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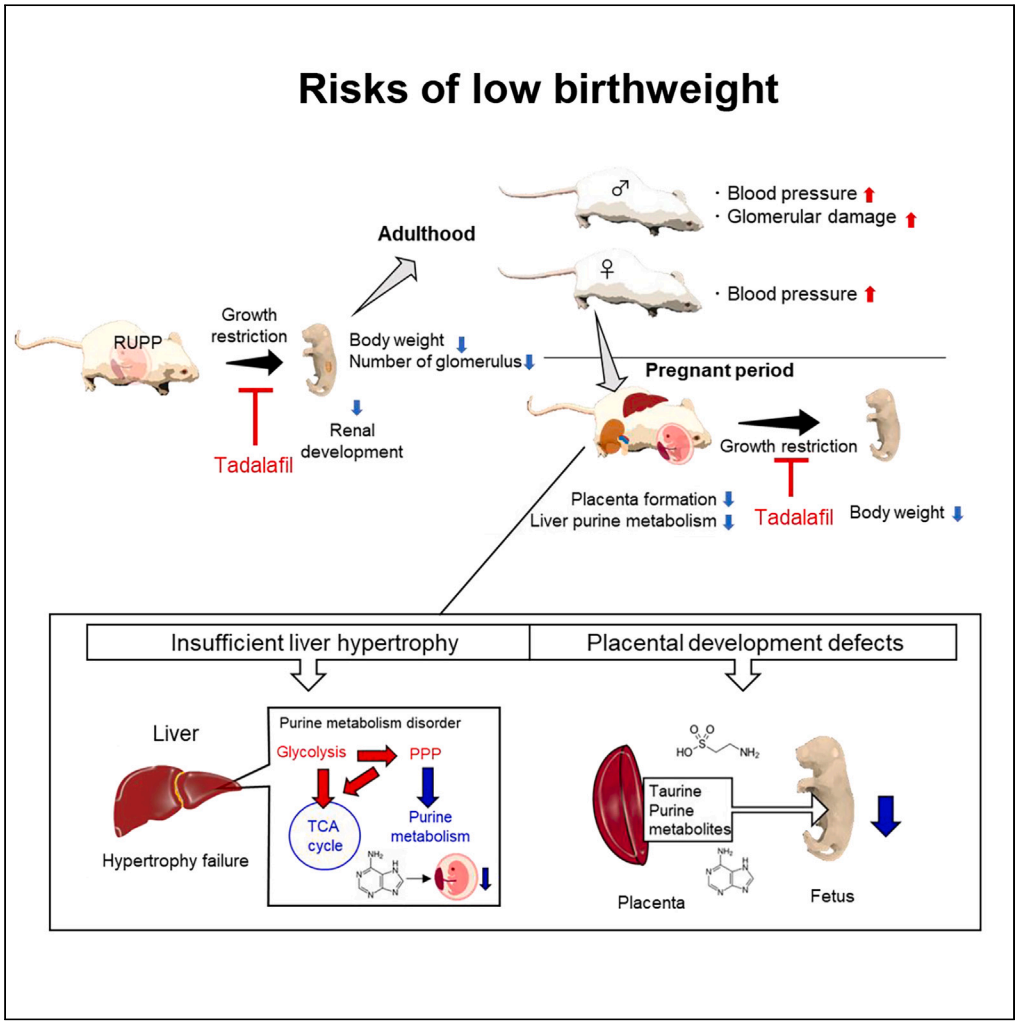


Akiyo Sekimoto, Yoko Takaso, Haruka Saruyama, ..., Nobuyuki Takahashi, Eikan Mishima, Emiko Sato

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## Highlights

Established a low birth weight mouse model by reducing uterine perfusion pressure

The mice had immature renal development and persistently reduced glomerular numbers

Maternal tadalafil treatment mitigates immature kidney and low birth weight

Pregnant mice born with low birth weight also have smaller fetuses

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# Impacts of low birthweight on kidney development and intergenerational growth of the offspring

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## SUMMARY

**Low birthweight (LBW) increases the risk of adult-onset diseases, including kidney diseases, with intergenerational consequences; however, the underlying mechanisms and effective interventions are unclear. To examine the cross-generational effects of LBW, we established an LBW mouse model through reduced uterine perfusion pressure (RUPP) and investigated the therapeutic potential of tadalafil, a phosphodiesterase 5 inhibitor, on LBW-associated consequences. RUPP-pups (R1) had lower fetal and birth weights, delayed renal development, and fewer glomeruli than Sham-pups. In adulthood, R1 mice exhibited persistently fewer glomeruli and elevated blood pressure, while Tadalafil-R1 mice showed reduced hypertension in both sexes and improved renal pathological changes in males. Additionally, pregnant R1 mice displayed inadequate gestational liver hypertrophy, impaired hepatic purine metabolism, and diminished placental angiogenesis, resulting in fetal growth restriction in the subsequent generation. These findings underscore the lasting impact of LBW on adult health and future generations and suggest tadalafil's potential to mitigate LBW-associated risks.**

## INTRODUCTION

The World Health Organization (WHO) defines low birthweight (LBW) as a birth weight of less than 2,500 g[1] In 2020, an estimated 19.8 million live newborns worldwide, corresponding to or 14.7%, were classified as having an LBW.[2] Given the association with the Developmental Origins of Health and Disease hypothesis, which emphasizes the influence of environmental factors during the fetal and early postnatal period on vulnerability to disease in later life,[3] LBW is closely associated with an increased future health issues, such as hypertension and chronic kidney disease (CKD), including end-stage kidney disease.[4,5] Additionally, women born with LBW face an elevated risk of developing hypertensive disorders during pregnancy.[6] Moreover, their offspring are at higher risk of fetal growth retardation and LBW, perpetuating a cycle of intergenerational risk factors.[7–10] To mitigate the prolonged risk of the diseases associated with LBW, proactive interventions are required.

Since adequate nephron formation occurs in the third trimester,[11] conditions such as LBW, small for gestational age, fetal growth restriction (FGR), and preterm birth although temporally and mechanistically distinct processes adversely affect a considerable proportion of the nephrogenic period, thereby contributing to the future risk of hypertension and CKD.[12,13,14] Thus, maintaining an appropriate nephron count in LBW infants could potentially mitigate these risks. To comprehensively investigate the relationship between LBW and adult-onset diseases and to develop potential therapeutic interventions, it is imperative to establish an animal model that replicates the LBW phenotype and the related pathologies that emerged in both adulthood and subsequent generations. Such an experimental animal model, suitable for studying intergenerational changes within a relatively short time frame compared to humans, holds the potential to become a valuable tool for developing prevention and treatment strategies for LBW and its associated intergenerational risks.

FGR and small for gestational age, which are characterized by impaired fetal development *in utero*, are major contributors to LBW. Preeclampsia, characterized by maternal hypertension and vascular endothelial damage occurring after 20 weeks of gestation, affects 2–5% of pregnant women and is a contributing factor to FGR.[15–18] We have previously demonstrated that tadalafil, a phosphodiesterase 5 (PDE5) inhibitor approved for the treatment of erectile dysfunction and pulmonary hypertension, shows therapeutic potential in improving elevated

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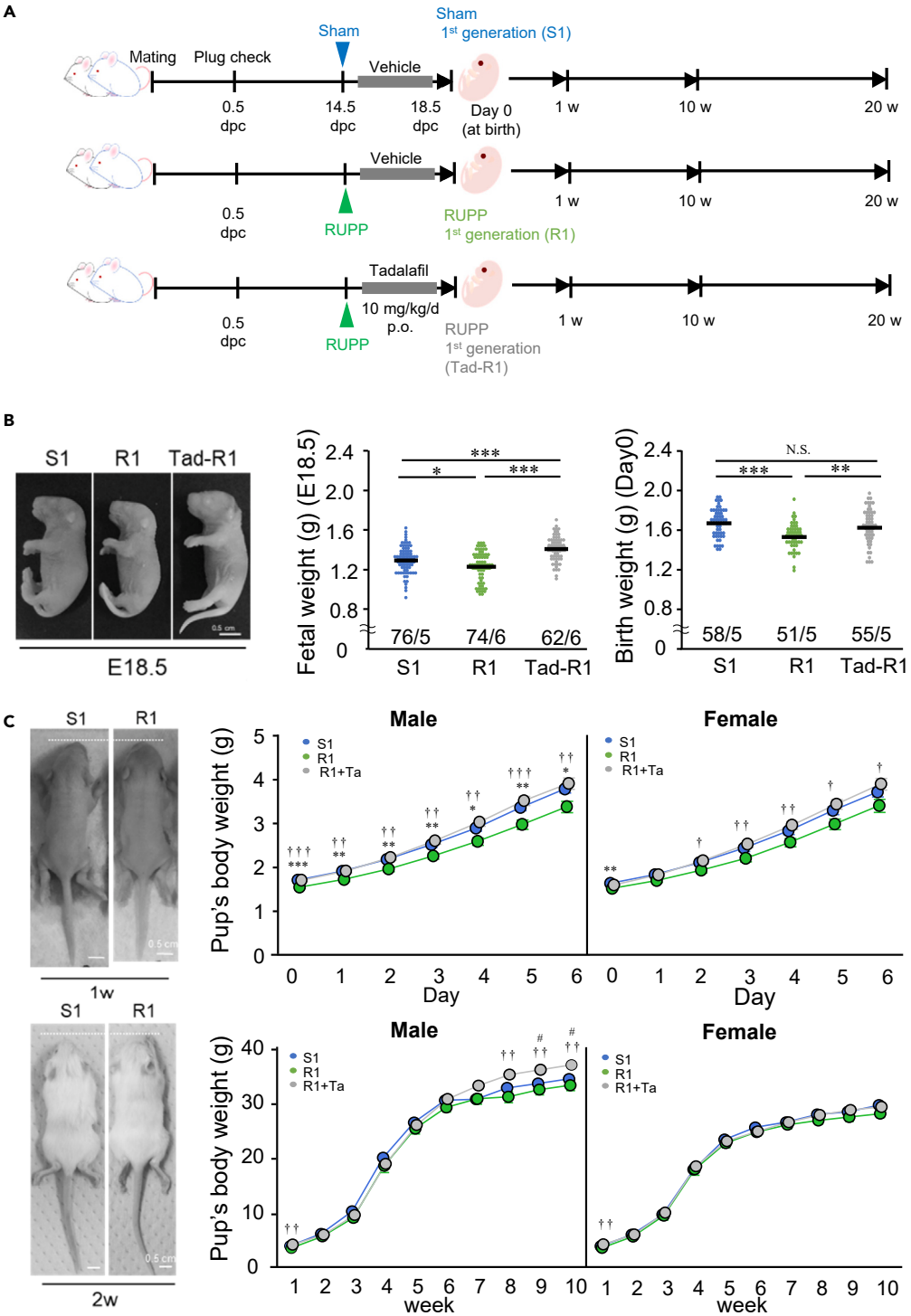


**Figure 1. Reduced uterine perfusion pressure (RUPP) operation causes lower R1 fetal and birth weights, but maternal tadalafil administration counteracts this**

(A) Experimental schedule. Fetuses and pups after sham or RUPP operations were designated as sham 1st generation (S1) and RUPP 1st generation (R1), respectively. Tadalafil (10 mg/kg/day) or vehicle (0.5% methyl cellulose 400) was orally administered daily starting the day after the operation.

