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ARTICLE

AT_2R deficiency in mice accelerates podocyte dysfunction in diabetic progeny in a sex-dependent manner

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Abstract

Aims/hypothesis The angiotensin II receptor type 2 (AT_2R) may be a potential therapeutic target for the treatment of hypertension and chronic kidney disease (CKD). The expression and function of AT_2R in the vasculature and kidney appear sexually dimorphic. We hypothesised that *Agtr2* knockout dams (AT_2RKO) with gestational diabetes would program their offspring for subsequent hypertension and CKD in a sex-dependent manner.

Methods Age- and sex-matched offspring of non-diabetic and diabetic dams of wild-type (WT) and AT_2RKO mice were followed from 4 to 20 weeks of age and were monitored for development of hypertension and nephropathy; a mouse podocyte cell line (mPOD) was also studied.

Results Body weight was progressively lower in female compared with male offspring throughout the lifespan. Female but not male offspring from diabetic AT_2RKO dams developed insulin resistance. Compared with the offspring of non-diabetic dams, the progeny of diabetic dams had developed more hypertension and nephropathy (apparent glomerulosclerosis with podocyte loss) at 20 weeks of age; this programming was more pronounced in the offspring of AT_2RKO diabetic dams, particularly female AT_2RKO progeny. Female AT_2RKO offspring had lower basal ACE2 glomerular expression, resulting in podocyte loss. The aberrant ACE2/ACE ratio was far more diminished in glomeruli of female progeny of diabetic AT_2RKO dams than in male progeny. Knock-down of *Agtr2* in mPODs confirmed the in vivo data.

Conclusions/interpretation AT_2R deficiency accelerated kidney programming in female progeny of diabetic dams, possibly due to loss of protective effects of ACE2 expression in the kidney.

Keywords AT_2R · Kidney programming · Maternal diabetes · Sexual dimorphism

Abbreviations

ACR	Albumin/creatinine ratio
Ang II	Angiotensin II
AT_1R	Angiotensin II receptor type 1
AT_2R	Angiotensin II receptor type 2
AT_2RKO	*Agtr2* knockout (mice)
CAKUT	Congenital abnormalities of the kidney and urinary tract
CKD	Chronic kidney disease
DHE	Dihydroethidium
IF	Immunofluorescence
IHC	Immunohistochemistry
IST	Insulin sensitivity test
KO	Knockout
KO-Con (-M/F)	Knockout control offspring from non-diabetic dams (male/female)
KO-Dia (-M/F)	Knockout offspring from diabetic dams (male/female)
KW	Kidney weight
MAS1	Mas1 receptor
mPOD	Mouse podocyte cell line
8-OHdG	8-Hydroxy-2-deoxyguanosine
PAS	Periodic acid Schiff
qPCR	Real-time quantitative PCR

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Research in context

What is already known about this subject?

- Loss of the angiotensin II receptor type 2 (AT_2R) is associated with congenital abnormalities of the kidney and urinary tract in both humans and mice
- AT_2R is a potential therapeutic target for the treatment of hypertension and chronic kidney disease
- The expression and function of AT_2R in the vasculature and kidney appear to be sexually dimorphic

What is the key question?

- What is the role of AT_2R in kidney programming (giving rise to later hypertension and kidney damage) in offspring, mediated by maternal gestational diabetes, and does it occur in a sex-dependent manner?

What are the new findings?

- Loss of AT_2R leads to a higher risk of adverse kidney programming in the progeny of diabetic dams
- Loss of AT_2R promotes adverse kidney programming and has a greater impact on female compared with male offspring
- The mechanism of adverse kidney programming may be through loss of the protective effects of ACE2 expression in the kidney

How might this impact on clinical practice in the foreseeable future?

- Novel therapeutic strategies to prevent AT_2R loss may provide a means to decrease the heightened risks for adverse kidney programming in the offspring of diabetic mothers

RAS	Renin–angiotensin system
SBP	Systolic blood pressure
STZ	Streptozotocin
Synpo	Synaptopodin
TL	Tibia length
WT	Wild-type
WT-Con (-M/F)	Wild-type control offspring from non-diabetic dams (male/female)
WT-Dia (-M/F)	Wild-type offspring from diabetic dams (male/female)
WT1	Wilms tumour 1

Introduction

Diabetes during pregnancy (maternal diabetes) in both humans and experimental animal models, whether gestational or pre-gestational (type 1 diabetes mellitus or type 2 diabetes mellitus) is associated with congenital abnormalities of the kidney and urinary tract (CAKUT) [1–6]. CAKUT occurs in 3–6 per 1000 live births and is responsible for 34–59% of chronic kidney disease (CKD) in childhood and for 31% of paediatric end-stage renal failure in the USA and Canada [7, 8]. It has been estimated that 4% of CAKUT cases may be associated with maternal diabetes [4, 5].

Maternal diabetes also results in offspring with a two- to fivefold greater risk for various debilitating diseases in adulthood, for example diabetes, obesity, cardiovascular disease, hypertension and CKD [3–6]. The dysmetabolic features of maternal diabetes and their impact vary with the sex of the offspring. The gene expression and molecular basis of these variations are poorly understood.

The angiotensin II receptor type 2 (AT_2R) is highly associated with CAKUT in both humans and mice [9–11]. However, the underlying mechanisms that cause kidney malformations related to AT_2R variants remain unclear, particularly with respect to the possible molecular and in utero environmental interactions, such as maternal diabetes, which lead to observed kidney abnormalities [7, 8, 12]. The AT_2R is expressed in multiple kidney compartments and cells [13, 14]. We [15–17] and others [18, 19] have reported aberrant features in the kidney in young *Agtr2* knockout (KO) mice.

The intrarenal renin–angiotensin system (RAS) is a key regulator of blood pressure and fluid/electrolyte homeostasis [20–22]. In particular, the AT_2R via the ACE2–Mas1 receptor (MAS1) pathway counteracts the generally detrimental actions of angiotensin II receptor type 1 (AT_1R) such as vasoconstriction, inflammation and proliferation [20–22]. Importantly, compelling evidence suggests that AT_2R activation is cardio- and renoprotective. Thus, the AT_2R may be a potential therapeutic target for the treatment of hypertension, cardiovascular disease

and CKD [20–22]. Further, the actions of the AT_2R in the vasculature and kidneys appear to be sexually dimorphic [23–26].

Given the rising incidence of diabetes in pregnancy, we speculated that AT_2R deficiency in dams that have diabetes mellitus could result in offspring with heightened risks for hypertension and CKD across their lifespan. Previously, we have reported that loss of AT_2R results in podocyte loss (mean, 30% fewer) without affecting total nephron number [17]. Here, we studied age- and sex-matched offspring of non-diabetic and diabetic dams, hypothesising that AT_2R deficiency resulting in podocyte loss accelerates adverse kidney programming (hypertension and nephropathy) in the progeny of dams with diabetes in a sex-dependent manner, mechanistically mediated by loss of the protective effects of ACE2 expression in the kidney.

Methods

Animal models

Agtr2 KO (AT_2RKO) mice (C57BL/6) [9, 10] were generated and kindly provided by T. Inagami (Vanderbilt University School of Medicine, Nashville, TN, USA) as we reported elsewhere [17, 27]. Our murine maternal diabetes model [28–31], which is induced by a single intraperitoneal streptozotocin (STZ, 150 mg/kg) injection on gestational day E13 is designed to mimic human gestational diabetes with onset late in the second trimester and avoids overt teratogenicity in the offspring [32]. We have documented that STZ administration per se had no toxic effect on kidney development [30] but that the offspring of diabetic dams had a phenotype of low birthweight and low nephron number [28–31]. Diabetic dams with blood glucose over 16.6 mmol/l (299 mg/dl) post STZ injection were included in the present study.

Age- and sex-matched offspring (male, M and female, F) from the different litters of non-diabetic and diabetic dams of wild-type (WT, C57BL/6) (Charles River, Montreal, QC, Canada) and AT_2RKO mice were studied from of 4 weeks until 20 weeks of age. Eight subgroups of mice were included: the offspring of non-diabetic and diabetic WT dams, respectively abbreviated as WT-Con (M/F) and WT-Dia (M/F); and the offspring of non-diabetic and diabetic AT_2RKO dams, as KO-Con (M/F) and KO-Dia (M/F), respectively.

All animals were euthanised at 20 weeks (75 mg/kg sodium pentobarbital i.p.) and the kidneys were removed immediately. The biological samples were processed, collected and stored for analysis. Kidneys were either quickly frozen in optimal cutting temperature (OCT) compound or fixed overnight in 4% paraformaldehyde at 4°C before paraffin-embedding.

Animal care and experimental procedures were approved by the Animal Care Committee at the Centre de recherche du centre hospitalier de l'Université de Montréal (CRCHUM). The animals were housed in ventilated cages in specific pathogen-free (SPF) conditions under a 12 h, light–dark cycle with free access to chow and water at the CRCHUM's animal facility.

