Intrauterine Low-Protein Diet Exacerbates Abnormal Development of the Urinary System in *Gen1*-Mutant Mice

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Keywords

Intrauterine low-protein diet · Intrauterine growth retardation · Congenital anomalies of the kidney and urinary tract · *Gen1*

Abstract

Background: *Gen1* mutation can cause various phenotypes of congenital anomaly of the kidney and urinary tract (CAKUT). An intrauterine low-protein isocaloric diet can also cause CAKUT phenotypes in offspring. However, single factors such as gene mutation or abnormal environmental factor during pregnancy can only explain part of the pathogenesis of CAKUT. **Objectives:** A low-protein isocaloric diet was fed to *Gen1*-mutant mice throughout pregnancy to establish a *Gen1*-mutant mouse model exposed to a low-protein isocaloric intrauterine environment. The mice were divided into 4 groups: normal (22%) protein diet (ND) + wild-type mice (CON group), ND + *Gen1*^{PB/+} mice (*Gen1*^{PB/+} group), low (6%)-protein isocaloric diet (LD) + wild-type mice (LD group), and the LD + *Gen1*^{PB/+} groups. **Methods:** The experimental design included observing proportion and distribution of CAKUT phenotypes of neonatal mice; evaluating the number of ureteric buds (UBs) on embryonic day (E) 11.5, the lo-

cation of UBs on E11.5, and length of the common nephric duct (CND); isolating embryonic kidneys on E11.5 from the *Gen1*^{PB/+} group and culturing embryonic kidneys in medium containing 10% serum or serum-free medium to observe the branching of UBs; and detecting the p-PLCγ, p-Akt, and p-ERK1/2 in UBs and CND on E11.5, as well as the apoptosis and proliferation of tissues by immunofluorescence staining. **Results:** We found that the incidence of CAKUT in offspring of *Gen1*^{PB/+} mice under an intrauterine low-protein isocaloric diet environment was significantly increased, and a duplicated collecting system was the dominant phenotype of CAKUT. During the early stage of metanephric development, ectopic protrusion of UBs may appear and lower locations of UBs in *Gen1*^{PB/+} mice under an intrauterine low-protein isocaloric diet environment and the number of UB branches in the serum-free culture condition significantly decreased. Further examination revealed that p-PLCγ signaling and tissue apoptosis were abnormal in UBs and the CND at the early stage of kidney development. **Conclusions:** The aforementioned findings suggest that an intrauterine low-protein isocaloric diet can aggravate the occurrence of CAKUT in *Gen1*-mutant mice, which might affect key steps in the meta-

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nephric development, such as the protrusion of UBs, which might be related to mediate UBs and CND apoptosis through p-PLC γ signaling.

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Introduction

Congenital anomaly of the kidney and urinary tract (CAKUT) is a common congenital malformation and is the leading cause of chronic kidney disease and end-stage renal disease in children [1, 2]. It can account for 20–30% of fetal malformations in prenatal diagnoses, and the incidence of CAKUT is approximately 3–6 per 1,000 live births [3, 4].

CAKUT often has the characteristics of familial aggregation and is congenital. CAKUT may be a part of the phenotype of syndromes caused by single-gene mutation, and the CAKUT phenotype has been seen in a single-gene-mutation animal model. Therefore, some scholars have speculated that CAKUT can be caused by single-gene mutations. However, the most common mutations of CAKUT can only explain 14–20% of CAKUT, while copy number variation can explain 16% of CAKUT. In addition, a retrospective analysis with a large population size also found some adverse factors during pregnancy that were associated with CAKUT, such as an intrauterine low-protein diet [5], intrauterine hypoxia [6], and intrauterine hyperglycemia [7]. It is believed that the pathogenic factors of CAKUT include gene mutations and environmental factors [8–10], but gene mutation or environmental factors themselves cannot fully explain the pathogenic mechanism of CAKUT [11, 12]. In animal models of gene mutations, the differences in the CAKUT phenotype are also very large, which further suggests that the interaction between genes and environment and that between genes and genes may partially explain the pathogenesis of CAKUT [13].