(B) Representative fetal images and comparisons of fetal embryonic day 18.5 (E18.5) and birth weight (day 0). Numbers indicate the number/maternal number of the fetuses or pups. The central line indicates the mean value. Tukey-Kramer's test; *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

**Figure 1. *Continued***

(C) Successive changes in pup body weight from day 0–10 weeks of age. The sample numbers of S1, R1, and Tad-R1 between days 0 and 6 were $n = 31$, $n = 30$, and $n = 24$ males, and $n = 27$, $n = 21$, and $n = 28$ females, respectively. The sample numbers of S1, R1, and Tad-R1 between 1 and 10 weeks were $n = 26$, $n = 25$, and $n = 22$ males, and $n = 22$, $n = 16$, and $n = 25$ females, respectively. Representative images of pups at 1 week (sex unspecified) and 2 weeks (male) of age. Data are shown as the mean ± standard error of the mean. Two-way analysis of variance followed by Bonferroni analysis; $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ (S1 vs. R1), $\dagger p < 0.05$, $\dagger\dagger p < 0.01$, $\dagger\dagger\dagger p < 0.001$ (R1 vs. Tad+R1), $\#p < 0.05$ (S1 vs. Tad+R1).

blood pressure, reducing proteinuria, and mitigating FGR in experimental animal models of preeclampsia.[19,20] Additionally, tadalafil has shown therapeutic efficacy in alleviating the symptoms of preeclampsia and subsequent FGR in a phase I clinical study.[21–23] In this study, we established a mouse model of LBW based on a preeclampsia model by reducing uterine perfusion pressure (RUPP) to examine the impact of LBW on fetal renal development in adulthood and its effects for the subsequent generations. Furthermore, we investigated the therapeutic potential of tadalafil to address these issues using the LBW model.

## RESULTS

### Establishment of the LBW model

To establish an animal model of LBW, we used mice with RUPP, an experimental preeclampsia model achieved by ligating the ovarian and uterine vessels with nylon thread to constrict the vessels (Figure S1), as previously reported.[24] We also examined the therapeutic potential of the maternal tadalafil treatment on the offsprings of this model.

Initially, we compared pups born to three groups of mice: the sham group (S1), mice subjected to the RUPP procedure (R1), and mice subjected to the RUPP procedure and maternal tadalafil treatment (Tad-R1) (Figure 1A). The R1 group exhibited significantly lower fetal weight at 18.5 days postcoitum (dpc) and lower birth weight than the S1 group (Figure 1B). In addition, the body weights of R1 pups remained lower than those of S1 pups until one week of age in both males and females; however, after one week, no significant differences in body weight were observed between the S1 and R1 groups in either males or females (Figure 1C). In contrast, maternal tadalafil treatment improved the low body weight of RUPP model pups (Figure 1B). Furthermore, the male offspring showed a significant increase in weight compared to the S1 male pups after 9 weeks of age (Figure 1C). These results indicated that the RUPP model led to an LBW phenotype in the pups and that the maternal tadalafil treatment demonstrated therapeutic potential in mitigating the lower body weight of pups in this model. Regarding the influence of sex, there was no significant difference in the weight between male and female mice from day 0 to week 4. The weights of male and female mice differed significantly between the three groups 5–6 weeks post birth (Figure S2). Therefore, in subsequent analyses, the data of male and female were not separated until 4 weeks of age, and the data of male and female were analyzed separately after 5 weeks of age.

### Effects of LBW on fetal kidney development

Next, we evaluated fetal kidney development on embryonic day 18.5 (E18.5) across the three groups. During kidney development, the ureteric bud invades the metanephric mesenchyme, which aggregates to form a structure on the cap. The aggregated posterior renal mesenchymal cells epithelialize and transform into a comma (C)-shaped or S-shaped body, which differentiate into nephron segments.[25,26] Analysis of hematoxylin and eosin (H&E)-stained kidney sections revealed a higher abundance of C-shaped or S-shaped bodies, indicative of immature nephrons, in the kidneys of R1 mice compared to those of S1 mice (Figure 2A). The transcription factor *Six2*, which is expressed in nephron progenitors during fetal kidney differentiation,[27] was more highly expressed in the E18.5 kidneys of the R1 group mice than in the S1 group mice. This increased expression suggests an inappropriate differentiation of nephron progenitor cells in the fetal kidneys of R1 mice. In addition, the area positive for lotus tetragonolobus lectin (LTL), a marker of mature proximal tubules in the renal cortex, decreased in the fetal kidneys of R1 mice compared to that of S1 mice. Maternal tadalafil treatment ameliorated these associated pathologies, including the abnormal abundance of C- and S-shaped bodies, abnormally increased expression of *Six2* in the E18.5 kidney, and reduced LTL-positive area in the fetal kidneys (Figures 2A and 2B). Similarly, at 1 week of age, both the number of glomeruli and glomerular density in the cortex were significantly lower in R1 kidneys than in S1 kidneys, whereas the glomerular number in Tad-R1 mice was comparable to that in S1 mice (Figure 2C). However, the glomerular density in Tad-R1 mice kidneys was significantly lower than that in S1 mice kidneys, possibly due to increased kidney weight and cortical area to correct in Tad-R1 mice (Figure S3). These results demonstrate that kidney development was delayed during the embryonic period in the RUPP-induced LBW model and that maternal tadalafil treatment improves this developmental delay.

### Effects of LBW on glomerular damage in adult mice

Next, we assessed the kidneys of adult mice in the three groups. In 20-week-old male mice, the glomerular number remained lower in the R1 group than that in the S1 group, whereas tadalafil treatment restored the glomerular number in R1 mice to the same level as that in S1 mice (Figure S4A). In addition, the kidney weight was significantly higher in the Tad-R1 group than in the R1 group. To assess glomerular damage in adult mice, we examined open capillary and mesangial periodic acid-Schiff (PAS)-positive areas in kidney sections (Figure 3). In the R1 group, the open capillary area was significantly decreased, and the mesangial area was increased compared with that of the S1 group. Tadalafil treatment improved these glomerular changes in the Tad-R1 group (Figure 3A).

Because reduced nephron numbers and glomerular damage are associated with hypertension, we evaluated the systolic blood pressure (SBP) of male mice at 4, 10, and 20 weeks of age. The SBP of mice in the R1 group was higher than that of mice in the S1 group at 10 weeks of

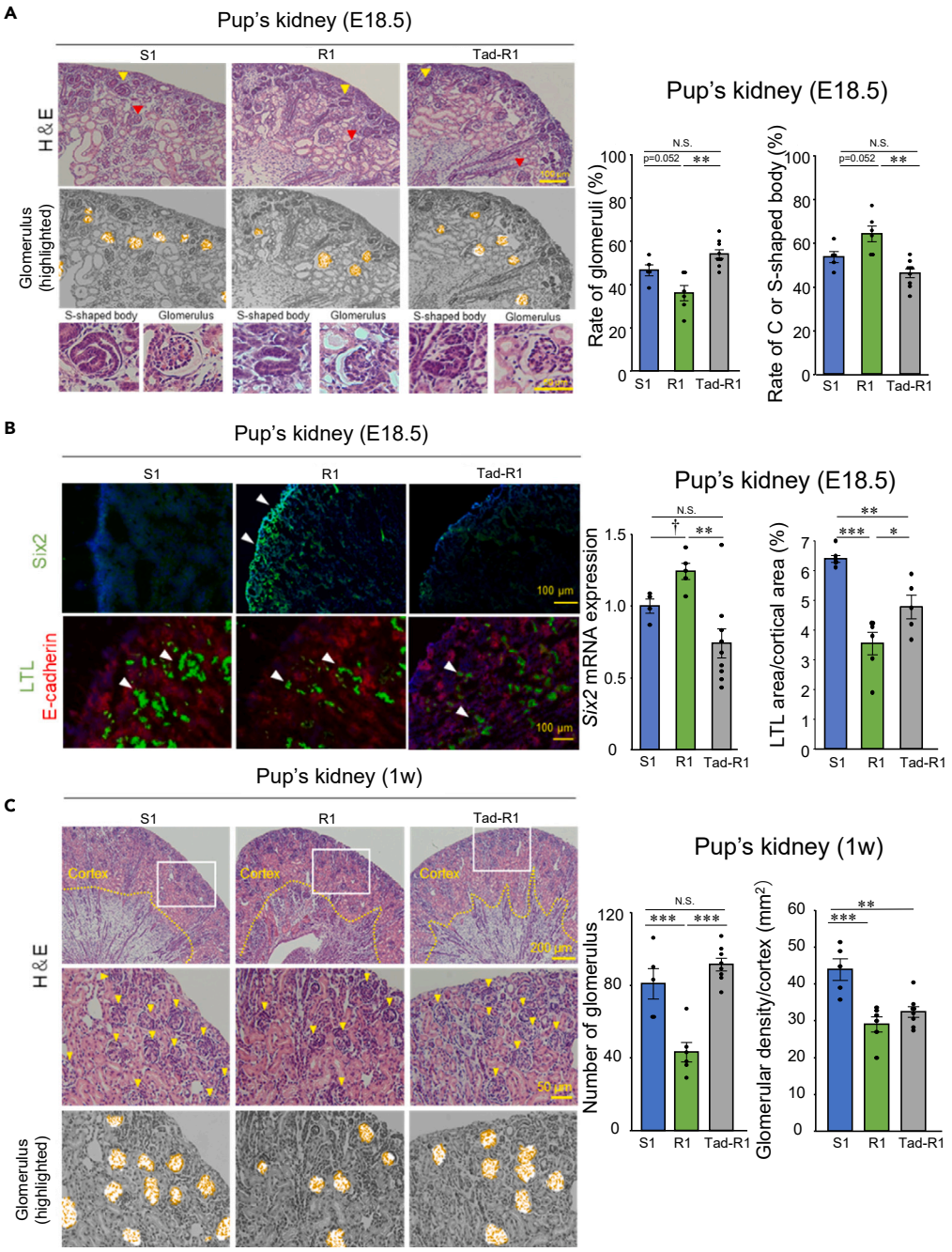


**Figure 2. Maternal tadalafil treatment improves fetal renal development**

(A) Representative images of hematoxylin and eosin (H&E)-stained kidneys on embryonic day (E18.5). ▽ (yellow) indicates C- or S-shaped body. ▽ (red) indicates glomerulus. Scale bar, 100 μm. The sample numbers of S1, R1, and Tad-R1 were $n = 5$, $n = 6$, and $n = 8$, respectively.