Physiological studies

Body weight (g) was measured weekly. Kidney weight (KW, g) and tibia length (TL, mm) were recorded when the animals were euthanised. Longitudinal systolic blood pressure (SBP) (from 14 until 20 weeks of age following 1 week of pre-training) was monitored by the tail-cuff method with a BP-2000 Blood Pressure Analysis System (Visitech Systems, Apex, NC, USA), as reported elsewhere [31, 33–36]. An IPGTT and an insulin sensitivity test (IST) were performed with 6 and 4 h fasting periods, respectively, at the age of 19 weeks [31, 37]. The GFR was measured by the FITC–inulin method [31, 33–36] before euthanising at the age of 20 weeks.

Urinary measurements

Urine samples were collected from mice individually housed in metabolic cages. Urinary albumin level was estimated by Coomassie Blue gel staining of electrophoresed urine samples, as reported previously [17]. In brief, 5 μl urine samples from individual mice were temperature denatured and separated using a 10% SDS-PAGE gel and a mouse albumin standard (mouse serum albumin; Exocell, Philadelphia, PA, USA), then stained with Coomassie Blue.

The albumin/creatinine ratio (ACR) (mg/mmol) (Albuwell and Creatinine Companion, Exocell, Philadelphia, PA, USA), urinary angiotensin II (Ang II)/creatinine ratio (pmol/μmol) (Immuno-Biological Laboratories, IBL America, Minneapolis, MN, USA), and angiotensin 1–7 (Ang 1–7)/creatinine ratio (pmol/μmol) (Immuno-Biological Laboratories, IBL America, Minneapolis, MN, USA), were assayed by ELISA and normalised by urinary creatinine levels as described [34–36].

Kidney morphology and podocyte number

Kidney morphology was assessed with periodic acid Schiff (PAS) and Masson trichrome staining [17, 31, 33–36]. Two major features of nephropathy—glomerulosclerosis (based on PAS images, semi-quantitative scale from 0 to 4) and glomerular fibrosis (based on Masson staining)—were scored by a person blinded to the experimental group [17, 31]. Relative immunohistochemistry (IHC)/immunofluorescence (IF) staining intensity and area were quantified using the 2-D staining images ($n = 8 \sim 15$ per animal, 6–11 mice/group) were analysed and quantified in a blinded fashion [17, 31, 33–36]. Brightness and contrast were adjusted on displayed images

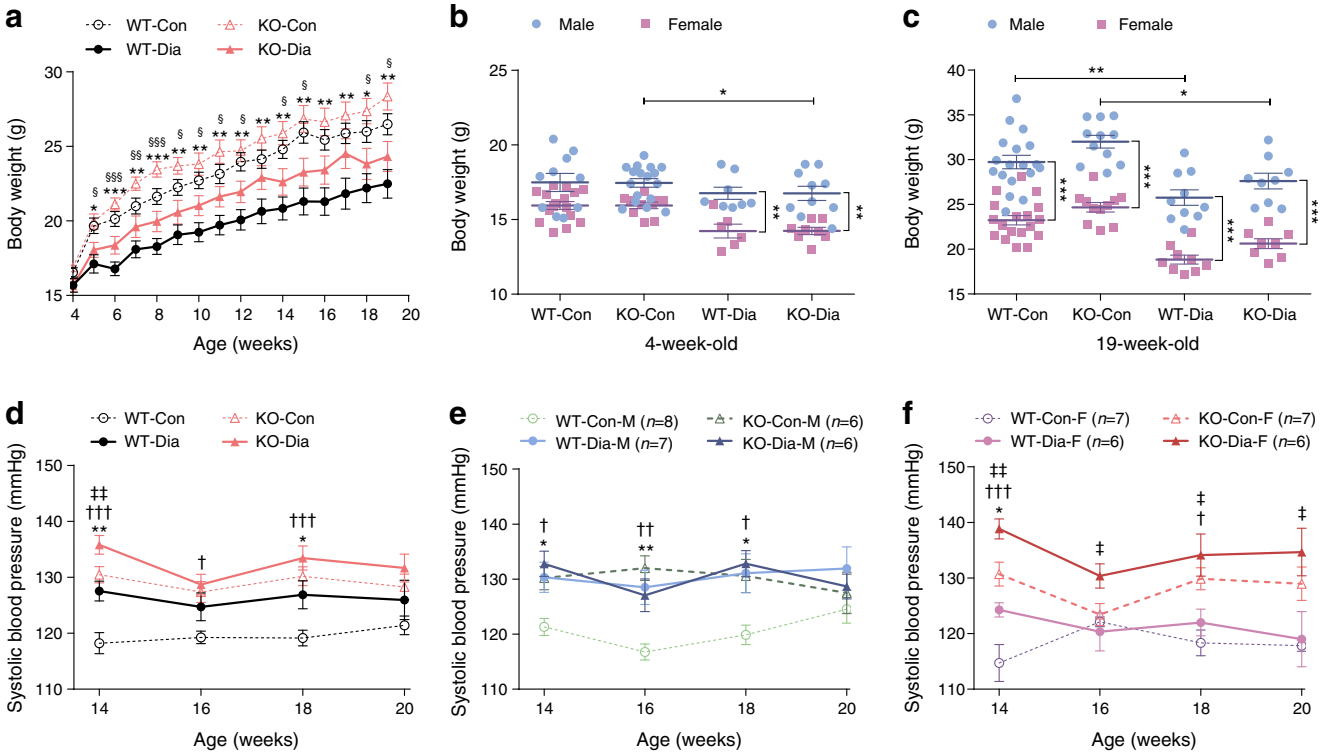


Fig. 1 (**a**) Body weight progression in age-matched offspring 4–19 weeks of age (male + female; WT vs AT_2RKO mice; non-diabetic vs diabetic dams). Data shown as mean ± SEM; one-way ANOVA, followed by Bonferroni's post hoc test. $^{*}p \le 0.05$, $^{**}p \le 0.01$, $^{***}p \le 0.001$, WT-Dia vs WT-Con; $^{\S}p \le 0.05$, $^{\S\S}p \le 0.01$, $^{\S\S\S}p \le 0.001$, KO-Dia vs KO-Con. (**b**, **c**) Body weight in the offspring at 4 (**b**) and 19 (**c**) weeks of age. Data shown as mean ± SEM; one-way ANOVA, followed by Bonferroni's post hoc test in comparison of four subgroups in total (WT-Con, KO-Con, WT-Dia and KO-Dia); two-way ANOVA, followed by Bonferroni's post hoc test in comparison of eight subgroups in a sex-specific manner [WT-Con (−M/F), KO-Con (-M/F), WT-Dia (-M/F) and KO-Dia (-M/F)]. $^{*}p \le 0.05$, $^{**}p \le 0.01$, $^{***}p \le 0.001$. (**d**–**f**) Longitudinal SBP measurement (**d**, in total; **e**, male; **f**, female) in mice 14–20 weeks of age. Data shown as mean ± SEM; one-way ANOVA followed by Bonferroni's post hoc test. $^{*}p \le 0.05$, $^{**}p \le 0.01$, WT-Dia vs WT-Con; $^{\dagger}p \le 0.05$, $^{\dagger\dagger}p \le 0.01$, $^{\dagger\dagger\dagger}p \le 0.001$, WT-Con vs KO-Con; $^{\ddagger}p \le 0.05$, $^{\ddagger\ddagger}p \le 0.01$, WT-Dia vs KO-Dia

(identically for compared image sets) and quantified (identical threshold settings for compared image sets) using NIH Image J software (Bethesda, MD, USA) [17, 31, 38, 39]. Podocytes were identified by IF staining of podocyte markers including synaptopodin (Synpo), Wilms tumour 1 (WT1) and p57 in mouse kidney sections [17]. Podocyte density per glomerular area (number per μm^2) was performed in a blinded fashion by counting both WT1- and p57-positive nuclear stained cells in glomerular-cross sections, 30–35 glomeruli/mouse, $n = 6$) [17, 40].

Real-time quantitative PCR

Real-time quantitative PCR (qPCR) (The Fast SYBR green master mix kit and the 7500 Fast real-time PCR system, Applied Biosystems, Life Technologies, Foster City, CA, USA) was performed in the isolated glomeruli [17] by using iron oxide beads (2.5 mg/ml) with the aid of a magnet concentrator [17, 41]. mRNA change in each gene was determined and normalised to its own *Actb* mRNA, and the percentage change was compared with the expression of the corresponding gene in male WT-Con (1 in fold change) (vs the rest of the subgroups) by using the $2^{-\Delta\Delta C_t}$ method. The primers used are shown in electronic supplementary material (ESM) Table 1.