Our group has used a low (6%)-protein isocaloric diet to successfully construct a mouse model of intrauterine growth retardation and confirmed that the intrauterine low-protein isocaloric diet causes an increase in the number of CAKUT mice in the offspring [5]. These offspring have multiple phenotypes of CAKUT, such as duplex kidney and hydronephrosis. In addition, to explore the interaction between genes and environment, a 6% protein isocaloric diet has been used to successfully construct an intrauterine low-protein isocaloric diet *roundabout guidance receptor 2* (*Robo2*)-mutant mice [14]. The study confirmed that the intrauterine low-protein isocaloric

diet increased the incidence of CAKUT in offspring of *Robo2*-mutant mice, accompanied by abnormally expressed key signaling molecules in metanephric development. These findings suggest that an adverse intrauterine environment can increase the incidence of gene mutation to a certain extent, resulting in a significantly increased risk of CAKUT in offspring. Does an intrauterine adverse environment increase the incidence of CAKUT in the offspring of mice with other gene mutation?

Gen1 (Holliday junction 5' flap endonuclease) is a nuclease that is responsible for the opening of Holliday junctions during DNA homologous recombination [15, 16]. Our research group insert the piggyBac (PB) transposon into the second intron of *Gen1* to suppress *Gen1* expression, and *Gen1*^{PB/PB} and *Gen1*^{PB/+} mice were obtained. We also found, for the first time, that *Gen1*^{PB/PB} and *Gen1*^{PB/+} mice had multiple CAKUT phenotypes [17–19]. Does an intrauterine adverse environment increase the incidence of CAKUT in the offspring of mice with *Gen1* gene mutation?

Therefore, this study aimed to establish an intrauterine low-protein isocaloric diet-fed *Gen1*-mutant mouse model to observe postnatal urinary system malformations and abnormalities throughout the early stage of kidney development to determine whether the intrauterine low-protein isocaloric diet would increase the incidence of CAKUT in *Gen1*-mutant mice, as well as to explore its underlying mechanism.

Materials and Methods

Intrauterine Low-Protein Diet Gen1-Mutant Mouse Model

In *Gen1*-mutant FVB/NJ mice, PB was inserted into the second intron of the *Gen1* gene. *Hoxb7*/myr-Venus mice expressing a fluorescent protein were donated by Dr. Costantini (Department of Genetics and Development, Columbia University, USA). *Gen1*^{PB/+} mice were hybridized with *Hoxb7*/myr-Venus mice to obtain *Gen1*-heterozygous-mutant mice (*Gen1*^{PB/+}; *Hoxb7*/myr-Venus, abbreviated as *Gen1*^{PB/+}; *Hoxb7*) and wild-type mice (*Gen1*^{+/+}; *Hoxb7*/myr-Venus, abbreviated as *Gen1*^{+/+}; *Hoxb7*) in which epithelial cells of the urinary system were labeled with fluorescent protein. The genotype was identified as previously described [17–19]. The experimental mice were bred in a specific pathogen-free-grade animal room. The protocol was approved by the welfare and use of experimental animals issued by the Animal Care and Use Committee of the Institute of Developmental Biology and Molecular Medicine at Fudan University (Permit number: SYXK [hu]2020-0011). And, 6- to 8-week-old mice with the same genetic type were housed in the same cage at a female-to-male ratio of 3:1–4:1. The female mice were observed for pregnancy at 8 a.m./p.m. The earliest time when a vaginal plug was observed was recorded as embryonic day (E) 0.5. After the vaginal plug was observed, the female mice were randomly divided into the experimental group and the

Fig. 1. Effect of diets and *Gen1*-mutant on embryos, newborn pups, and pregnant mice. **a** Body weight of newborn pups. **b** Number of pups at birth. **c** Crown-rump length of the embryonic mice at E 11.5. **d** Number of embryos at E11.5. **e** Pregnancy cycle. **f** Pregnancy rate in the following 4 groups: the CON group (*Gen1*^{+/+}; Hoxb7 mice with routine 22% protein diet), the LD group (*Gen1*^{+/+};

Hoxb7 mice with 6% low-protein isocaloric diet), the LD + *Gen1*^{PB/+} group (*Gen1*^{PB/+}; Hoxb7 mice with 6% low-protein isocaloric diet), and the *Gen1*^{PB/+} group (*Gen1*^{PB/+}; Hoxb7 mice with routine 22% protein diet). Data are shown as means ± SE. ns, not significant; E, embryonic day.