(B) Images of Six2-immunoshistochemistry, and lotus tetragonolobus lectin (LTL) (green)/E-cadherin (red) staining in the kidneys of E18.5 fetuses. The sample numbers of S1, R1, and Tad-R1 were $n = 4$, $n = 6$, and $n = 9$, respectively.

(C) Images of H&E-stained kidneys from 1-week-old mice. Extended images of the square area in white are shown below. ▽ (yellow) indicates glomerulus. The sample numbers of S1, R1, and Tad-R1 were $n = 5$, $n = 6$, and $n = 8$, respectively. Data are shown as the mean ± standard error of mean, Tukey-Kramer's test; *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$. Student's t test †$p < 0.05$.

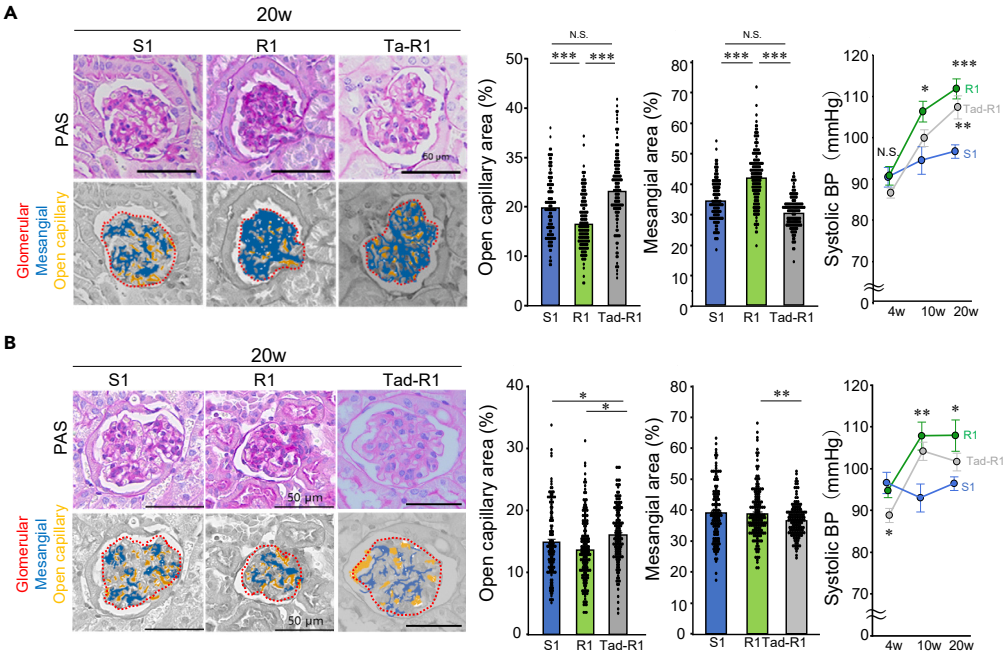


**Figure 3. Comparison of glomerular open capillary area, mesangial area, and systolic blood pressure in 20-week-old adult male and female mice**

(A) Representative periodic acid-Schiff (PAS)-stained kidney sections from 20-week-old adult male mice. Open capillary and mesangial areas at 20-week. Comparison of the mean systolic blood pressure (SBP) at 4, 10, and 20 weeks of age in male mice. Scale bar = 50 μm. The numbers of mice with SBP measured in S1, R1, and Tad-R1 were $n$ = 11, $n$ = 13, and $n$ = 10, respectively. Two-way analysis of variance followed by Bonferroni analysis. *$p$ < 0.05, **$p$ < 0.01. Data are shown as the mean ± standard error of the mean.

(B) Representative PAS-stained kidney sections from 20-week-old adult female mice. Open capillary and mesangial areas at 20-week. Scale bar = 50 μm. The numbers of mice with SBP measured in S1, R1, and Tad-R1 were $n$ = 11, $n$ = 12, and $n$ = 10, respectively. Tukey-Kramer's test or Steel-Dwass test; *$p$ < 0.05, **$p$ < 0.01, ***$p$ < 0.001 Student's t test; †$p$ < 0.05. Data are shown as the mean ± standard error of the mean. $N$ = 120 (S1), 160 (R1), and 140 (Tad-R1) glomeruli were evaluated for the analysis in (A) and (B).

age. Moreover, the differences in SBP between the S1 and R1 mice were more evident at 20 weeks of age. In contrast to the findings observed in the male mice, the open capillary area and mesangial areas of female mice did not differ between the S1 and R1 groups at 20 weeks of age (Figure 3B). At 20-week, female Tad-R1 mice showed a significant increase in the open capillary area and a significant decrease in the mesangial area compared to the R1 groups (Figure 3B). Although females in the R1 group showed no glomerular damage, SBP was significantly higher in R1 mice at 10 and 20 weeks than in S1 mice in both males and females. Urinary albumin excretion did not differ between S1 and R1 mice at 10 or 20 weeks of age (Figures S4B and S4C). These results demonstrated that LBW caused pathological glomerular changes and elevated blood pressure in males. Tadalafil treatment ameliorated glomerular damage and reduced blood pressure in male mice, with no adverse effects observed in female mice.

## Effects of LBW on the next generation and placental angiogenesis

Furthermore, we investigated the effects of LBW on the subsequent generations and explored its underlying mechanisms. After mating S1 or R1 mice aged 7–13 weeks, fetal weight at E13.5 and E18.5 were measured (Figures 4A and 4B) and the maternal tissue weight and placental weights were assessed. Although the number of fetuses were not significantly different between S1 and R1 mice (Figure 4C), the weight of the fetus (R2) of R1 mice was significantly lower than that of the fetus (S2) of S1 mice at both E13.5 and E18.5 (Figures 4D and 4E). At E13.5, there was no significant difference in placental weight between S2 and R2 mice; however, the fetus-to-placenta weight ratio was significantly lower in R2 mice (Figure 4D). By E18.5, placental weight of R2 mice was significantly lower than that of S2 mice, resulting in a significantly higher fetus-to-placenta weight ratio in R2 mice (Figure 4E). Notably, the weight of R2 mice was significantly lower than that of S2 mice at both E13.5 and E18.5 (Figures 4D and 4E), despite comparable renal status and blood pressure during pregnancy (18.5 dpc) in S1 and R1 mice (Figures S5A–S5C).

Considering the lower fetal weight of R2 mice compared to that of S2 mice, we analyzed the influence of the placenta on the potential developmental defects. The placental weight was significantly lower in the R2 group than in the S2 group (Figure 5A); although H&E staining results did not show obvious histological differences between S1 and R1 mice (Figure 5A). Moreover, the gene expression of angiogenesis-related factors, such as *Vegfa*, *Plgf*, *Kdr*, *Tie2*, *Angpt1*, and *CD31*, was upregulated in the placental labyrinth of R2 mice (Figure 5B). In addition, placental angiogenesis evaluation by isolection B4 staining, which binds to fetal endothelial cells in the labyrinth, also suggested that the placental vascular network in the labyrinth zone of R2 mice was sparser than that in S2 mice (Figure 5C). Immunohistochemical staining for HIF-2α, a hypoxia response factor, suggested the elevated HIF-2α expression in the placental labyrinth of R2 mice compared to that in S2