Immunohistochemical studies and reagents

Both IHC and IF staining were performed using standard protocols [17, 31, 33–36]. The antibodies used included: anti-β-actin antibody (1:20000) (Sigma-Aldrich, Oakville, ON, Canada); anti-Ang II antibody (1:200) (Phoenix Pharmaceuticals, Burlingame, CA, USA); antibodies to AT_1R (1:100), Synpo (P-19) (1:100) and cyclin-dependent kinase inhibitor p57 (H-91) (1:100) (Santa Cruz Biotechnology, Santa Cruz, CA, USA); antibodies to ACE2 (1:400), WT1 (C-19) (1:100); 8-hydroxy-2-deoxyguanosine (8-OHdG) (1:400) (Abcam, Toronto, ON, Canada); anti-ACE antibody (1:200) (Invitrogen, Burlington, ON, Canada); and anti-MAS1 antibody (1:200) (Novus Biologicals, Toronto, ON, Canada).

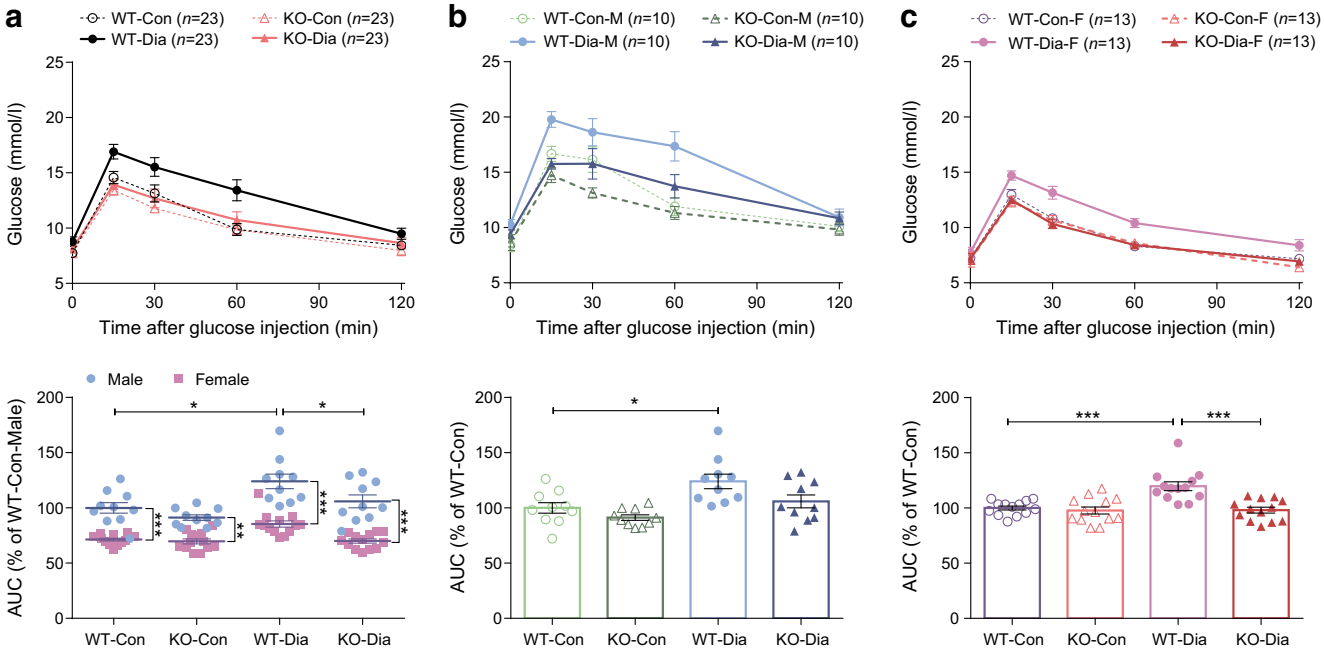


Fig. 2 IPGTT and IPGTT AUC (% of WT-Con) quantification (0–120 min) in age- and sex-matched offspring of non-diabetic and diabetic dams (WT vs AT_2RKO mice) at 19 weeks. (**a**) The offspring in total; data shown as mean ± SEM; one-way ANOVA, followed by Bonferroni's post hoc test in comparison of four subgroups in total (WT-Con, KO-Con, WT-Dia and KO-Dia); two-way ANOVA, followed by Bonferroni's post hoc test in comparison of eight subgroups in a sex-specific manner [WT-Con (-M/F), KO-Con (-M/F), WT-Dia (-M/F) and KO-Dia (-M/F)]. $*p \le 0.05$, $**p \le 0.01$, $***p \le 0.001$. (**b**) Male offspring. (**c**) Female offspring. (**b**, **c**) Data shown as mean ± SEM; one-way ANOVA followed by Bonferroni's post hoc test; $*p \le 0.05$, $***p \le 0.001$

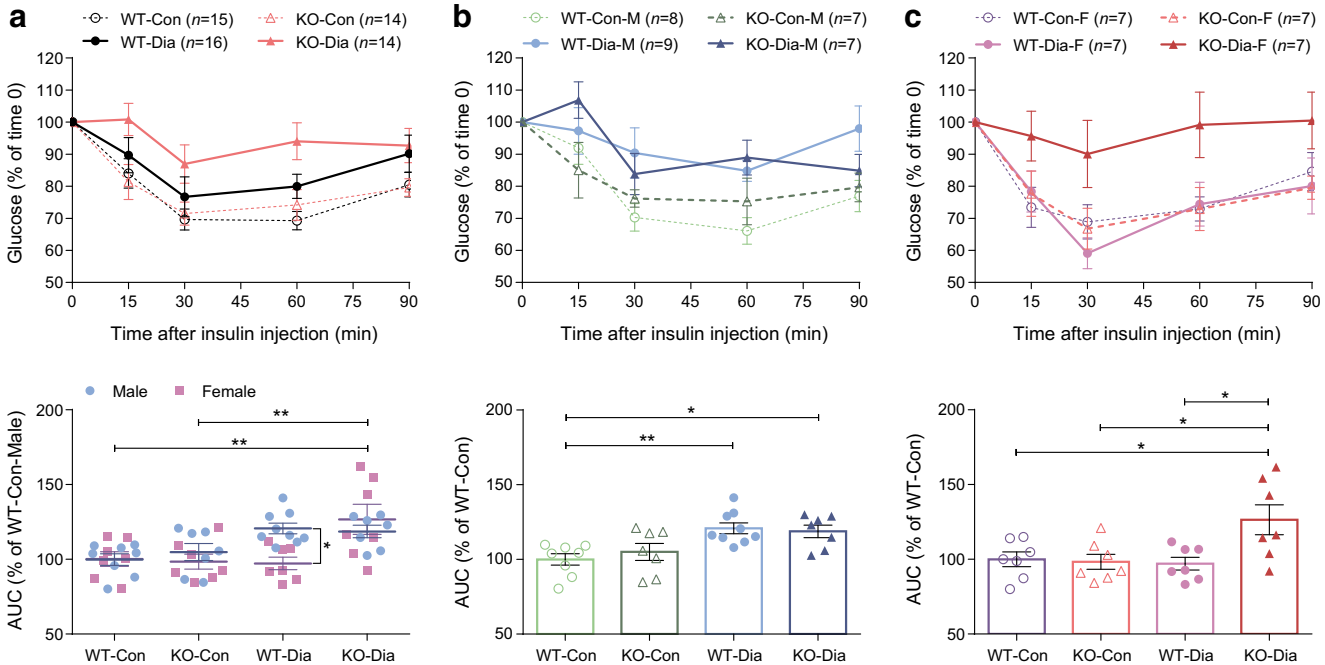


Fig. 3 IST and IST AUC (% of WT-Con) quantification (0–90 min) in age- and sex-matched offspring of non-diabetic and diabetic dams (WT vs AT_2RKO mice) at 19 weeks. (**a**) The offspring in total; data shown as mean ± SEM; one-way ANOVA, followed by Bonferroni's post hoc test in comparison of four subgroups in total (WT-Con, KO-Con, WT-Dia and KO-Dia); two-way ANOVA, followed by Bonferroni's post hoc test in comparison of eight subgroups in a sex-specific manner [WT-Con (-M/F), KO-Con (-M/F), WT-Dia (-M/F) and KO-Dia (-M/F)]. $*p \le 0.05$, $**p \le 0.01$. (**b**) Male offspring. (**c**) Female offspring. (**b**, **c**) Data shown as mean ± SEM; one-way ANOVA followed by Bonferroni's post hoc test; $*p \le 0.05$, $**p \le 0.01$

Podocyte cell line

An immortalised mouse podocyte cell line (mPOD) was kindly donated by S. J. Shankland (University of Washington, WA, USA) [42] as we previously reported [17]. Briefly, mPODs were grown on collagen I-coated plates in a DMEM medium (5 mmol/l glucose) supplemented with 10% FBS (Invitrogen, Burlington, ON, Canada), 100 U/ml penicillin–streptomycin (Invitrogen), and 10 U/ml recombinant mouse IFN-γ (Sigma) at 33°C (defined as a permissive condition). The differentiated mPODs were then cultured under non-permissive conditions; i.e., without IFN-γ at 37°C for 10 days before the specific experiments.

