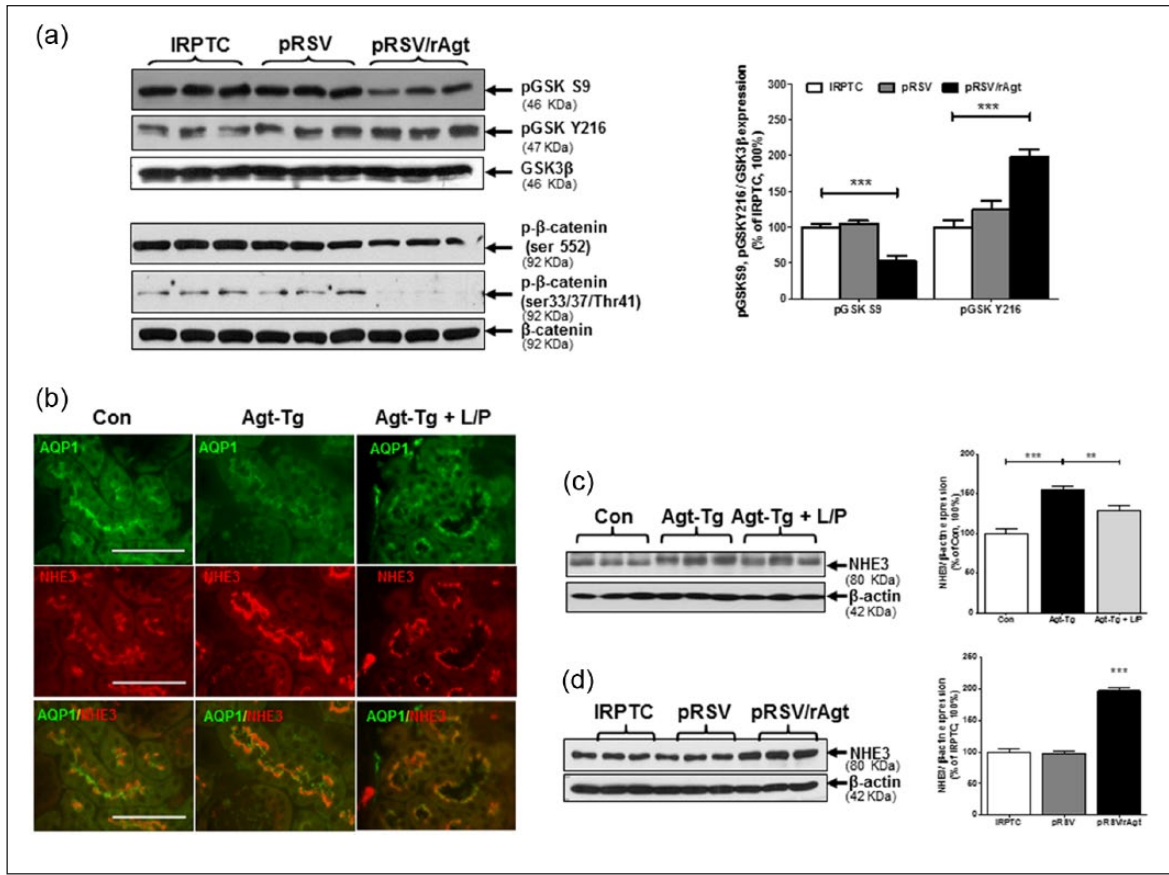
Both angiotensinogen and AQP1, which are mainly expressed in RPTCs, are important for maintaining normal fluid homeostasis; however, how they interact has not been fully delineated; whether their interaction has a regulatory role in the development of hypertension and nephropathy remains elusive. Notably, substantial inhibition of AQP1 and HO-1 protein expression in the RPTCs was observed in the kidney of Agt-Tg mice, implicating their possible role in the pathogenesis of hypertension and nephropathy. This possibility is supported by the observation that significantly decreased renal AQP1 content was observed in the obstructed kidneys of rats with unilateral ureteral obstruction, suggesting that downregulation of AQP1 might be associated with tubule-interstitial fibrosis.<sup>9</sup> Moreover, in the mouse model of hypertension induced by a high-salt diet, the reduction of cardiac AQP1 might be associated with hypertension and cardiac injury, because

angiotensin-receptor blocker treatment (valsartan) partially reversed the effects of a high-salt diet on hypertension with cardiac damage (fibrosis and inflammatory cell infiltration) and normalised cardiac AQP1 expression.<sup>10</sup>