CON group. The experimental group was given 6% low-protein isocaloric diet (Double Lion Experimental Animal Feed Technology Co., Ltd.) throughout pregnancy [5, 14], and the CON group was given the normal 22% protein diet (Suzhou Su-Hang Science and Technology Equipment Co., Ltd.) throughout pregnancy until natural birth. Both groups of pregnant mice had free access to food and water. Starting from E16.5, the pregnant mice were observed at 8 a.m./p.m. every day to see whether pups had been delivered. The earliest time when the newborn pups were observed was marked as postnatal day 0.5 (day P0.5).

Postnatal Gross Phenotype Analysis

After the newborn mice in the 4 groups were euthanized using CO₂, the urinary tract was dissected through a midline incision in the anterior abdomen. The position, morphology, and number of kidneys; the morphology and number of ureters; and the morphology of the bladder of the newborn pups were observed under a fluorescence microscope.

Embryonic Kidney Phenotype Analysis

Pregnant mice at E11.5 were anesthetized using CO₂. Dissection was performed layer by layer, the uteri were isolated, the embryos were removed, and the fetal membranes were retained for

genotype identification. Genotypes were determined as previously described [17–19]. The embryonic kidneys were photographed and observed for the number and location of ureteric buds (UBs), common nephric duct (CND) length, and number of UB branches under a fluorescence stereomicroscope.

Embryonic Kidney Culture

The E11.5 embryonic kidneys from the *Gen1*^{PB/+} group were isolated and cultured in the absence of serum [20, 21] or in the presence of 10% fetal bovine serum with Dulbecco's modified Eagle's medium/Nutrient Mixture F-12 (DMEM/F-12) (Gibco) in Transwell for 72 h. The culture condition was 5% CO₂ at 37°C. Pictures were taken every 24 h under a fluorescence microscope (Leica).

Immunofluorescence Staining

The E11.5 embryonic kidneys from mice were isolated under a stereomicroscope and fixed in formaldehyde overnight. After washing with 0.3% Triton X-100, tissues were incubated in a shaker at 4°C overnight in 5% donkey serum to block nonspecific antigen binding. The tissues were incubated with anti-caspase-3 antibody (CST, #9662, 1:400), anti-Phh3 antibody (CST, #3377, 1:800), phosphorylated (p-)Akt antibody (CST, #9272, 1:200), phosphory-

Fig. 2. Analysis of new born mice phenotypes in 4 groups at P 0.5d. **a** LD + Gen1^{PB/+} group (LD + Gen1^{PB/+}). **b** LD group (LD). **c** Gen1^{PB/+} group (Gen1^{PB/+}). **d** Gen1^{+/+} group (CON). P, postnatal day.

lated extracellular signal-regulated kinase 1/2 (p-ERK1/2) antibody (CST, #4695, 1:200), and phospho-PLCγ (p-PLCγ) antibody (R & D systems, #MAB74542, 1:100) in a shaker at 4°C overnight. The tissues were washed with PBS and incubated with Cy5-donkey anti-rabbit antibody (Jackson, 1:1,000). DAPI (1:1,000) was used to stain the nuclei. Five embryonic kidneys from each group were used. The samples were mounted with anti-fade reagent. Images were taken under a confocal microscope. NIH ImageJ software was used for data analysis.

Statistical Analysis

All data were analyzed by GraphPad7 and SPSS. All results were expressed as means ± standard error of the mean. $p > 0.05$ was not significantly different, while $p < 0.05$ was considered significantly different. An unpaired t -test was used for data analysis between groups.

Results

Successful Construction of Intrauterine Low-Protein Isocaloric Diet Gen1-Mutant Mouse Model

Gen1^{PB/+}; Hoxb7 pregnant mice were given a low-protein isocaloric diet throughout pregnancy to successfully construct an intrauterine low-protein isocaloric diet Gen1-mutant mice model (LD + Gen1^{PB/+}). The LD + Gen1^{PB/+} neonates weighed significantly less than the Gen1^{PB/+} neonates (0.9526 ± 0.1174 g vs. 1.277 ± 0.121 g, $p < 0.0001$) (shown in Fig. 1a). The number of neonatal mice at birth in the LD + Gen1^{PB/+} group was lower than that in the Gen1^{PB/+} group (4 [3, 7] vs. 8 [8, 9], $p = 0.0065$) (shown in Fig. 1b). The intrauterine low-protein isocalo-