Statistical analysis

For the animal studies, groups of 7 to 18 mice were used (the precise number of animals used for each specific experiment is either labelled in the figures or as individual data points on column scatter graphs). Statistical significance between the experimental groups was analysed by using Prism 6.0 software (GraphPad, San Diego, CA). Statistical significance between the experimental groups was analysed by Student's *t* test (in vitro studies) or one-way ANOVA and/or two-way ANOVA, followed by the Bonferroni's post hoc test (in vivo studies), whenever appropriate. A probability level of $p \leq 0.05$ was considered to be statistically significant [17, 31, 33–37].

Results

Offspring growth curves

We compared the growth pattern of age- and sex-matched offspring of non-diabetic and diabetic dams (WT vs AT_2RKO mice) from the age of 4 to 20 weeks (Fig. 1a, body weight). During follow-up, mean body weight in the age-matched WT vs KO progeny from non-diabetic and diabetic dams did not differ significantly. Compared with control offspring, the offspring from diabetic dams had lower body weight throughout life (Fig. 1a).

The body weight progression in the offspring of both WT and KO dams showed a sexually dimorphic pattern (Fig. 1b,c). At 4 weeks of age (Fig. 1b), both sexes of control offspring (WT and AT_2RKO) had similar body weights. By contrast, females clearly weighed less than male offspring from diabetic dams (mean body weight: WT-Dia-M vs WT-Dia-F, $p < 0.01$; KO-Dia-M vs KO-Con-F, $p < 0.01$).

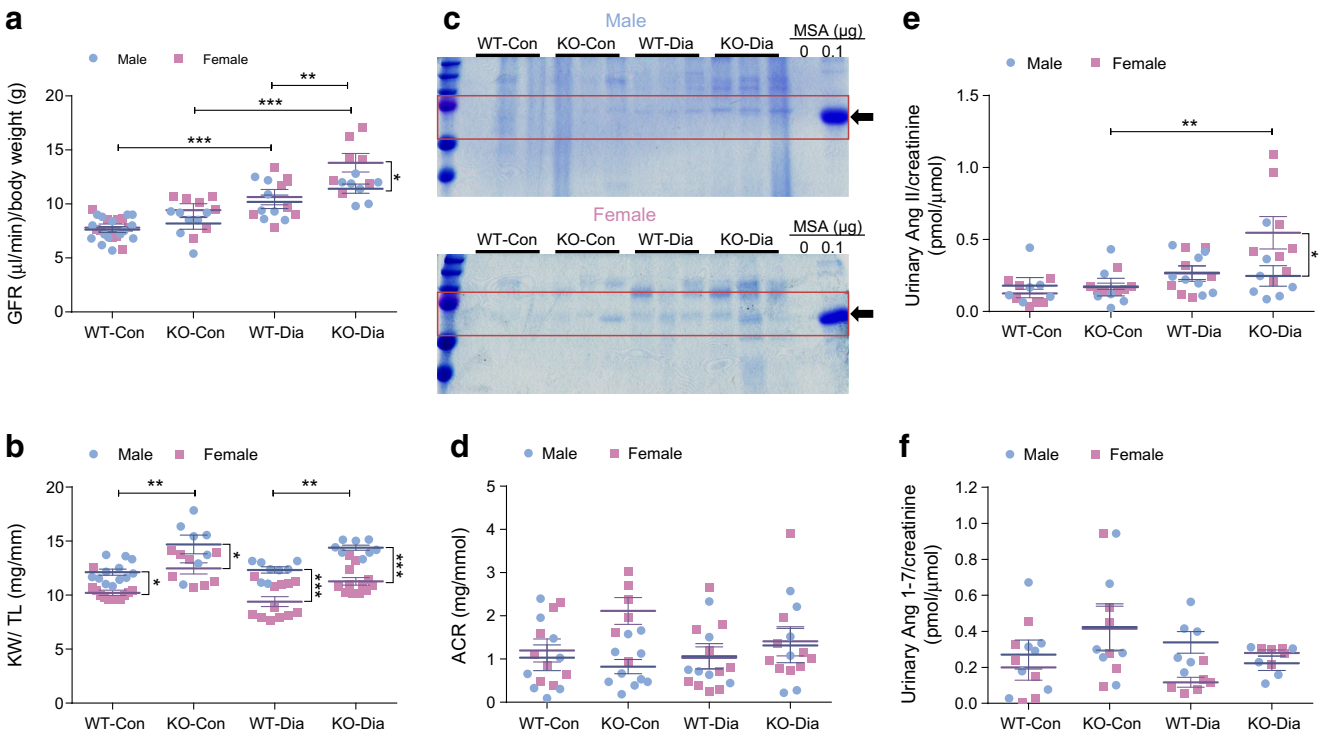


Fig. 4 Physiological measurements (**a**, **b**) and urine measurements (**c**–**f**) in age- and sex-matched offspring of non-diabetic and diabetic dams (WT vs AT_2RKO mice) at 20 weeks. (**a**) GFR measurement. (**b**) KW/TL (mg/mm) ratio. (**c**) Coomassie Blue gel staining. Mouse serum albumin (MSA, µg) was used as a standard. (**d**) Urinary ACR. (**e**) Urinary Ang II/creatinine (pmol/µmol) levels; (**f**) Urinary Ang 1–7/creatinine (pmol/µmol) levels. (**a**, **b**; **d**–**f**) Data shown as mean ± SEM; one-way ANOVA, followed by Bonferroni's post hoc test in comparison of four subgroups in total (WT-Con, KO-Con, WT-Dia and KO-Dia); two-way ANOVA, followed by Bonferroni's post hoc test in comparison of eight subgroups in a sex-specific manner [WT-Con (-M/F), KO-Con (-M/F), WT-Dia (-M/F) and KO-Dia (-M/F)]; *$p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$