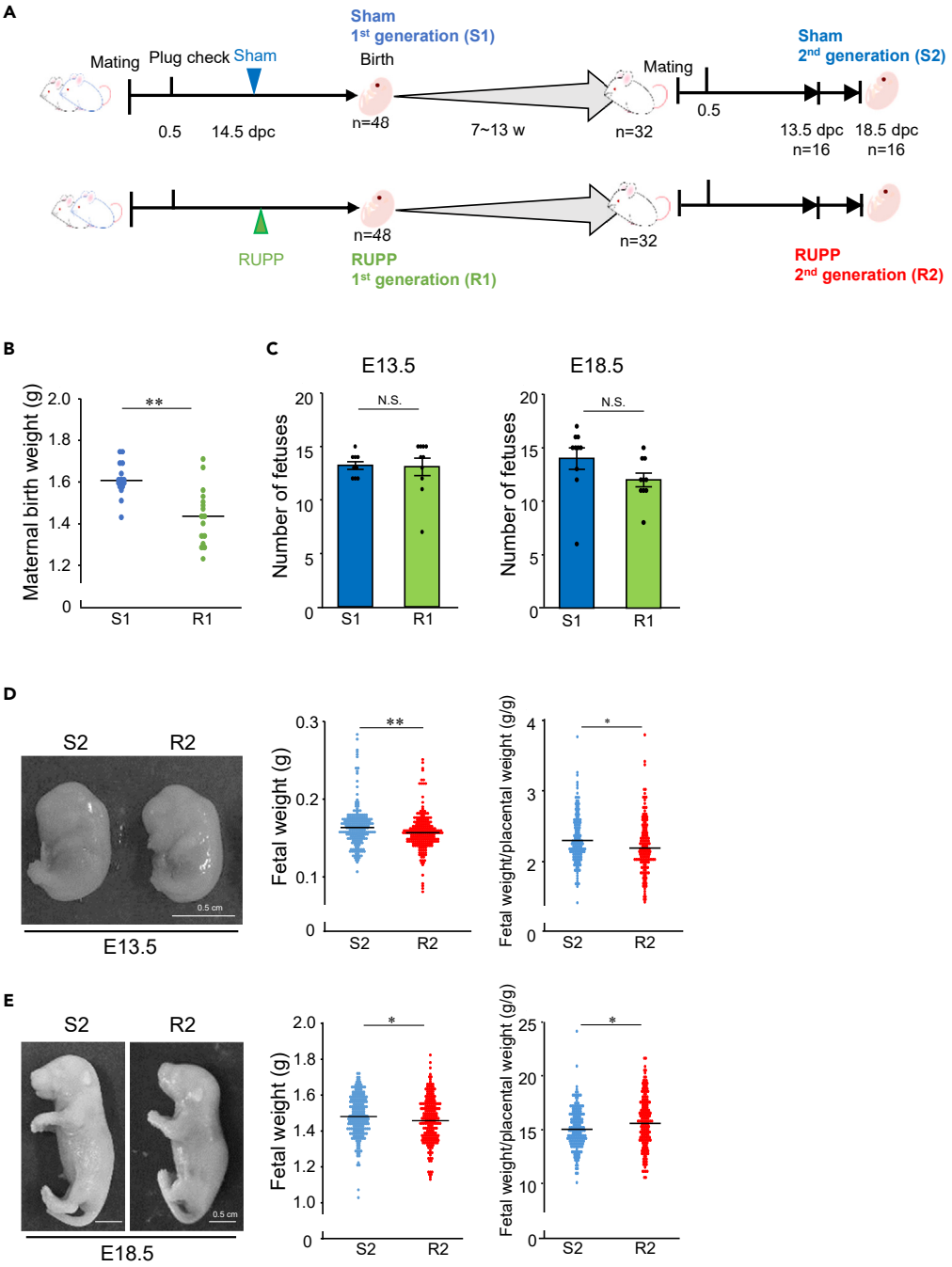


**Figure 4. Fetuses from low birthweight female mice have a lower fetal weight**

(A) Experimental schedule. The fetuses of S1 and R1 were designated as sham 2nd generation (S2) and RUPP 2nd generation (R2), respectively.

(B) Maternal (S1 and R1) birth weight. The sample numbers of S1 and R1 were $n = 16$ and $n = 16$, respectively.

(C) Comparison of the number of fetuses at 13.5 and 18.5 days postcoitum. Data are shown as the mean ± standard error of the mean.

(D and E) Representative images of the fetus and comparison of the fetal weight and fetal weight/placental weight ratio at embryonic day (E)13.5 and E18.5. The sample numbers of S2 and R2 on E13.5 were $n = 223$, and $n = 223$, respectively. The sample numbers of S2 and R2 on E18.5 were $n = 222$, and $n = 210$, respectively. Student's t test; $*p < 0.05$, $**p < 0.01$. The central line indicates the mean value.

mice (Figure 5C). An unbiased metabolomic analysis of the labyrinth was conducted to determine the changes in metabolic pathways in the placenta. Enrichment analysis revealed significant alterations in taurine and hypotaurine metabolism, along with increased taurine levels in the placentas of R2 mice (Figure 5D).

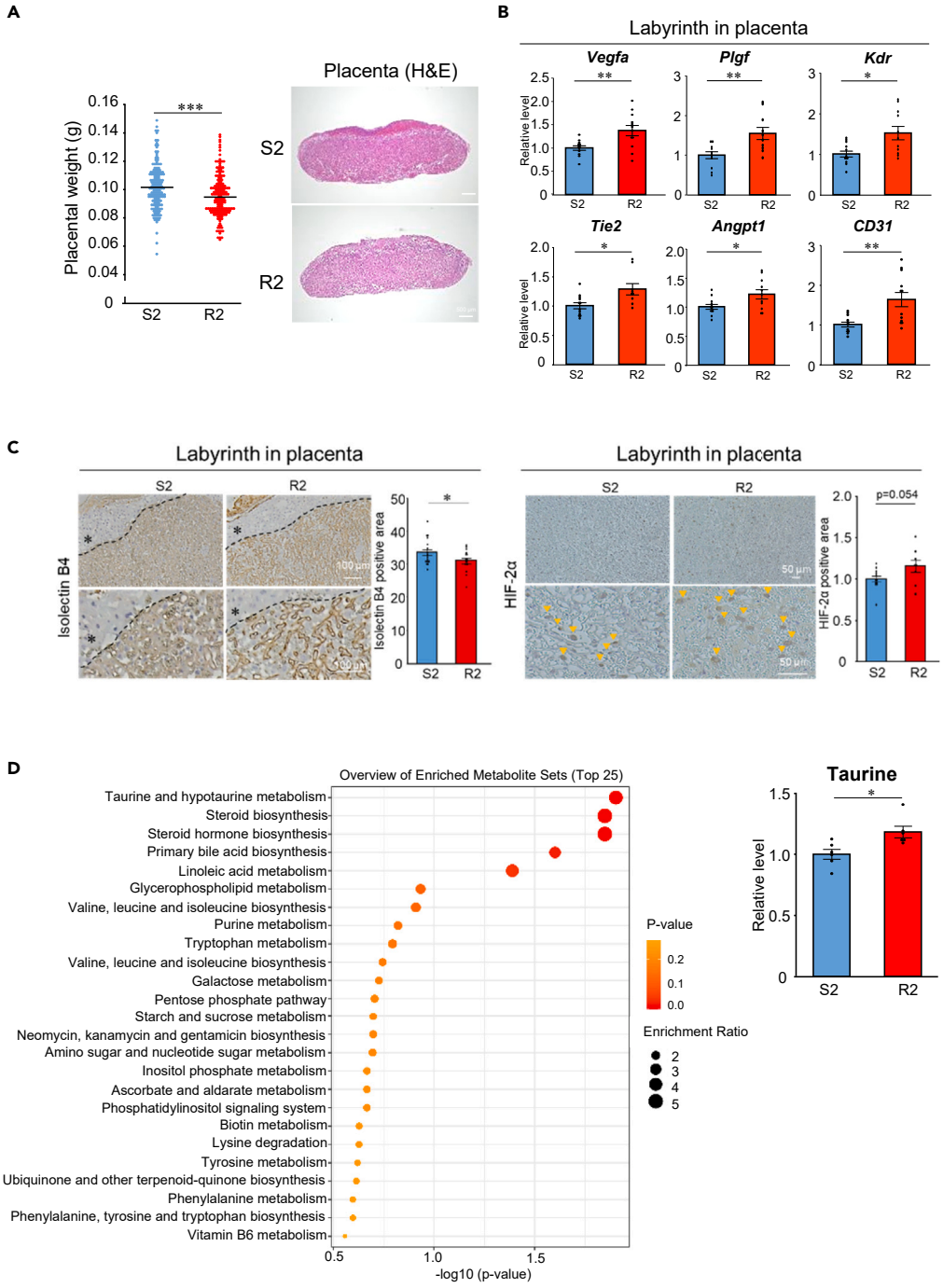


**Figure 5. Angiogenesis defects in the placenta of low birth weight female mice**

(A) Placental weight and images of hematoxylin and eosin (H&E)-stained placentas of the S2 and R2 mice (18.5 days postcoitum). The sample numbers of S2 and R2 were $n = 184$, and $n = 170$, respectively. The central line indicates the mean value.

(B) Expression of genes related to angiogenic factors in the placental labyrinth on embryonic day 13.5 (E13.5). The sample numbers of S2 and R2 were $n = 12$, and $n = 12$, respectively.

(C) Isolectin B4 and HIF-2α immunostaining of placental sections at E18.5. The lower panels show magnified images of the upper panels. * Indicates junctional zones. The isolectin B4 staining sample numbers of S2 and R2 were $n = 18$, and $n = 16$, respectively. The HIF-2α sample numbers of S2 and R2 were $n = 11$, and $n = 12$, respectively.

(D) Enrichment analysis of the metabolomics dataset of the placental labyrinth at E13.5. Comparison of taurine levels in the labyrinth at E13.5. The sample numbers of S2 and R2 were $n = 6$, and $n = 6$, respectively. Data are shown as the mean ± standard error of the mean. Student's t test; $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

### Effects of LBW on maternal liver

In addition to the differences in the placenta, during pregnancy (at 18.5 dpc), the liver weights of R1 mice were lower than those of S1 mice, whereas the liver weights of S1 and R1 mice were comparable under non-pregnant conditions (Figure 6A). H&E staining results showed no obvious histological differences between S1 and R1 mice 18.5 dpc (Figure 6A). In general, during pregnancy, liver size is enlarged due to the increased metabolic demands to support fetal growth.[28–30] To elucidate the mechanism underlying the insufficient enlargement of the liver of pregnant R1 mice, we examined gene expression and metabolites in the liver. Gene expression of *Tnf*, a factor related to liver hypertrophy,[28] was significantly lower in the livers of pregnant R1 mice. In contrast, the expressions of *Hif1a* and *Vegf*, markers of hypoxia and angiogenesis, respectively, were higher in the livers of pregnant R1 mice (Figure 6B).

Metabolic changes were also evaluated using metabolomic analysis of the livers of pregnant R1 and S1 mice. Enrichment analysis indicated marked alterations in the purine metabolism pathway in the R1 group, with a significant decrease in the levels of purine metabolites such as adenine, adenosine, guanine, and guanosine, compared to the S1 group (Figures 6C and 6D). Additionally, adenosine diphosphate (ADP) and adenosine monophosphate (AMP) levels were lower (Figure 6D), whereas spermidine levels were higher in the livers of R1 mice.

In the purine metabolism pathway, the gene expression of enzymes involved in both the *de novo* and salvage pathways of purine nucleotide synthesis (*Prps1*, *Hprt*, and *Aprt*) was significantly increased in the livers of R1 mice (Figure 6E). These results suggested that the livers of R1 mice exhibit decreased levels of purine nucleotide metabolites, accompanied by a compensatory upregulation of the salvage pathways for purine nucleotide synthesis.

Purine nucleotide synthesis depends on the production of metabolites though the pentose phosphate pathway. Within metabolic pathways that include the pentose phosphate pathway, glycolysis, and the TCA cycle, the expression of the genes such as *Gapdh*, *Pgd*, and *Taldo1* was upregulated in the liver of R1 (Figure 6F). The levels of 6-phosphogluconate (6-PG), pyruvate, 2-ketogrutarate (2-KG), and fumarate decreased, whereas those of erythrose 4-phosphate (erythrose 4-P) and sedoheptulose 7-phosphate (sedoheptulose 7-P) increased in the livers of R1 mice (Figure 6F). These findings suggested that during pregnancy, the pentose phosphate pathway is upregulated in the liver, owing to the increased demand for nucleic acid synthesis. However, in R1 mice, impaired angiogenesis leads to tissue hypoxia, prompting a shift toward glycolysis for energy production. Consequently, metabolites in the liver of R1 mice are redirected from the pentose phosphate pathway to glycolysis, rather than being utilized in purine metabolism (Figure 6G).