Our in vitro studies demonstrated that CoPP, an activator of HO-1, dose-dependently stimulates HO-1 and AQP1 and inhibits angiotensinogen protein expression in IRPTCs, suggesting an inverse relationship between the expression of angiotensinogen and HO-1/AQP1 in RPTCs. How HO-1 and AQP1 interact is not fully understood. A possible link between AQP1 and HO-1 might be via the Kruppel-like protein, since the AQP1 promoter contains Kruppel-like sequences,<sup>28</sup> and Kruppel-like factor 2 dependently induced HO-1 expression.<sup>29</sup>

HO-1 is a stress-inducible protein that induces cellular protection in the event of injury, inflammation, oxidative stress, etc. Exogenous induction of HO-1 has been shown to have renal and/or cardiovascular protective functions<sup>30,31</sup> and to attenuate the development of hypertension and to decrease blood pressure in models of established



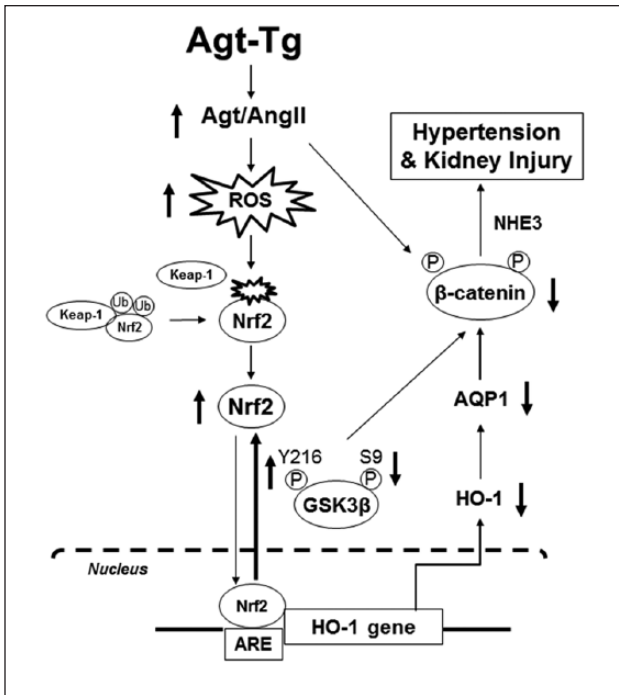


**Figure 6.** The phosphorylation of  $\beta$ -catenin and NHE3 expression in vivo and in vitro. (a) Phosphorylation of GSK3 $\beta$  and  $\beta$ -catenin in IRPTC and IRPTCs stable transformants analysed by western blot (WB). The relative densities of pGSK3 $\beta$  S9 and pGSK3 $\beta$  Y216 were compared with total GSK3 $\beta$ . The values in naive IRPTC cells were considered as 100%. Each point represents the mean  $\pm$  SEM of three independent experiments. \*\*\* $P$  < 0.001 vs. naive IRPTC. (b) Co-localisation of immunofluorescence (IF)-AQP1 and IF-NHE3 (magnification 200 $\times$ ) in three groups of mice (Con, Agt-Tg and Agt-Tg + L/P) at 20 weeks old. Scale bar 50  $\mu$ m. (c) WB in the isolated RPTCs in three groups of mice (Con, Agt-Tg and Agt-Tg + L/P) at 20 weeks old. The relative density of NHE3 was compared with its own  $\beta$ -actin. Control value was considered as 100%. Each point represents the mean  $\pm$  SEM of three independent experiments. \* $P$  < 0.05 vs. Con. (d) NHE3 protein expression in IRPTC stable transformants analysed by WB. The relative density of NHE3 was compared with its own  $\beta$ -actin. The value in naive IRPTC cells was considered as 100%. Each point represents the mean  $\pm$  SEM of three independent experiments. \*\*\* $P$  < 0.001 vs. naive IRPTCs.

hypertension.<sup>30,31</sup> HO-1 expression is modulated by Nrf2, a transcription factor that is highly expressed in the kidney.<sup>32,33</sup> It is thought that the Nrf2/Keap1-HO-1 defence system is renoprotective and that its induction might even improve kidney function.<sup>32,33</sup> Thus, we tested the intrarenal expression pattern of Nrf2/Keap1 in our three groups of animals.

By means of elevated ROS generation, Agt-Tg mice displayed augmented RPTC Nrf2 accumulation, primarily in the cytosol, with less nuclear staining, and this Nrf2 translocation pattern was further confirmed in our pRSV/rAgt-IRPTC stable transformants. These data suggested that while overexpression of angiotensinogen in RPTCs resulted in activated Nrf2 expression, it still failed to promote sufficient HO-1 and AQP1 expression in RPTCs to prevent or diminish ROS-induced kidney damage and hypertension occurring in Agt-Tg mice. Compelling