Fig. 3. Analysis of new born mice phenotypes in the LD + Gen1^{PB/+} group. **b, d, f** Visualized by Hoxb7/myr-Venus expression. **a** Solitary kidney. **b** Normal kidney. **c** Unilateral duplicated collecting system. **d** Unilateral duplicated collecting system. **e** Unilateral duplicated collecting system with hydronephrosis and megaureter.

f Unilateral duplicated collecting system with hydronephrosis and megaureter. White arrowheads point to the normal Ur, red arrowheads point to duplicated Ki, or double Urs, or hydronephrosis, or megaureter. Bl, bladder; Ki, kidney; Ur, ureter.

ric diet did not have a significant effect on the pregnancy rate, gestation period, crown-rump length on E11.5, the number of E11.5 embryos, the number of mice at birth, or the gestation period in Gen1^{PB/+} mice (shown in Fig. 1c-f).

Increased Incidence of CAKUT in Neonatal Mice of the LD + Gen1^{PB/+} Group

The proportion of neonatal mice with CAKUT in the LD + Gen1^{PB/+} group was slightly higher than that in the Gen1^{PB/+} group (37.74% [20/53] vs. 25.69% [28/109], $p = 0.0620$), while the proportion of duplicated collecting systems was significantly higher (28.30% [15/53] vs. 13.76%

Fig. 4. Comparison of the number and location of UB in 4 groups. **a** Analysis at E11.5 shows different types of ectopic UBs and normal UB in LD + Gen1^{PB/+} group embryos (visualized by Hoxb7/myr-Venus expression). **b** Culturing the ectopic UBs of E11.5 LD + Gen1^{PB/+} embryonic kidney under 10% serum condition for 72 h. The nephric duct is shown in lateral view (dorsal to the right).

White arrow line points to the normal UB. Red arrow line points to ectopic UBs. **c** Analysis at E11.5 shows a lower located in the LD + Gen1^{PB/+} group, red dashed lines represent length of the CND; each value is expressed as mean $\pm$ SE. CND, common nephric duct; ND, nephric duct; UB, ureteric bud.

[15/109], $p = 0.0300$). The proportion of neonatal mice with CAKUT in the LD + Gen1^{PB/+} group was significantly higher than that in the LD group (37.74% [20/53] vs. 18.03% [11/61], $p = 0.0180$), and the incidence of duplicated collecting systems was also significantly higher (28.30% [15/53] vs. 11.48% [7/61], $p = 0.0170$) (shown in Fig. 2, 3).

Increased Duplicated Budding of UBs, Lower UB Location, and Fewer UB Branches

Duplicated budding on E11.5 was significantly more in the LD + Gen1^{PB/+} group than the Gen1^{PB/+}, LD, and CON groups (respectively, 39.13% [18/46] vs. 19.70% [13/66], $p = 0.0240$; 39.13% [18/46] vs. 5.41% [2/37], $p <$

0.0001; and 39.13% [18/46] vs. 0.91% [1/110], $p < 0.0001$) (shown in Fig. 4a). Culturing the E11.5 embryonic kidneys of the duplicated budding for 72 h in vitro, the ectopic budding could branch stepwise (shown in Fig. 4b). Compared with the location of UB protrusions in the Gen1^{PB/+}, LD, and CON groups of single UB protrusion on E11.5, except for the duplicated budding, we found the location of UB protrusions in the LD + Gen1^{PB/+} group was lower, and the length of CND in the LD + Gen1^{PB/+} group was longer (respectively, $293.9 \pm 85.29 \mu\text{m}$ vs. $185.6 \pm 81.29 \mu\text{m}$, $p = 0.0004$; $293.9 \pm 85.29 \mu\text{m}$ vs. $227.6 \pm 52.84 \mu\text{m}$, $p = 0.0046$; and $293.9 \pm 85.29 \mu\text{m}$ vs. $205 \pm 40.14 \mu\text{m}$, $p < 0.0001$) (shown in Fig. 4c).

Fig. 5. Comparison of branching in 2 groups in vitro. **a, b** In vitro culture of the E11.5 embryonic kidney of the Gen1^{PB/+} group under serum-free or 10% serum-containing. White arrow points to the

UB tips. Red arrow points to the BP. Each value is expressed as mean $\pm$ SE. CND, common nephric duct; ND, nephric duct; UB, ureteric bud; BP, branching point; TIPS, UB tips.