### Effects of maternal tadalafil treatment on reduced fetal weight in female mice with LBW

In the present study, we found that the fetal weight of R1 mice (R2) was lower than that of S1 mice (S2) at both E13.5 and E18.5 (Figures 4D and 4E). Thus, we further investigated whether maternal tadalafil treatment in R1 mice could prevent the reduction in fetal weight observed in R2 mice (Figures 7A–7C). The maternal birthweight of R1 was significantly lower than that of S1 mice (Figure 7B). Although the number of fetuses was not significantly different among the three groups, the fetal weight of the fetuses of R1 mice (R2) was significantly lower than that of the fetuses of S1 mice (S2) at E18.5 (Figures 7B and 7C). In contrast, the fetal weight of the Tad-R2 group was significantly higher than that of the R2 group and comparable to that of the S2 group (Figure 7C). These findings suggest that maternal tadalafil treatment effectively prevents fetal weight reduction in offspring of female mice with LBW.

### Effects of salt loaded diets in pregnant female mice

Finally, since we did not observe significant glomerular damage in female R1 mice at either the adult stage or during pregnancy, we investigated the effect of salt loading during pregnancy on glomerular damage in female R1 mice (Figures S5A and S5B; Figure S6A). Female mice from the S1 and R1 groups, aged 8–10 weeks were fed a high-salt diet containing 4% salt for 4 weeks prior to pregnancy (Figure S6A). The salt-loaded R1 female group exhibited a significantly reduced open capillary area and an increased mesangial-positive area in the kidney compared to the S1 group (Figure S6B). To further evaluate the changes that occur under severe conditions, a high-salt diet containing 8% salt was administrated to S1 and R1 mice before and during pregnancy (Figure S7A). This salt-loading protocol produced comparable results, showing a reduced open capillary area and increased mesangial-positive area in the kidneys of the R1 female group (Figure S7B). However, there was no significant difference in the number of fetuses between the S1 and R1 groups, and fetal weight, placental weight, and fetus-to-placenta weight ratio did not significantly differ between S2 and R2 fetuses in either salt-loading experiments (Figures S6C and S7C). These results indicated that renal vulnerability is manifested by salt-loading stress in the R1 mice.

## DISCUSSION

In the present study, we established a mouse model of LBW and explored its impact on fetal kidney development, long-term effects in adulthood and in subsequent generations, and the therapeutic potential of tadalafil. First, the LBW mouse model causes immature kidney development in fetuses and pups, resulting in a lower glomerular number and glomerular damage in adulthood. Second, maternal tadalafil treatment ameliorated the impaired kidney development in fetuses and pups, promoted fetal growth, and prevented an increase in SBP in adult mice. Third, during pregnancy, mice born with LBW exhibit insufficient liver enlargement and placental angiogenesis with impaired metabolic flow, leading to lower fetal weight in the next generation. Fourth, the administration of tadalafil to pregnant mice born with LBW prevented their fetuses from being born with lower fetal weight. Our study demonstrates the mechanisms underlying intergenerational consequences of LBW, highlighting the importance of therapeutic interventions targeting immature organ development to mitigate the

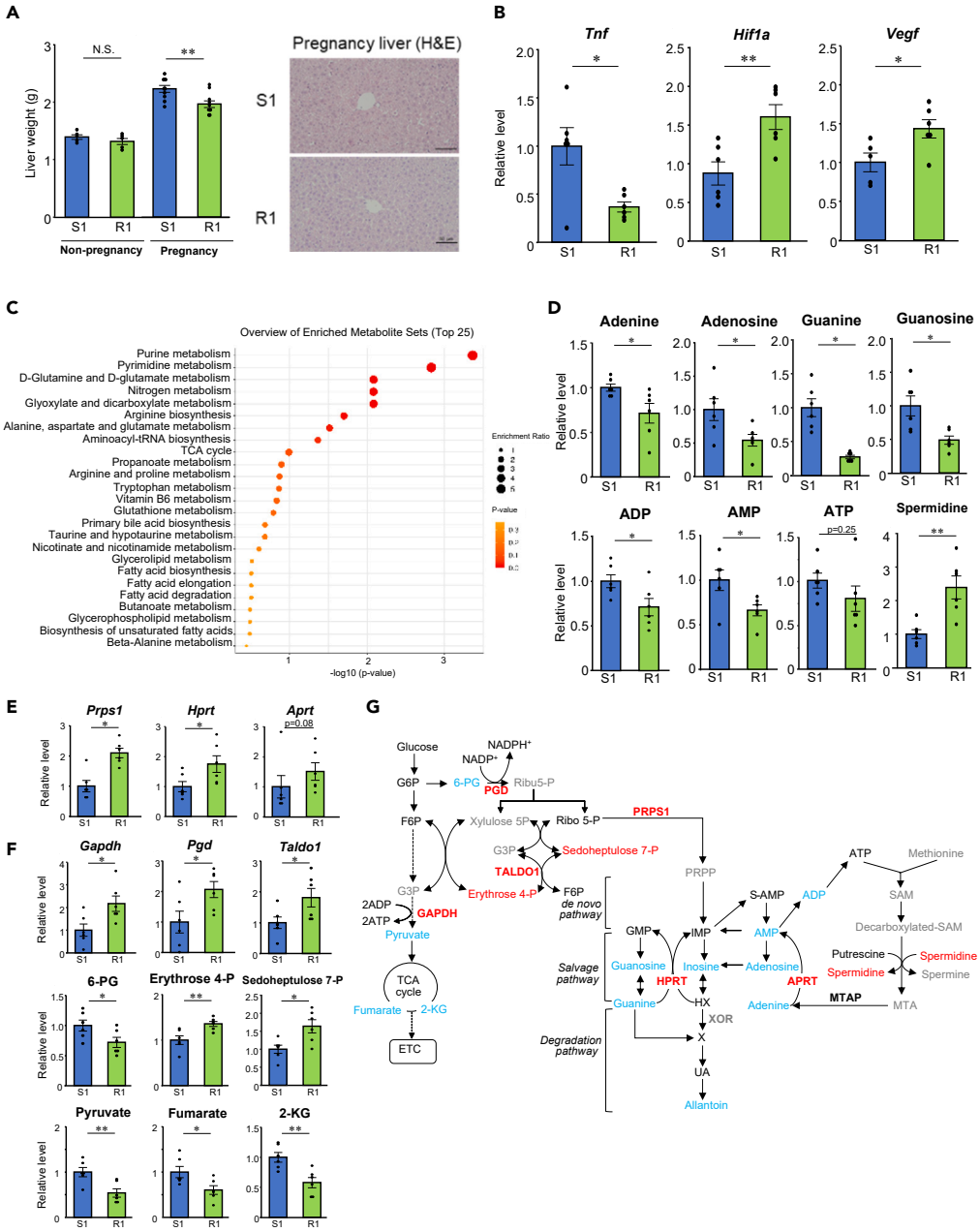


**Figure 6. Changes in purine metabolism in the liver of low birthweight female mice during pregnancy**

(A) Liver weights of S1 and R1 mice at 10 weeks of age (not pregnant) and 18.5 days post coitm (dpc; pregnant). Images of hematoxylin and eosin (H&E)-stained livers from pregnant mice. The sample numbers of non-pregnant S1 and R1 were $n = 5$ and $n = 5$, respectively. The sample numbers of pregnant S1 and R1 were $n = 16$, and $n = 16$ respectively.

(B) Gene expression levels of Tnf (13.5 dpc), Hif1a (18.5 dpc), and Vegf (18.5 dpc) in the livers of the pregnant S1 and R1 mice. Expression levels were normalized using Rps18. The sample numbers of S1 and R1 were $n = 6$, and $n = 6$ respectively.

(C) Enrichment analysis of metabolite sets in the livers of the pregnant mice at 18.5 dpc. The sample numbers of S1 and R1 were $n = 6$ and $n = 6$, respectively.

(D) Levels of purine metabolites and polyamines in the livers of S1 and R1 at 18.5 dpc. The sample numbers of S1 and R1 were $n = 6$ and $n = 6$, respectively.

(E) Gene expression levels of Prps1, Hprt, and Aprt, which are involved in purine synthesis, in the livers of the pregnant mice (18.5 dpc). The sample numbers of S1 and R1 were $n = 6$ and $n = 6$, respectively.

(F) Gene expression levels of Gapdh, Pgd, and Taldo1, which are involved in glycolysis and the pentose phosphate pathway. Metabolic pathways and the levels of the measured metabolites involved in glycolysis, the pentose phosphate pathway, the TCA cycle, and purine metabolism in the livers of the pregnant mice

**Figure 6. *Continued***

(18.5dpc). The sample numbers of S1 and R1 were $n = 6$ and $n = 6$, respectively. Data are shown as the mean ± standard error of the mean. Student's t test; $^{*}p < 0.05$, $^{**}p < 0.01$.

(G) Metabolic pathway changes assumed in the liver during R1 pregnancy. Metabolites in blue letters indicate a decrease, and metabolites or gene expression in red letters indicate an increase.

future risks associated with LBW. Furthermore, our RUPP-based LBW animal model provides a valuable tool for developing therapeutic strategies for LBW infants with low nephron counts and their associated pathologies.

Our results showed that maternal tadalafil treatment ameliorated the immature kidney development associated with LBW. Maintaining a balance between the proliferation and differentiation of nephron progenitor cells is crucial for proper renal development during the embryonic stage.[31–35] The transcription factor Six2, an essential for maintaining the progenitor state, is expressed in nephron progenitors and gradually decreases as nephron progenitors differentiate.[27,31,36,37] Persistent *Six2* expression in the R1 kidney suggests inappropriate differentiation of nephron progenitor cells.

A low nephron count is a risk factor for the development of hypertension and CKD in adulthood.[32,38,39] Mechanistically, low nephron numbers lead to compensatory glomerular hyperfiltration, accelerating kidney dysfunction.[40,41] We demonstrated that LBW mice exhibited lower nephron counts at birth, hypertrophy of the glomerulus, and higher blood pressure in adult male mice. In adult female mice, LBW was associated with higher blood pressure without obvious glomerular damage. Thus, our RUPP-induced LBW mouse model can serve as a valuable system for studying LBW infants with low nephron numbers and for developing therapies targeting low nephron counts. This is exemplified by the potential therapeutic effects of maternal tadalafil treatment on immature nephron development in LBW mice. In this study, pathological glomerular changes due to LBW were not extensively observed in adult female mice, unlike in male mice, although glomerular changes emerged under the high salt-loading conditions. In general, men are at a higher risk of CKD progression with a more rapid decline than women,[42] and LBW has a greater association with CKD progression in men.[43] Mechanistically, female hormones, such as estrogen, have been considered renoprotective factors.[44] Thus, with regards to the effect of sex on kidney damage, our LBW model also imitated the phenotype observed in the clinical setting.