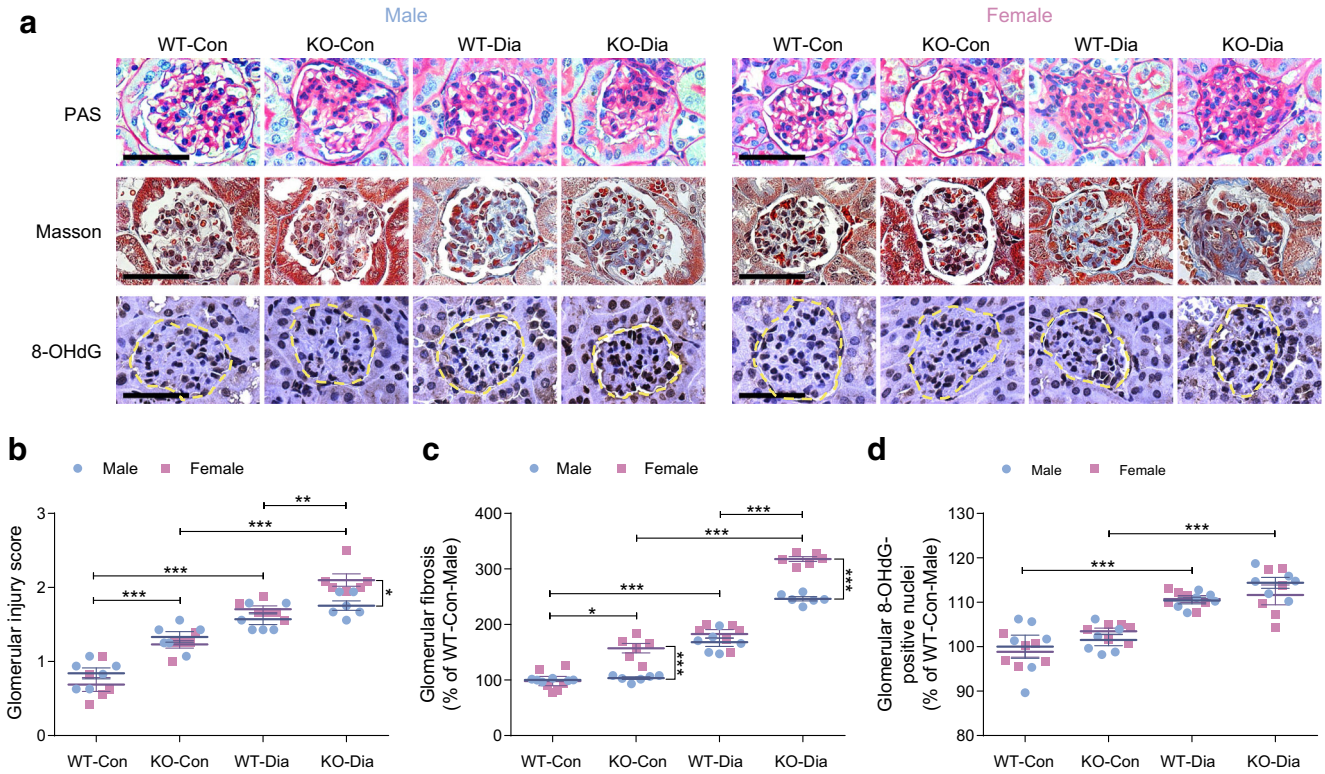


Fig. 5 Kidney morphology in age- and sex-matched offspring of non-diabetic and diabetic dams (WT vs AT_2RKO mice) at 20 weeks. (**a**) Kidney morphologic images of PAS, Masson's trichrome and 8-OHdG IHC staining in male and female mice; scale bar, 50 μm. (**b**–**d**) Semi-quantification of staining. (**b**) Glomerulosclerosis injury score based on PAS staining (scale from 0 to 4: Grade 0, normal glomeruli; Grade 1, presence of mesangial expansion/thickening of the basement membrane; Grade 2, mild/moderate segmental hyalinosis/sclerosis involving less than 50% total glomerular area; Grades 3 and 4, glomeruli are solidified and smaller than Grade 0 glomeruli [Grade 3 as 50% to 75% sclerosis; and Grade 4 as 76% to 100% sclerosis]. (**c**) Glomerular fibrosis per glomerulus based on Masson staining, expressed as % of WT-Con-Male, set at 100%; (**d**) 8-OHdG IHC-positive nuclei per glomerulus, expressed as % of WT-Con-Male, set at 100%;. Data shown as mean ± SEM; one-way ANOVA, followed by Bonferroni's post hoc test in comparison of four subgroups in total (WT-Con, KO-Con, WT-Dia and KO-Dia); two-way ANOVA, followed by Bonferroni's post hoc test in comparison of eight subgroups in a sex-specific manner [WT-Con (-M/F), KO-Con (-M/F), WT-Dia (-M/F) and KO-Dia (-M/F)]; *$p \le 0.05$; ** $p \le 0.01$. *** $p \le 0.001$

Moreover, female offspring from both non-diabetic and diabetic dams weighed less than male offspring throughout life, for example, mean body weight at 19 weeks (Fig. 1c) was lower (WT-Con-M vs WT-Con-F, $p < 0.001$; KO-Con-M vs KO-Con-F, $p < 0.001$; WT-Dia-M vs WT-Dia-F, $p < 0.001$; KO-Dia-M vs KO-Con-F, $p < 0.001$).

Physiological measurements and biomarkers

Blood pressure Longitudinal measurement of SBP (mmHg) was carried out in the offspring (WT vs AT_2RKO; non-diabetic vs diabetic dams) from 14 to 20 weeks of age (Fig. 1d; ESM Table 2). Compared with WT-Con, the WT-Dia and AT_2RKO offspring (both the KO-Con and KO-Dia) had significantly increased SBP over the follow-up period (Fig. 1d, in total; Fig. 1e, male; Fig. 1f, female). Compared with the sex-matched WT-Con offspring, the increased SBP in WT-Dia offspring is mainly driven by the SBP of male WT-Dia over time ($p \le 0.05$), while female WT-Dia remained normotensive (Fig. 1e,f). Strikingly, when compared with the WT-Dia progeny, female KO-Dia developed significant hypertension ($p \le 0.05$), while male KO-Dia had similar SBP to male WT-Dia (Fig. 1e,f).

IPGTT and IST At 19 weeks, an IPGTT (Fig. 2a, in total; Fig. 2b, male; Fig. 2c, female) and an IST (Fig. 3a, in total; Fig. 3b, male; Fig. 3c, female) were conducted. Both WT-Con and KO-Con mice showed the similar baseline of fasting glucose levels (Fig. 2a). Male offspring had higher AUC values than female offspring within the same subgroup (Fig. 2a). Regardless of sex, WT-Dia progeny had significantly impaired glucose tolerance compared with WT-Con mice; however, IPGTTs were the same in KO-Con and KO-Dia mice (Fig. 2b,c). Only KO-Dia, but not WT-Dia, mice showed insulin resistance compared with WT-Con offspring (Fig. 3a). Notably, WT-Dia offspring showed a sexual dimorphic IST response—e.g., male WT-Dia mice developed insulin resistance but female WT-Dia mice had a normal insulin sensitivity (Fig. 3b,c). In contrast, the IST on female KO-Dia mice revealed clear insulin impairment while male KO-Dia mice had normal insulin responses (Fig. 3b,c).

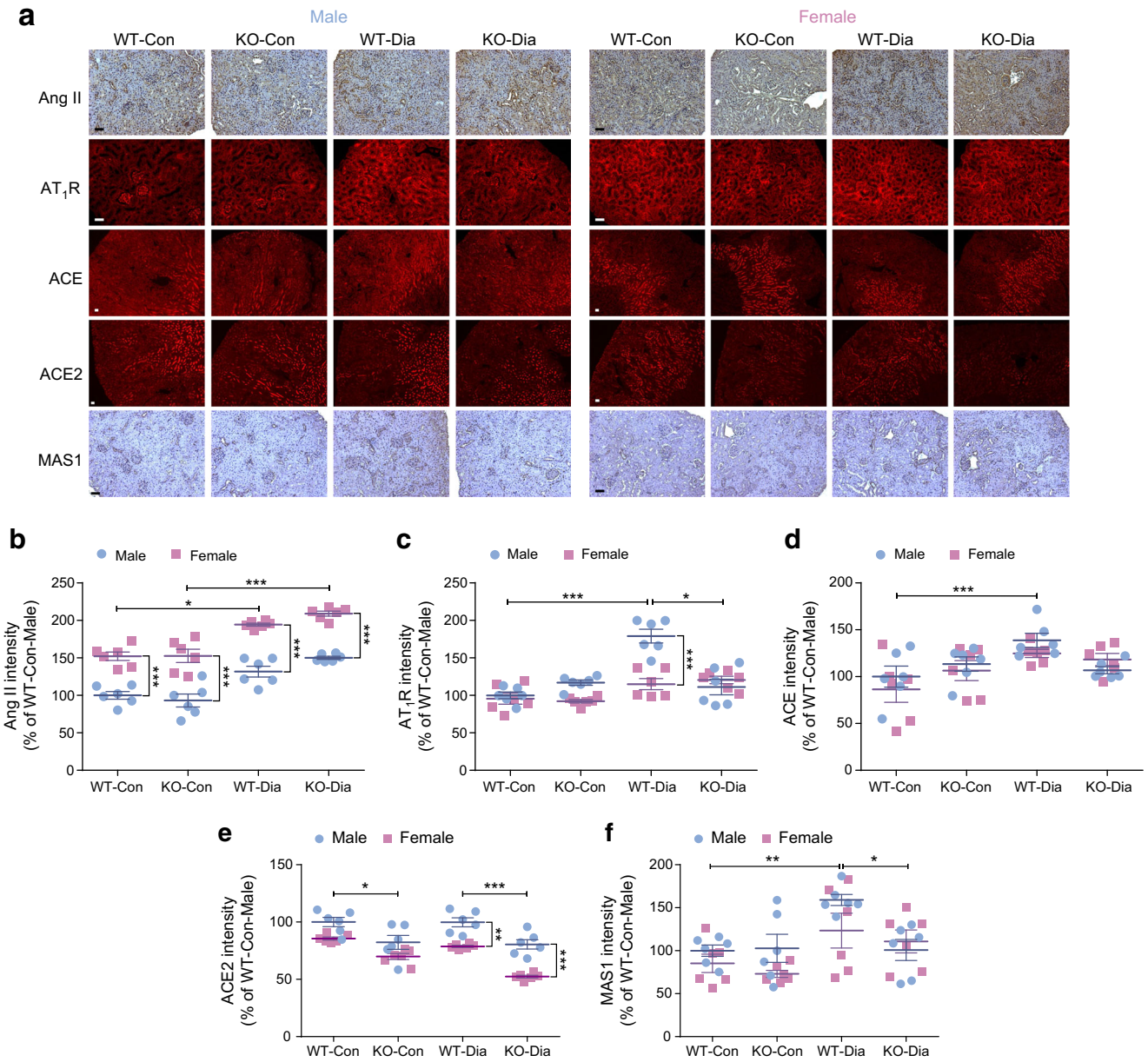


Fig. 6 Intrarenal RAS protein expression in the kidney of age- and sex-matched offspring of non-diabetic and diabetic dams (WT vs AT_2RKO mice) at 20 weeks. (**a**) IHC and IF images in male and female mice: Ang II-IHC, AT_1R-IF, ACE-IF, ACE2-IF and MAS1-IHC staining; scale bar, 50 μm. (**b**–**f**) Semi-quantification of IHC/IF staining. (**b**) Ang II-IHC; (**c**) AT_1R-IF; (**d**) ACE-IF; (**e**) ACE2-IF; and (**f**) MAS1-IHC. Data shown as mean ± SEM; one-way ANOVA, followed by Bonferroni's post hoc test in comparison of four subgroups in total (WT-Con, KO-Con, WT-Dia and KO-Dia); two-way ANOVA, followed by Bonferroni's post hoc test in comparison of eight subgroups in a sex-specific manner [WT-Con (-M/F), KO-Con (-M/F), WT-Dia (-M/F) and KO-Dia (-M/F)]; *$p \le 0.05$, ** $p \le 0.01$, *** $p \le 0.001$

Kidney variables Both WT-Con and KO-Con offspring had similar basal GFR at the age of 20 weeks (Fig. 4a), regardless of sex. Consistently, GFR levels in the offspring of diabetic dams (both WT-Dia and KO-Dia) increased significantly in both sexes, profoundly so in KO-Dia offspring (vs WT-Dia, $p < 0.01$), especially in the female KO-Dia progeny (vs KO-Dia-M, $p < 0.05$).