studies suggested that Nrf2 accumulation/activation is countered by two major Nrf2 degradation mechanisms – e.g. Keap1-induced Nrf2 proteasomal degradation in the cytosol; and/or GSK3 $\beta$ -mediated nuclear export and degradation of Nrf2.<sup>32–36</sup> As renal Keap1 expression did not differ among the three groups of animals, Keap1-associated Nrf2 degradation appears to be normal in our model. In contrast, the activated GSK3 $\beta$  (i.e. pGSK Y216 phosphorylation)<sup>33,34,37</sup> has been reported to phosphorylate Fyn tyrosine kinase, leading to enhanced nuclear export of Nrf2 and proteasomal degradation, probably via the adaptor protein  $\beta$ -TrCP independent of Keap1.<sup>33,34,38,39</sup> By conducting both in vivo and in vitro experiments, we observed that overexpression of angiotensinogen in RPTCs indeed promoted and inhibited the phosphorylation of pGSK Y216 (active form) and pGSK S9 (inactive form), respectively. This suggests that the enhanced nuclear export of



**Figure 7.** Our working model. In brief, overexpression of the intrarenal angiotensinogen gene in RPTCs via elevated ROS generation mediates Nrf2 activation with an impaired Nrf2 translocation pattern. Angiotensinogen overexpression in RPTCs promotes and inhibits the phosphorylation of pGSK Y216 (active form) and pGSK S9 (inactive form), respectively. Consequently, nuclear export of Nrf2 activity is enhanced, resulting in the accumulation of Nrf2 in the cytosol, and decreased Nrf2 expression in nuclei, which fails to trigger HO-1 expression as a defence mechanism and subsequently diminishes AQP1 expression in RPTCs. Concomitantly, the depleted AQP1 expression through  $\beta$ -catenin-dependent signalling further contributes to intrarenal RAS-induced nephropathy and hypertension (via NHE3).

Nrf2 was associated with an accumulation of Nrf2 in the cytosol, while lower nuclear levels of Nrf2 failed to trigger HO-1/AQP1 induction-mediated renoprotection in RPTCs in Agt-Tg mice.

Evidence suggests that  $\beta$ -catenin might be one of mediators that links AQP1 and Ang II functionally.<sup>40–42</sup> For example, AQP1 acts as a scaffold in interaction with GSK3 $\beta$  to promote  $\beta$ -catenin degradation by increasing  $\beta$ -catenin phosphorylation; vice versa, loss of AQP1 inhibits  $\beta$ -catenin degradation and facilitates the translocation of free  $\beta$ -catenin to the nucleus to enhance Wnt signalling, consequently triggering cystic dilation of RPTs in polycystic kidney disease.<sup>42</sup> In addition, Ang II via AT1R appears to promote the accumulation of  $\beta$ -catenin protein, correlated with GSK3 $\beta$  phosphorylation, contributing to the development of renal fibrosis and hypertension.<sup>40,41</sup> In the current study, we observed the depleted AQP1 in our Agt-Tg and pRSV/rAgt-IRPTC stable transformants activated and inhibited phosphorylation of GSK3 $\beta$  (Y216) and

$\beta$ -catenin (Ser33/37/Thr41 and Ser552), respectively, suggesting that loss of AQP1 might trigger the Wnt/ $\beta$ -catenin pathway, resulting in RPTs damage and hypertension.

Finally, our data both in vivo and in vitro also suggest that overexpression of angiotensinogen in RPTCs and the related hypertension might be due to decreased water absorption via AQP1 and increased sodium reabsorption via NHE3. Indeed, this observation is in line with recent findings – e.g. when compared with wild-type mice (NHE3<sup>+/+</sup>), AQP1 is significantly increased in RPTCs of NHE3 knockout mice (NHE3<sup>-/-</sup>), which completely blunted Ang II-induced hypertension, underscoring the importance of AQP1 and NHE3 interaction.<sup>43</sup> In fact, Ang II-dependent hypertension mediated by an increased NHE3 abundance in RPTCs has been reported in AT1a receptor-deficient mice<sup>44–46</sup> and in oxidative stress-modulated AT1R signalling in Sprague–Dawley rats,<sup>47,48</sup> although other Ang II infusion models have provided variable results<sup>49–51</sup> (NHE3 has been reported as increased<sup>50</sup> or decreased<sup>49</sup> or not changed).<sup>51</sup>

In conclusion, our data suggest that angiotensinogen/Nrf2-mediated downregulation of AQP1 and HO-1 expression in the proximal tubule plays a key role in Ang II-induced hypertension and kidney injury.

#### Author contributions

SYC, CSL, XPZ, MCL and IC performed all experiments and contributed to discussion. RB, JRJ, JSDC and SLZ participated in the interpretation of the results and reviewed/edited the paper. SLZ drafted the manuscript. All authors have read and critically revised the final version of the manuscript.

#### Declaration of conflicting interest

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