Tadalafil is an inhibitor of PDE5, an intracellular secondary messenger that degrades cyclic guanosine monophosphate (cGMP), which is involved in vascular smooth muscle relaxation. By inhibiting the action of PDE5, tadalafil increases arterial blood flow by relaxing the vascular smooth muscle cells. Several studies have reported that tadalafil does not cross the human placental barrier and is degraded by trophoblasts. Supporting this notion, animal experiments have reported that tadalafil improves the width of maternal blood sinuses in the placenta but does not significantly alter fetal capillaries, suggesting that tadalafil functions only on the maternal side and not on fetal tissues.[20,45,46] Thus, tadalafil administration to the mother improves blood flow in the placenta and may also improve blood flow in the fetus. This suggests that tadalafil improved blood flow in the placenta of pregnant women with preeclampsia, which in turn promoted fetal kidney development. However, in the present study, Tad-R1 male mice showed greater weight gain than S1 male mice and insufficient control of elevated blood pressure in adulthood. These results suggest the potential sex differences in the effects of maternal tadalafil administration. The safety of tadalafil in pregnant women has been reported in phase I and II clinical trials. Phase I trial, which evaluated the safety and dose-finding for tadalafil use in pregnant women with FGR, showed that a favorable safety profile for both pregnant women and fetuses.[21] Phase II trial evaluated the therapeutic safety of tadalafil for fetuses with early-onset growth restriction and found that tadalafil reduced fetal and infant deaths associated with FGR.[45] However, neither clinical trial evaluated the influence of fetal sex differences.

Tadalafil is used for the treatment of pulmonary hypertension, and there are concerns about its adverse effects on pregnant women without pulmonary hypertension and on normally growing fetuses. However, a previous study investigated the cardiovascular effects of tadalafil in pregnant women without pulmonary hypertension and reported no adverse cardiovascular effects.[47] Therefore, maternal tadalafil treatment may be a promising therapy for effective intervention in infants with LBW.

However, a previous clinical trial reported that sildenafil, another PDE5 inhibitor, failed to demonstrate a beneficial effect on FGR.[48] One possible explanation for this outcome is that sildenafil can be transferred to the fetus, adversely affecting pulmonary circulation and increasing the risk of neonatal pulmonary hypertension.[46,49] In contrast, tadalafil exhibits lower fetal transfer rates and primarily targets the maternal placenta. Our previous data indicated that although the maternal vascular sinus of the placenta dilates with tadalafil administration, there is no dilatation of the fetal microvasculature.[20] Moreover, sildenafil administration has been shown to decrease fetal arterial prefusion pressure within the placenta, whereas tadalafil did not exhibit a similar effect.[46] Based on these findings, tadalafil may have a distinct impact on FGR compared to sildenafil. Further clinical trials investigating the use of tadalafil for the management of FGR are currently underway (jRCTs041190065).

During pregnancy, the maternal liver undergoes hypertrophy to meet the developmental and growth demands of the placenta and fetus.[28–30] Proinflammatory factors, especially tumor necrosis factor alpha (TNFα), and new blood vessel formation are considered crucial for regulating the early reparative response required for gestational liver hypertrophy.[28,50] Our study found that the liver of R1 mice exhibited impaired hypertrophy signaling and angiogenesis. In addition, gestational liver hypertrophy is associated with increased nucleic acid content[28] with purine metabolites serving as essential building blocks for nucleic acids and playing a vital role in fetal development.[51] In addition, adenosine, a purine metabolite, acts as a vasodilator and is involved in maintaining placental blood flow.[52] Interestingly, our results revealed that the liver of R1 mice exhibited altered hepatocyte metabolism and stagnation of purine metabolism. The decrease in adenine and increase in polyamines observed in the livers of R1 mice suggest reduced MTAP activity, which is an essential enzyme that controls the salvage synthesis of adenine from the substrate methylthioadenosine.[53–55] As this pathway plays an important role in cellular

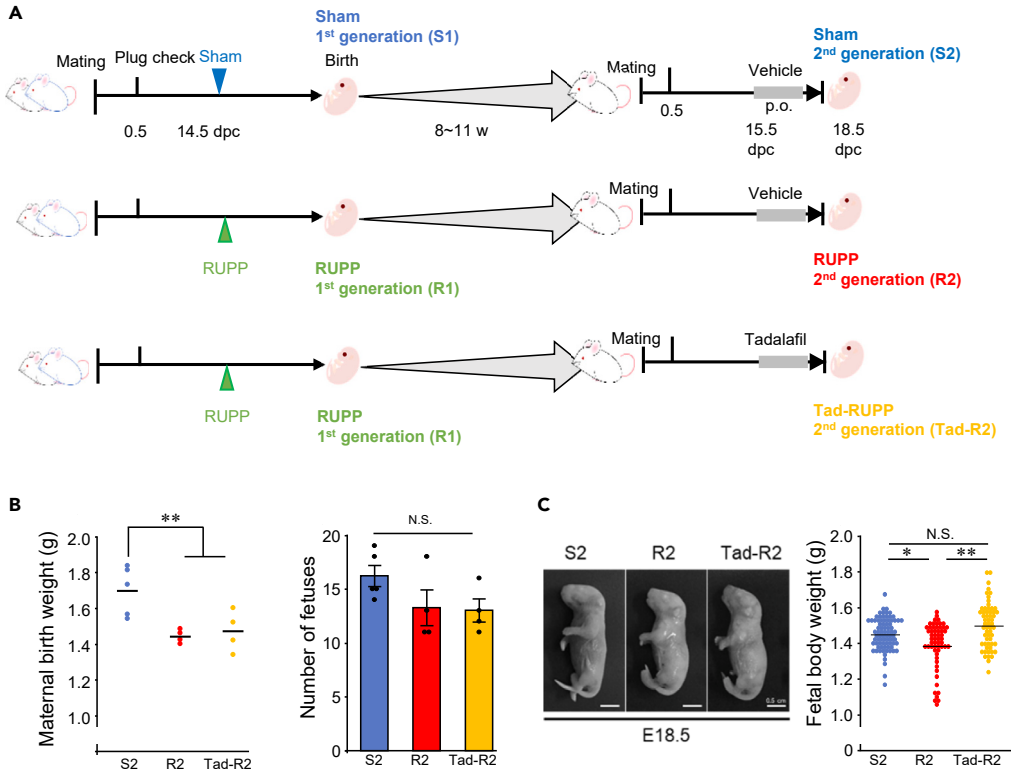


**Figure 7. Tadalafil ameliorated smaller fetal weight in low birthweight female mice**

(A) Experimental schedule. Female S1 and R1 mice were born spontaneously after the sham or RUPP surgery. The fetuses of S1 or R1 are designated as sham 2nd generation (S2) and RUPP 2nd generation (R2), respectively. Half of the S1 and R1 mice were orally administered tadalafil (10 mg/kg/day) daily starting from 15.5 dpc. The remaining S1 and R1 pregnant mice were orally administered 0.5% methyl cellulose 400 daily starting from 15.5 dpc. Mice were dissected at 18.5 dpc and fetal weights at embryonic day 18.5 (E18.5) and placental weights were recorded.

(B) Maternal birth weight (S1, R1, and Tad-R1). The maternal sample numbers of S2, R2, and Tad-R2 were $n = 5$, $n = 4$, and $n = 4$, respectively. Data are shown as the mean ± standard error of the mean. Student's t test; $^{**}p < 0.01$.

(C) Representative (E18.5) fetal images. Comparison of the fetal weights. The sample numbers of S2, R2, and Tad-R2 were $n = 79$, $n = 57$, and $n = 58$, respectively. The central line indicates the mean value. Steel-Dwass test; $^{*}p < 0.05$, $^{**}p < 0.01$.

energy homeostasis and DNA and protein synthesis,[56] the impaired purine metabolism pathway appears to be associated with the gestational liver hypertrophy failure in R1 mice. Impaired purine metabolism may be attributed to the hypoxia response in the liver of R1 mice because HIF-mediated responses promote energy flow to glycolysis[57] and the pentose phosphate pathway,[58] leading to an impaired purine synthesis pathway.[59] Indeed, our findings demonstrated the upregulation of genes involved in glycolysis and the pentose phosphate pathway in the liver of R1 mice.

Taurine is an important amino acid nutrient for fetal organ development, promoting the maturation of the fetal central nervous system, retina, and kidneys.[60,61] Maternal taurine in circulating blood is actively transported to the placenta and gradually transferred to the fetal organs. Because the fetus and placenta have a low capacity for taurine synthesis,[62] the taurine provided by the mother to the fetus via the placenta is essential for fetal organ development.[63] The present study showed an increase in the retention of taurine in the placenta, which might have contributed to the failure of taurine transport from the placenta to the fetus, thereby affecting the development of fetal organs, including the kidneys. In addition to the liver, the placenta of R1 mice showed defective angiogenesis and an upregulated hypoxia response. This pathological hypoxic response likely triggers metabolic changes in each organ, resulting in inadequate delivery of purines and taurine to the fetus, which could be a factor in the insufficient development of the fetus, including impaired kidney development.

In conclusion, our study highlights the importance of LBW-induced immature kidney and organ development by establishing an LBW animal model and demonstrating implications for adulthood, pregnancy, and subsequent generations. Developing therapeutic interventions for LBW is crucial for mitigating the adverse effects of LBW and immature organ development, thereby reducing the future risks associated with LBW (Figure 8).