The KW/TL ratio was higher in AT_2RKO offspring than in WT offspring ($p < 0.01$); and in male offspring than in female offspring (Fig. 4b) (WT-Con-M vs WT-Con-F, $p < 0.05$; KO-Con-M vs KO-Con-F, $p < 0.05$; WT-Dia-M vs WT-Dia-F, $p < 0.001$; KO-Dia-M vs KO-Dia-F, $p < 0.001$). There was no apparent difference in the offspring from non-diabetic or diabetic dams. KW and glomerular numbers per kidney cortex area among all the subgroups are shown in ESM Fig. 1 and ESM Fig. 2, respectively.

Urinary measurements Compared with respective control offspring from non-diabetic dams, both WT-Dia and KO-Dia offspring had detectable albuminuria by Coomassie

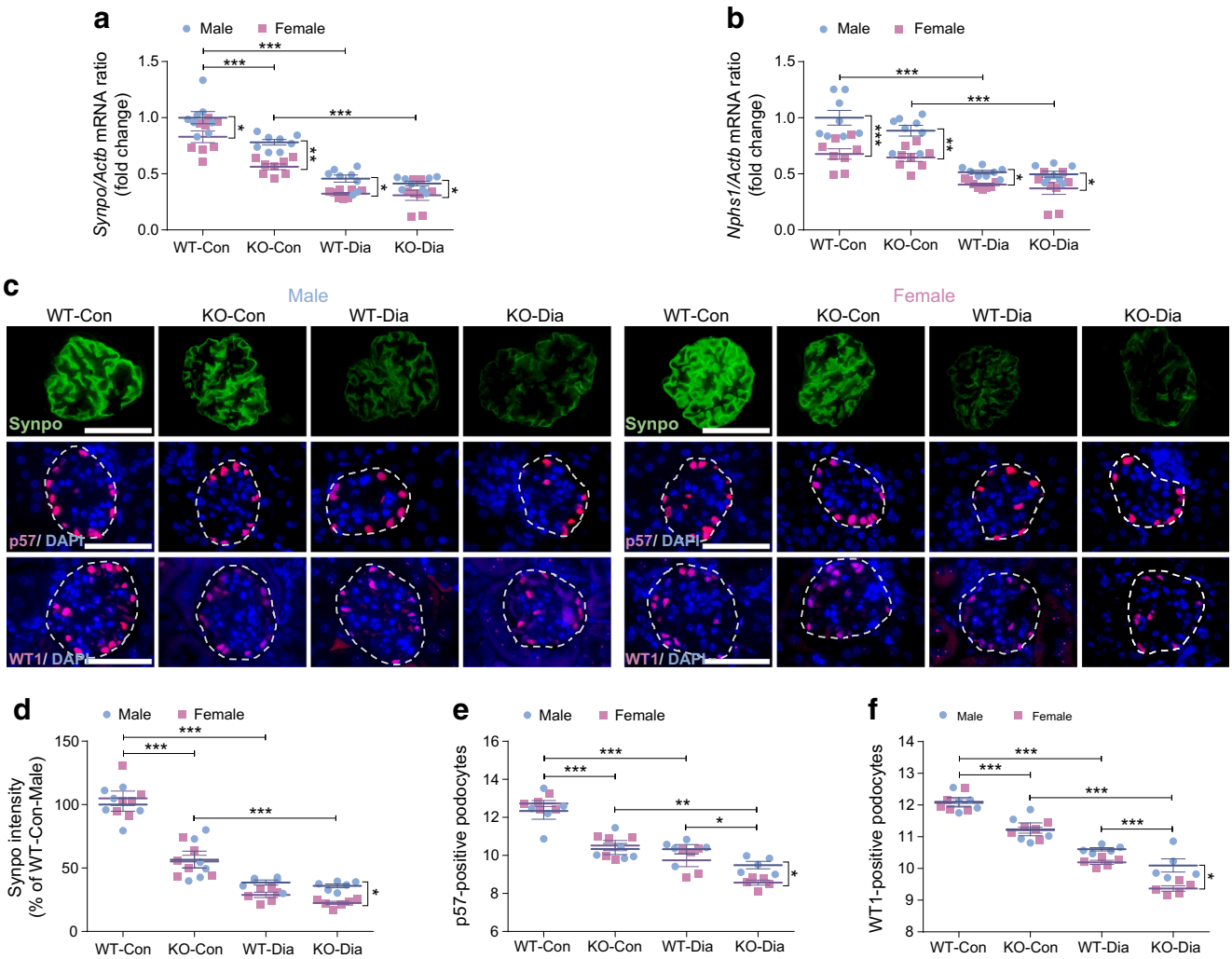


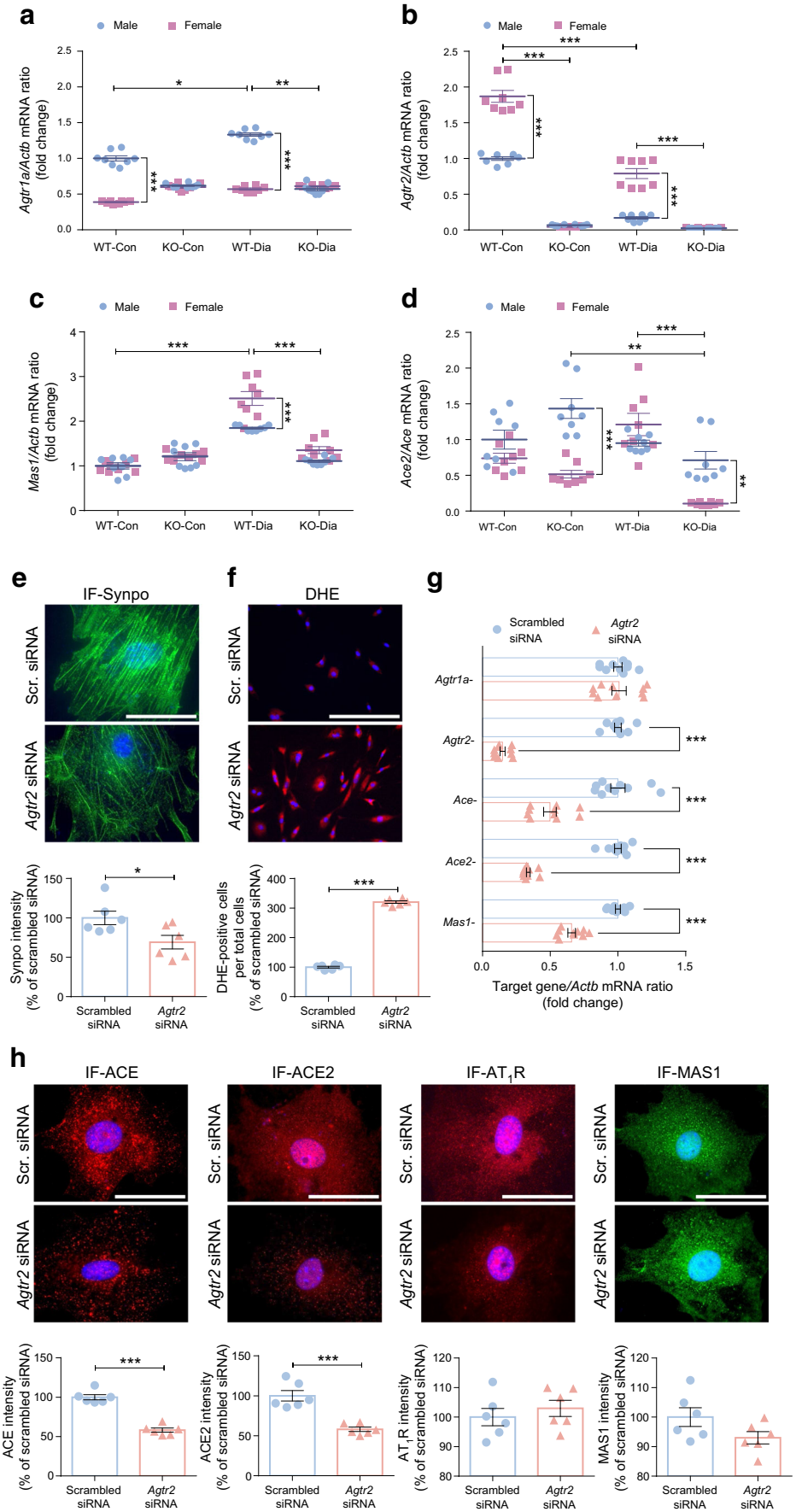
Fig. 7 Podocyte studies in age- and sex-matched offspring of non-diabetic and diabetic dams (WT vs AT_2RKO mice) at 20 weeks. (**a**, **b**) qPCR in isolated glomeruli. (**a**) *Synpo* and (**b**) *Nphs1* mRNA expression. (**c**) IF staining images in male and female mice: Synpo-IF (Synpo, green); p57-IF (p57, red; DAPI, blue); WT1-IF (WT1, red; DAPI, blue); scale bar, 50 μm; dashed circle, glomerulus. (**d**–**f**) Semi-quantification of IF staining. (**d**) Synpo; (**e**) p57; and (**f**) WT1. (**a**, **b**; **d**–**f**) Data shown as mean ± SEM; one-way ANOVA, followed by Bonferroni's post hoc test in comparison of four subgroups in total (WT-Con, KO-Con, WT-Dia and KO-Dia); two-way ANOVA, followed by Bonferroni's post hoc test in comparison of eight subgroups in a sex-specific manner [WT-Con (-M/F), KO-Con (-M/F), WT-Dia (-M/F) and KO-Dia (-M/F)]; $^*p \le 0.05$, $^{**}p \le 0.01$, $^{***}p \le 0.001$