## Limitations of the study

Our study has several limitations. One limitation of this study is that the results were obtained using a mouse model; therefore, further clinical studies in humans are necessary. Second limitation of this study is that because of the small size of the kidneys of fetal and post-natal mice,

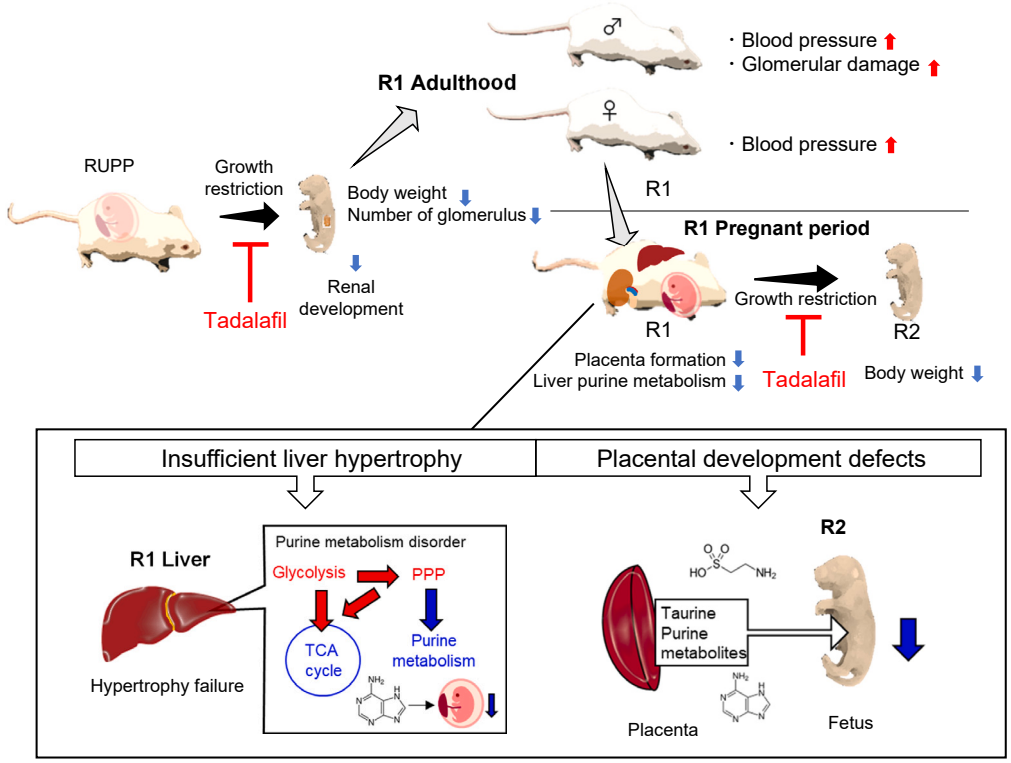


**Figure 8. Long-term impact of low birthweight mice and the subsequent effects on offspring**

Maternal mice subjected to RUPP operation give rise to low birthweight (LBW) pups with fetal growth restriction. The pups exhibit immature kidney development, with reduced glomerular count, glomerular damage, and elevated systolic blood pressure in adulthood. However, administration of tadalafil to the mother prevents these LBW-associated adverse effects. Furthermore, when LBW pups mature and become pregnant, they present with insufficient liver hypertrophy and placental developmental defects, leading to compromised fetal growth in the next generation.

the number of glomeruli was assessed by the number of glomerular profiles per unit area of tissue sections. However, this method may not accurately reflect the total number of glomeruli in the entire kidney. Although the physical scatter and fractionation method is considered a reliable method for analyzing glomerular number,[64] it is limitated in that it requires considerable time and effort for a single sample. Therefore, because the study required the analysis of multiple kidney samples, we used the glomerular profile per unit area of the tissue sections as a parameter. Third limitation of this study is that maternal administration of tadalafil improved LBW and subsequent weight gain in offspring, and reduced the increase in blood pressure during adulthood. However, these effects varied between the sexes. Notably, as these findings were obtained exclusively in mice, further research is needed to evaluate the safety and efficacy of tadalafil, including its differential effects on sex, before considering its clinical application in humans.

## RESOURCE AVAILABILITY

### Lead contact

Further information and any requests should be directed to and will be fulfilled by the lead contact, Emiko Sato (emiko.sato.b8@tohoku.ac.jp).

### Materials availability

This study did not generate new unique reagents.

### Data and code availability

GS-MS data files are available from DDBJ MetaboBank (https://mb2.ddbj.nig.ac.jp/study/MTBKS233.html). This paper dose not report original code. Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

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## AUTHOR CONTRIBUTIONS

A.S., T.Toyohara., E.M., and E.S. designed the study. A.S., Y.T., H.S., M.Ookawa, M.Y., M.Otsuka., D.S., T.F., Y.F., S.Y., and E.S. performed the experiments and analyzed the data. A.S., S.Y., K.T., T.I., T.Toyohara, T.Tanaka, N.T., E.M., and E.S. interpreted the data. A.S., E.M., and E.S. wrote the manuscript. T.I., T.Tanaka., and N.T. edited the manuscript. A.S., D.S., T.F., E.M., and E.S. provided funding for the project. All authors read the manuscript, provided feedback, and approved the final manuscript.

## DECLARATION OF INTERESTS

The authors declare that they have no conflicts of interest.

## STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

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## SUPPLEMENTAL INFORMATION

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# STAR★METHODS

## KEY RESOURCES TABLE

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---|---|---|
| Antibodies | | |
| Six2 | Proteintec | Cat # 11562-1-AP ; RRID: AB_2189084 |
| *Griffonia simplicifolia Lectin I-B4 Isolectin, Biotin Conjugate* | VECTORS lab | Cat #B1205; RRID: AB_2314661 |
| HIF-2 alpha/EPAS1 | NOVUS Biologicals | Cat#NB100-122; RRID: AB_10002593 |
| Chemicals, Peptides, and Recombinant Proteins | | |
| Methyl Cellulose 400 | Fujifilm | 132–05055 |
| Tadalafil | Sigma Aldrich | PHR1810 |
| Critical Commercial Assays | | |
| LIBIS Mouse Albumin ELISA Kit | Fujifilm Wako Shibayagi | AKRAL-121 |
| Deposited Data | | |
| https://ddbj.nig.ac.jp/public/metabobank/study/ | DDBJ MetaboBank | MTBKS233 |
| Experimental Models: Organisms/Strains | | |
| ICR strain | Clea-Japan | ICR strain |
| Oligonucleotides | | |
| See Table S1 for Primer | | |
| Software and Algorithms | | |
| JMP software | SAS Institute Inc | version 15.0.0 |
| ImageJ software | NIH | |
| SPSS software | IBM Corp | |

## EXPERIMENTAL MODEL AND STUDY PARTICIPANT DETAILS

Female mice aged 2–3 months were cohabitated with male mice, and the date of vaginal plug confirmation, an indicator of mating success, was set at 0.5 dpc.

Female mice were weighed daily and subjected to sham or RUPP operation at 14.5 dpc with anesthetization with isoflurane. The RUPP operation was performed with a slight modification to that which has been previously reported.[19,24] The RUPP operation was performed by ligating the uterine and ovarian vessels distal to the ovarian branches with a nylon threads 0.75 mm in diameter. The nylon thread was immediately pulled out to leave a small space and reduce the blood flow to the uterus (Figure S1). This model showed a slight increase in SBP and glomerular damage, but no urinary albumin excretion.[19] RUPP had little effect on the fetus and did not significantly affect survival among the three groups.

All animal experiments were approved by the Animal Committee of Tohoku University (Approval No. 2021PhA-001-02). Experimental protocols and animal care were performed according to the guidelines for the care and use of animals established by Tohoku University. ICR mice were purchased from CLEA Japan, Inc. (Tokyo, Japan). Animals were housed individually at 24°C on a 12:12 h light-dark cycle and fed a regular chow (MR stock: Nosan Corporation, Kanagawa, Japan) and water *ad libitum*.

## METHOD DETAILS

### Animal experiments

Half of the RUPP-operated mice were orally administered tadalafil (10 mg/kg/day) daily starting on the day after the operation. The remaining RUPP- and sham-operated mice were orally administered 0.5% methylcellulose 400 solution daily starting on the day after the operation. Tadalafil was administrated orally once in the morning based on our previous report.[19] Assignment to the RUPP and tadalafil treatment groups was random. The group allocations at different stages of the experiment were known to the animal operators. For the sham operation, the laparotomy was opened for the same amount of time required for the RUPP operation without stenosis, followed by suturing. Postoperatively, buprenorphine hydrochloride (0.002 mg) and antibiotics were administered. Immediately after the operation and at 15.5 dpc, 0.5 mL of rehydration solution containing 0.3% NaCl and 4% sucrose was administered orally.

After the operation, the pups were born spontaneously, and the pups of mothers who had undergone the sham or RUPP operation were designated as the sham 1st generation (S1) and RUPP 1st generation (R1) groups, respectively. In addition, pups derived from mice treated with tadalafil and RUPP were designated as the Tad-R1 group. After birth, rearing was continued. At 1 week of age, 1–3 pups/litter (total six pups/three litters), and at 20 weeks of age, 1 pup/litter (total 6–8 pups/6–8 litters) were dissected, and the kidneys were removed, weighted, and stored. A portion of the pregnant mice were dissected at 18.5 days of gestation, fetal weights were measured at E18.5, and the kidneys were removed and stored. SBP measurements were performed at 4, 10, and 20 weeks of age according to a previous method.[24] The experimental schedule is shown in Figure 1A. Sample sizes were determined based on our previous studies that provided statistically significant results.[19] Confounders were not controlled for in the study. The humane endpoints for the study were established as follows: prompt euthanasia was administered in cause of a weight loss of 20% or more, a decrease in drinking water intake due to wounds, a decrease in activity, or signs of abnormal breathing. Observations were performed daily.

### Blood pressure measurement

SBP was noninvasively measured between 10 a.m. and noon by determining tail blood volume using a volume pressure recording sensor and an occlusion tail cuff (CODA System; Kent Scientific, Torrington, CT, USA). Blood pressure was measured in the same mice at weeks 4, 10, and 20 weeks.

### Effects of LBW on the next generation

Spontaneously born female S1 and R1 mice were mated with ICR male mice when they were 7–13 weeks old, and then dissected at 13.5 dpc or 18.5 dpc, and the placenta, all fetuses (S2 or R2), liver, and kidneys were harvested (Figure 4A). The fetuses of S1 and R1 mice were defined as sham 2nd generation (S2) and RUPP 2nd generation (R2), respectively.

### Tadalafil treatment effects on fetal weight

Spontaneously born female S1 and R1 mice were mated with ICR male mice at 7–13 weeks of age. Half of the R1 pregnant mice were orally administered tadalafil (10 mg/kg/day) daily starting from 15.5 dpc. The remaining S1 and R1 pregnant mice were orally administered 0.5% methylcellulose 400 solution daily starting from 15.5 dpc. Mice were dissected at 18.5 dpc and fetal weights at E18.5 and placental weights were recorded (Figure 7A).

### High-salt diet loading experiments

Female S1 and R1 pups spontaneously born to sham or RUPP-operated mice were fed a 4% high-salt diet for approximately 4 weeks from 4 weeks of age, and then mated and fed a normal diet until 18.5 dpc (Figure S6A). Female S1 and R1 pups spontaneously born to sham or RUPP-operated mice were fed an 8% high-salt diet for approximately 4 weeks from 4 weeks of age, and then mated and fed an 8% high-salt diet until 18.5 dpc (Figure S7A). At 18.5 dpc, maternal mice were dissected, and the placenta, all fetuses (male and female), and maternal kidneys were harvested.

### Quantitative real-time PCR analysis

The liver, kidneys, and placenta were randomly selected from 6 to 12 samples in each group. One milliliter of TRI Reagent (Molecular Research Center Inc., Cincinnati, OH, USA) was used for RNA extraction, according to the manufacturer's protocol. For reverse transcription, an Advanced cDNA Synthesis Kit for RT-qPCR (Bio-Rad Laboratories, Inc., Hercules, CA, USA) was used, according to the manufacturer's protocol. For quantitative real-time PCR (qRT-PCR), primers for hypoxanthine-guanine phosphoribosyl transferase (*Hprt*), *Six2,* and 6-phosphogluconate dehydrogenase (*Pgd*), *Tnf,* ribosomal protein S18 (*Rps18*), transaldolase 1 (*Taldo*1), and glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*) were purchased from Takara (Kusatsu, Japan), and primers for *Plgf*, *Angpt1*, *Kdr*, *Tie2*, *Vegfa*, *CD31*, *TauT*, *Hif1*, *Aprt,* and *Prps1* were synthesized by Fasmac Co., Ltd (Kanagawa, Japan), and their set IDs and sequence are listed in Table S1. Gene expression was evaluated using Luna Universal qPCR Master Mix (NEW ENGLAND BioLabs, Massachusetts, USA) on a Bio-Rad CFX real-time PCR system. *Hprt* or *Rps 18* was used as a reference. The cycling conditions were as follows: preliminary denaturation at 95°C for 60 s followed by 40 cycles of denaturation at 95°C for 15 s, and annealing and extension was 60°C for 30 s. The data were analyzed using the calibration curve method.

### Renal histological analysis

The kidneys were fixed with 2% paraformaldehyde, and paraffin embedding, and H&E and PAS staining were performed at the Laboratory Animal Pathology Platform, Common Equipment Office, Tohoku University Graduate School of Medicine. H&E– or PAS-stained sections were used for glomerular evaluation, and the sections were observed and photographed using a Keyence BZ-X800 (KEYENCE Corporation, Osaka, Japan). The number of glomeruli was counted and the cortical area was calculated using the following equation.

$$Glomerular\ density = \frac{Number\ of\ glomeruli}{Cortical\ area\ (mm^2)}$$

To evaluate the open capillary and PAS-positive areas, 20 glomeruli from one section were randomly selected. The open capillary area was calculated using the following formula:

$$\text{Open capillary area } (\%) = \frac{\text{Open capillary area}}{\text{Glomerular tuft area}} \times 100$$

The PAS-positive area was designated as the mesangial area, and the following formula was used to calculate the percentage of each mesangial area relative to the glomerular tuft area:

$$\text{Mesangial area } (\%) = \frac{\text{PAS} - \text{positive area}}{\text{Glomeruar tuft area}} \times 100$$

### Fetal kidney fluorescence immunostaining

The kidneys collected from E18.5 fetuses were embedded in Tissue-Tek O.T.C Compound (Sakura Finetek Japan Co., Ltd., Tokyo, Japan) and stored at −20°C. The 6.0 μm kidney sections were washed three times with phosphate buffered saline (PBS) and then incubated with Protein Block Serum-Free (Dako North America, Inc., Carpinteria, CA, USA). Next, 500-fold diluted Six2 (11562-1-AP; Proteintech Group, Inc., Rosemont, IL, USA) or 500-fold diluted LTL-fluorescein (LTL-FITC) (FL-1321; Vector Laboratories, Inc., Burlingame, CA, USA) and 500–fold diluted E-cadherin (610181; Becton Dickinson and Company, Franklin Lakes, NJ, USA) antibodies were added and incubated overnight at 4°C. Two secondary antibodies, Alexa Fluor 488 goat anti-rabbit IgG (H + L) and Alexa Fluor 555 goat anti-mouse IgG (H + L) (Life Technologies Corporation, Eugene, OR, USA), were diluted 2000-fold. Secondary antibodies were used in the combinations described in Table S2. The sections were encapsulated using DAPI Fluoromount-G and observed and photographed using a Keyence BZ-X800. Sections stained with LTL-FITC were quantified for the LTL-positive area using ImageJ software (National Institutes of Health, Bethesda, MD, USA). Proximal tubule development was assessed by calculating the percentage of LTL-positive area using the following formula:

$$\text{LTL positive area ratio } (\%) = \frac{\text{LTL} - \text{positive area}}{\text{cortical area}} \times 100$$

### Placenta immunohistochemistry staining

*Griffonia simplicifolia* Lectin I-B4 Isolectin, Biotin Conjugate antibody (1:50, GSL I-B4 Isolectin #B1205; VECTORS Lab) and HIF-2 alpha/EPAS1 antibody (1:100, #NB100-122; NOVUS Biologicals, CO, USA) were used. Heat-induced antigen retrieval was performed using sodium citrate buffer to detect GSL I-B4 Isolectin. Proteinase K (Dako) was used to detect HIF-2 alpha. The primary antibodies were incubated overnight at 4 °C. Streptavidin-HRP was used as a secondary antibody to detect GSL I-B4 Isolectin. An N-Histofine Simple Stain kit (Nichirei Biosciences, Tokyo, Japan) was used as the secondary antibody according to the manufacturer's protocol for detect HIF-2 alpha detection. Sections were incubated without primary antibody as a negative control. The positive areas of the GSL I-B4 Isolectin and HIF-2 alpha were assessed using ImageJ software.

### Sample preparation for GC-MS

Fifty micrograms of placenta or liver was homogenized with 250 μL of a solution containing 55% methanol and 22% chloroform dissolved in distilled water containing 0.5 mg/mL 2-isopropylmalate (10 mL; Sigma-Aldrich). The mixture was then, dissolved in distilled water and incubated in a Thermomixier C (Eppendorf) at 37°C with shaking ad 1200 rpm for 30 min. The samples were then centrifuged at 16,000 × *g* for 3 min at 4°C. Two hundred and twenty-five microliters of supernatant were collected and added to 200 μL of distilled water. The sample was dried using an evaporator under reduced pressure and lyophilized. For oximation, 20 mg/mL methoxyamine hydrochloride (80 μL; Sigma-Aldrich) dissolved in pyridine was mixed with the lyophilized sample, sonicated for 20 min, and shaken at 1,200 rpm for 90 min at 30°C. Next, 40 μL of N-methyl-N-trimethylsilyl-trifluoroacetamide (GL Sciences, Tokyo, Japan) was added for derivatization. The mixture was then mixed at 1,200 rpm for 30 min at 37°C, centrifuged at 16,000 × g for 5 min at 4°C, and the resulting supernatant (1 μL) was subjected to GC-MS/MS.

### Analysis of purine bases and nucleotides

This measurement was based on our previously reported methods.[65,66] GC-MS/MS analysis was performed using a GC-MS/MS TQ8040 (Shimadzu) with a fused silica capillary column (BPX-5; 30 m × 0.25 mm inner diameter, film thickness: 0.25 μm; Shimadzu), and a front inlet temperature of 250°C, and a helium gas flow rate through the column of 39.0 cm/s. The column temperature was held at 60°C for 2 min, then raised by 15°C/min to 330°C and maintained for 3 min. The interface and ion source temperatures were 280°C and 200°C, respectively. All data obtained by GC-MS/MS analysis were analyzed by TraverseMS (Reifycs Inc., Tokyo, Japan). The retention times indicated in the Smart Metabolites Database (Shimadzu) were used as references to create a library for the data analysis. To perform a semi-quantitative assessment, the peak area of each quantified ion was calculated and normalized using the 2-isopropylmalate peak area. Analysis of purine bodies were performed according to the previous methods.[67]

### Biochemical measurement of samples

Urine was collected as spot urine prior to dissection. Urinary albumin concentrations were determined with the LIBIS Mouse Albumin ELISA Kit (AKRAL-121, Fujifilm Wako Shibayagi, Gunma, Japan). Urine was diluted 50–100 times and measured according to manufacturer's protocol. Urinary albumin was corrected for urinary creatinine levels.

### Urinary creatinine measurement

For sample preparation, 150 μL of 0.1% formate methanol containing 2.0 μg/mL creatinine-$d_3$ were added to 50 μL of diluted urine and vortexed for 1 s. Then, the samples were sonicated for 5min and centrifuged at 16,400 ×g for 20 min at 4°C. The supernatant was filtered through membranes. Quantitative analysis of creatinine was performed using Nanospace SI-2 3033 coupled to a TSQ-Quantum Ultra mass spectrometer and operated in positive mode. For creatinine measurement, each sample (1 μL) was injected into 150 × 2.0 mm YMC-PACK Pro C18, 3-μm column (YMC Co., Ltd. Tokyo, Japan) with YMC semi-micro cartridge Pro C18 10 × 2.0 mm (YMC Co., Ltd.) at a flow rate of 0.1 mL/min. The transitions of the precursor ion to the product ion and collision energy were monitored: *m/z* 114 to 44, 17 V for creatinine; *m/z* 117 to 47, 17 V for creatinine-$d_3$.

## QUANTIFICATION AND STATISTICAL ANALYSIS

Data were analyzed using JMP software version 15.0.0 (SAS Institute Inc., Cary, NC, USA). Differences were considered statistically significant at $p < 0.05$. Student's t test was used for two variables comparisons. Statistical comparisons of multiple groups were performed using analysis of variance (ANOVA), followed by the Tukey–Kramer test for normal distribution. In the case of a nonnormal distribution, the Wilcoxon test, followed by the Steel–Dwass test was used. Values are presented as the mean ± standard error of the mean. Outliers were defined as observations that fell below Q1–1.5 interquartile range or above the Q3+1.5 interquartile range. two-way ANOVA followed by Bonferroni analysis was used to analyze the blood pressure assessment and weight change over time using SPSS software (IBM Corp., IL, USA). The detail of each statistical test is provided in each figure legend.