Blue staining of urine, regardless of sex (Fig. 4c). However, we did not detect any significant differences in the urinary ACR (Fig. 4d) in any of the subgroups. In addition, only KO-Dia offspring had increased urinary Ang II/creatinine (pmol/μmol) ratio, which was mainly contributed by female KO-Dia offspring (KO-Con-F vs KO-Con-M, $p < 0.05$) (Fig. 4e). Urinary Ang 1–7/creatinine ratios (pmol/μmol) were similar in all the subgroups (Fig. 4f).

Kidney pathology and intrarenal biomarker expression

Kidney morphology Kidney morphology was examined on sections stained with PAS (Fig. 5a,b) and Masson trichrome (Fig. 5a,c). Compared with the respective WT-Con offspring, the progeny (WT-Dia and KO-Dia) of both diabetic WT and AT_2RKO dams displayed more evidence of nephropathy (apparent glomerulosclerosis in combined with glomerular extracellular matrix protein accumulation) at 20 weeks. Those kidney changes were more pronounced in KO-Dia offspring, female KO-Dia offspring in particular (Fig. 5a–c). The kidney fibrosis among all the subgroups was further confirmed by *Tgfb1* mRNA expression (ESM Fig. 3) as well. 8-OHdG staining (Fig. 5a,d) showed an oxidative stress pattern in the glomeruli, similar to changes revealed in the kidney morphologic staining discussed above (Fig. 5a–c), but without sexual differences in KO-Dia offspring.

Ang II and intrarenal RAS gene expression in the kidney We examined Ang II (Fig. 6a,b) and several intrarenal RAS


a
Male
Female
Agtr1a/Actb mRNA ratio (fold change)
WT-Con
KO-Con
WT-Dia
KO-Dia
b
Agtr2/Actb mRNA ratio (fold change)
c
Mas1/Actb mRNA ratio (fold change)
d
Ace2/Ace mRNA ratio (fold change)
e
IF-Synpo
Scr. siRNA
Agtr2 siRNA
Synpo intensity (% of scrambled siRNA)
Scrambled siRNA
Agtr2 siRNA
f
DHE
DHE-positive cells per total cells (% of scrambled siRNA)
g
Scrambled siRNA
Agtr2 siRNA
Agtr1a-
Agtr2-
Ace-
Ace2-
Mas1-
Target gene/Actb mRNA ratio (fold change)
h
IF-ACE
IF-ACE2
IF-AT1R
IF-MAS1
ACE intensity (% of scrambled siRNA)
ACE2 intensity (% of scrambled siRNA)
AT1R intensity (% of scrambled siRNA)
MAS1 intensity (% of scrambled siRNA)

◀ **Fig. 8** In vivo (**a**–**d**) and in vitro (**e**–**h**) studies. (**a**–**d**) qPCR in isolated glomeruli from the age- and sex-matched offspring of non-diabetic and diabetic dams (WT vs AT_2RKO mice) at 20 weeks. mRNA expression of (**a**) *Agtr1a*; (**b**) *Agtr2*; (**c**) *Mas1*; and (**d**) the *Ace2/Ace* ratio. Data shown as mean ± SEM; one-way ANOVA, followed by Bonferroni's post hoc test in comparison of four subgroups in total (WT-Con, KO-Con, WT-Dia and KO-Dia); two-way ANOVA, followed by Bonferroni's post hoc test in comparison of eight subgroups in sex-specific manner [WT-Con (-M/F), KO-Con (-M/F), WT-Dia (-M/F) and KO-Dia (-M/F)]. $*p \le 0.05$, $**p \le 0.01$, $***p \le 0.001$. (**e**–**h**) The in vitro study of mPODs treated with 50 nmol/l scrambled (Scr.; blue bars) or 50 nmol/l *Agtr2* siRNA (pink bars). (**e**) Synpo-IF staining and its semi-quantification (scale bar, 50 μm); (**f**) DHE staining and its semi-quantification (scale bar, 50 μm). (**g**) qPCR of *Agtr1a*, *Agtr2*, *Ace*, *Ace2* and *Mas1* mRNA. (**h**) IF staining of AT_1R, ACE, ACE2 and MAS1 as well as its corresponding semi-quantification (scale bar, 50 μm). (**e**–**h**) Three to four separate experiments; data shown as mean ± SEM; an unpaired Student's *t* test; $*p \le 0.05$, $***p \le 0.001$ vs mPODs cultured with scrambled siRNA (set at 100%)

components including AT_1R (Fig. 6a,c), ACE (Fig. 6a,d), ACE2 (Fig. 6a,e) and MAS1 (Fig. 6a,f) IF/IHC-protein expression in the kidneys of these animal subgroups. Compared with the control offspring, the offspring of diabetic dams had heightened Ang II-IF protein expression, but this was pronounced in female progeny from diabetic dams (male vs female, $p < 0.001$), while there was no apparent difference between WT and AT_2RKO mice. Compared with WT-Con, AT_1R-IF, ACE-IF and MAS1-IHC protein expression were increased, while ACE2-IF protein expression was not affected in the kidney of WT-Dia offspring. By contrast, AT_2RKO offspring (KO-Con vs KO-Dia; male vs female) had similar levels of AT_1R-IF, ACE-IF and MAS1- IHC protein expression in their kidneys.

Notably, the increased AT_1R-IF protein was mainly driven by male WT-Dia offspring (WT-Dia-M vs WT-Dia-F, $p < 0.001$), while the similar MAS1-IHC protein expression was seen in both sexes of mice within the same subgroup. Strikingly, ACE2-IF protein expression was consistently lower in the kidneys of female but not male WT-Dia and KO-Dia offspring (WT-Dia-M vs WT-Dia-F, $p < 0.01$; KO-Dia-M vs KO-Dia-F, $p < 0.001$).

Podocyte morphology/numbers We examined several podocyte markers (Synpo, Nephrin, WT1, and p57) and their gene expression in these animal subgroups (Fig. 7). *Synpo* (Fig. 7a) and *Nphs1* (encoding nephrin) (Fig. 7b) mRNA expression in the glomeruli were decreased in KO-Con compared with WT-Con mice, and the levels were much lower in female than in male KO-Con offspring. In the presence of maternal diabetes, the expression of these genes was further decreased in the glomeruli of KO-Dia offspring (vs WT-Dia), more so in female offspring. These results were confirmed by podocyte loss estimated by Synpo (Fig. 7c,d), p57 (Fig. 7c,e) and WT1 IF staining (Fig. 7c,f).

Intrarenal RAS gene expression in the glomeruli We further examined intraglomerular mRNA levels of *Agtr1a*, *Agtr2*, *Ace*, *Ace2 and Mas1* in isolated glomeruli of all animal subgroups (qPCR of *Agtr1a* [Fig. 8a], *Agtr2* [Fig. 8b] and *Mas1* [Fig. 8c]) genes as well as the *Ace2/Ace* ratio [Fig. 8d]; ESM Fig. 4, qPCR of *Ace* and *Ace2* genes). Compared with WT-Con offspring, the increased mRNA expression of *Agtr1a* and *Mas1* genes, the decreased *Agtr2* mRNA expression and the unaffected *Ace2/Ace* ratio were shown in glomeruli of both sexes of WT-Dia offspring. Notably, lower *Agtr1a* and higher *Agtr2* mRNA expression were evident in the glomeruli of female compared with male offspring within the same WT subgroup. In contrast, the mRNA levels of *Agtr1a* and *Mas1* genes were completely unaffected in glomeruli of AT_2RKO mice, regardless of sex. Most importantly, both sexes of KO-Dia offspring had a significantly lower expression of the *Ace2/Ace* ratio compared with WT-Dia and KO-Con, more pronounced in female KO-Dia progeny.

Studies in cultured mouse podocytes We further validated several of our in vivo findings by knocking-down *Agtr2* using *Agtr2* siRNA in mouse podocytes (mPODs: Fig. 8e–h). Compared with scrambled siRNA (50 nmol/l), treatment with *Agtr*2 siRNA (50 nmol/l) inhibited Synpo protein expression and disoriented podocyte integrity (Fig. 8e, IF). It also increased oxidative stress revealed by dihydroethidium (DHE) staining (a cell-permeable fluorescent dye, redox indicator) in mPODs (Fig. 8f). In addition, *Agtr2* siRNA decreased gene expression of *Ace* and *Ace2* whereas *Agtr1a* gene expression was unaffected (Fig. 8g, qPCR; Fig. 8h, IF). Although *Mas1* mRNA was significantly decreased by *Agtr*2 siRNA, podocyte morphology remained relatively normal compared with mPODs treated with scrambled RNA.

Discussion

In the present study, we demonstrated that AT_2R deficiency led to more pronounced nephropathy in the progeny of diabetic dams. These changes were more pronounced in female offspring; we postulate this was possibly due to loss of the protective effects of ACE2 expression in their kidneys.

During the follow-up period, overall, AT_2RKO offspring had similar weights to WT offspring; the body weight of the offspring of diabetic dams was lower compared with that of the offspring of non-diabetic dams, and female offspring were significantly lighter than male offspring. This disparity in body weight led us to speculate that kidney programming might be a factor in the low-birthweight offspring in a sex-dependent manner given that low birthweight is associated with a higher risk of CKD in both experimental data [28, 29] and in humans [4, 43].

Body weight has been associated with additional sexually dimorphic variations, such as later metabolic changes [44]. Consistent with a previous report [29], both sexes of WT-Dia progeny showed significantly impaired glucose tolerance. Intriguingly, IPGTT results were unaltered in both sexes of KO-Dia offspring. KO-Dia mice developed significant insulin resistance that appeared driven by female KO-Dia mice, since male KO-Dia mice had a normal insulin response. These data are in agreement with the studies of Quiroga et al. [45], who reported unaltered IPGTT and impaired IST in female AT_2RKO mice. Since WT and AT_2RKO offspring had similar body weights, a key distinguishing factor might be related to sex hormones, which play a contributing role in AT_2R action. It has been known that AT_2R stimulation has had more pronounced effects in females [23–26]. While oestrogen upregulates AT_2R expression [26, 46], testosterone, via androgen receptor-mediated extracellular signal-regulated protein kinase (ERK)1/2/mitogen-activated protein (MAP) kinase-dependent mechanisms, could downregulate AT_2R expression [47].

Both WT and AT_2RKO offspring from non-diabetic and diabetic dams have similar kidney weight and glomerular numbers; however, the obvious kidney hypertrophy (revealed by KW/TL ratio) and higher GFR in KO-Dia offspring may lead to a higher risk of subsequent hypertension and injury [27, 48], compared with the mild hypertension phenotype in KO-Con animals [9, 13, 27]. Sex-specific differences in blood pressure levels related to maternal diabetes are known in both experimental and human studies [29, 49, 50]. For example, human studies have suggested that offspring of diabetic mothers seem to be at higher risk of developing hypertension than the offspring of non-diabetic mothers, and that this difference is more clearly evident in male than female offspring [49, 50]. While confirming that WT-Dia offspring are prone to developing hypertension in adulthood [29], we found that female KO-Dia offspring developed more pronounced SBP elevation with higher incidence of hyperfiltration compared with both male WT-Dia and male KO-Dia offspring over time. Although albuminuria is present, as is revealed by Coomassie Blue gel staining in both WT-Dia and KO-Dia offspring, the ACR was not statistically different among the subgroups, suggesting an adaptive capacity of mature C57BL/6 mice during an early stage of nephropathy.

In addition, both sexes of progeny of diabetic AT_2RKO dams (KO-Dia, M/F) develop greater nephropathy and elevated oxidative stress. Such kidney damage is far more evident in female KO-Dia offspring. The mechanisms underlying sexual dimorphism seen here in the progeny of diabetic dams remain poorly understood. The presence of intrarenal RAS dysfunction and activation of oxidative stress has been suggested [28, 29, 51, 52]. Here, we confirmed evidence of elevated oxidative stress in the kidneys of the offspring of diabetic dams [28, 29] and found that the level of Ang II was also increased. However, only female KO-Dia offspring had elevated Ang II (but not Ang 1–7) excretion in urine, hinting at a disturbance of intrarenal RAS.

Although AT_2R expression decreases significantly within days after birth [22, 53], notably, the decline in renal AT_2R expression is greater in males [53]. Moreover, the axis of renal *Agtr2*/*Ace2*/*Ang 1–7*/*Mas1* gene expression has been reported as greater in female (both adult [24, 26, 53] and ageing [24, 54]) rodents, suggesting the shift of depressor RAS pathways in females, which protects female rodents from hypertension and kidney injury. Consistent with previous reports [28, 29], compared with WT-Con offspring, AT_1R-IF, ACE-IF and MAS1-IHC expression were increased, while ACE2-IF expression was not affected in the kidney of WT-Dia offspring (WT-Con vs WT-Dia; male vs female). Notably, ACE2-IF expression was consistently lower in the kidneys of female but not male WT-Dia and KO-Dia offspring, while AT_1R-IF, ACE-IF and MAS1-IHC expression remained unchanged in AT_2RKO offspring (KO-Con vs KO-Dia; male vs female). Together, these data suggest that loss of ACE2 expression in the kidney might implicate the pronounced kidney programming in female KO-Dia offspring.

Podocytes express AT_2R and play a key role in maintaining the integrity and function of the glomerular filtration barrier [14, 17]. Consistent with our previous findings that AT_2R deficiency has more impact on podocyte integrity than on nephron numbers [17], we found that AT_2R deficiency resulted in podocyte loss in diabetic progeny in a sex-dependent manner, more pronounced in female KO-Dia offspring. We examined several podocyte markers (Synpo, Nephrin, WT1 and p57) and observed that their gene expression was further decreased in the glomeruli of KO-Dia offspring (vs WT-Dia), and more so in female KO-Dia offspring. Mechanistically, the *Ace2*/*Ace* ratio further declined in isolated glomeruli, which was more pronounced in female KO-Dia progeny, while *Agtr1a* and *Mas1* mRNA was not affected. Finally, we have validated podocyte morphology/integrity (Synpo-IF staining), oxidative stress (DHE staining) and the gene expression of these RAS components (qPCR and IF staining) in vitro by using *Agtr2* siRNA (vs scrambled siRNA) in mPODs to confirm our in vivo findings.

Our present studies have certain limitations. The level of intrarenal ACE2 activity requires further validation in the current model. Moreover, ACE2 is primarily expressed in the brush border of the renal proximal tubule [27, 33–35]. We have reported that basal *Ace2* mRNA and protein in isolated proximal tubules is decreased in AT_2RKO mice [27]; and ACE2, through decreasing renal oxidative stress-mediated signalling, protects diabetes-related glomerular and tubular injury and prevents the development of hypertension [27, 33–35]. Therefore, a contribution through ACE2 downregulation in the proximal tubule seems likely. To date, little is known about how AT_2R knockout leads to a diminution in ACE2 in the renal proximal tubule and how that influences kidney programming and related sexual dimorphism.

In conclusion, our data suggest that loss of AT_2R protective effects may lead to a higher risk of hypertension and kidney damage in the progeny of diabetic dams, particularly among female offspring. We propose that the dysfunction of intrarenal RAS and/or activation of oxidative stress mediate sexual dimorphism in KO-Dia offspring with pronounced kidney programming. Prospectively, novel therapeutic strategies to prevent AT_2R loss may provide a means to decrease the heightened risks for adverse kidney programming in the offspring of diabetic mothers.

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Data availability The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

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Authors' relationships and activities The authors declare that there are no relationships or activities that might bias, or be perceived to bias, their work.

Contribution statement SLZ is the guarantor of this work, had full access to all study data, and takes responsibility for data integrity and the accuracy of data analysis. JSDC and SLZ were principal investigators and were responsible for the study conception and design. SLZ drafted/reviewed/edited the manuscript. MCL, YCP, SYC, XPZ, IC and SLZ contributed to the experiments and data collection. JRI contributed to data discussion and reviewed/edited the manuscript. All authors were involved in the analysis and interpretation of data and contributed to the critical revision of the manuscript. All authors provided final approval of the version to be published.

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