After culturing the E11.5 embryonic kidneys of the Gen1^{PB/+} group for 24, 48, and 72 h in vitro, the protrusion and branching of UBs were observed. After culturing for 24 h, the number of UB branches was significantly decreased (BP: $p = 0.0309$) in serum-free culture compared to culture with medium containing 10% serum. After culturing for 48 h, the number of UB branches and UB tips was significantly decreased in serum-free culture compared to 10% serum culture (BP: $p = 0.0016$; UB tips: $p = 0.0015$). After culturing for 72 h, the number of UB branches and UB tips was more significantly decreased in serum-free culture compared to 10% serum culture (BP: $p = 0.0044$; UB tips: $p = 0.0017$) (shown in Fig. 5).

Reduced Activity of p-PLC γ and Increased Apoptosis during the Protrusion of UBs in the LD + Gen1^{PB/+} Group

Immunofluorescence was used to detect the levels of p-PLC γ , p-Akt, and p-ERK1/2 in the UBs and the CND on E11.5 in the LD + Gen1^{PB/+}, Gen1^{PB/+}, and LD groups. The results showed that p-PLC γ was downregulated in the LD + Gen1^{PB/+} group compared to the Gen1^{PB/+} group and the LD group (UB: $p = 0.0385$, $p < 0.0001$; CND: $p = 0.0482$, $p = 0.0064$, respectively) (shown in Fig. 6). p-Akt and p-ERK1/2 did not show significant differences between the LD + Gen1^{PB/+} group and the Gen1^{PB/+} group.

The activation of the p-PLC γ signal is associated with tissue cell proliferation and apoptosis. Therefore, immunofluorescence was used to detect the apoptosis and pro-

liferation of UBs and the CND on E11.5 in the LD + Gen1^{PB/+}, Gen1^{PB/+}, and LD groups. UB apoptosis was higher in the LD + Gen1^{PB/+} group ($p = 0.0377$) than in the Gen1^{PB/+} group. CND apoptosis was higher in the LD + Gen1^{PB/+} group than in the Gen1^{PB/+} and LD groups ($p = 0.0037$; $p = 0.0040$, respectively) (shown in Fig. 7). There was no significant difference in cell proliferation between the LD + Gen1^{PB/+} group and the Gen1^{PB/+} group.

Discussion/Conclusion

In this study, a low (6%)-protein isocaloric diet was provided throughout pregnancy to successfully construct an intrauterine low-protein isocaloric diet Gen1^{PB/+} neonatal mouse model. It was found that under environmental stimulation with an intrauterine low-protein isocaloric diet, the incidence of CAKUT was increased in offspring of Gen1-mutant mice, and a duplicated collecting system was the dominant CAKUT phenotype. In the early stage of metanephric development, ectopic protrusion of UBs may appear and lower locations of UBs. The intrauterine low-protein isocaloric diet environment was simulated in vitro using serum-free culture medium [20, 21]. The culture experiments with Gen1^{PB/+} embryonic kidneys showed that the number of UB branches in the serum-free culture was significantly decreased compared to when they were cultured in 10% serum medium. Further examination revealed that p-PLC γ signaling and tis-

Fig. 6. Detect the expression levels of p-Plcγ in UB and CND tissues at E11.5 embryonic kidney by immunofluorescence. **a–c** Detect the p-Plcγ in 4 groups at E11.5. Each value is expressed as mean ± SE. CND, common nephric duct; ND, nephric duct; UB, ureteric bud. Five embryonic kidneys from each group were used.

Fig. 7. Detect the apoptosis in UB and CND tissues at E11.5 embryonic kidney by immunofluorescence. **a–c** Detect the apoptosis in groups. Each value is expressed as mean $\pm$ SE. CND, common nephric duct; ND, nephric duct; UB, ureteric bud. Five embryonic kidneys from each group were used.

sue apoptosis were abnormal in UBs and the CND at the early stage of kidney development. The aforementioned findings suggest that an intrauterine low-protein isocaloric diet can aggravate the occurrence of CAKUT in *Gen1*-mutant mice, which might affect key steps in the metanephric development, such as the protrusion of UBs, and mediate UB and CND apoptosis through p-PLC γ signaling, which might be one possible mechanism underlying the increase in CAKUT occurrence in *Gen1*-mutant mice due to the intrauterine low-protein isocaloric diet.

Currently, most scholars believe that the pathogenic factors of CAKUT include gene mutations and environmental factors [8]. However, genetic factors or environmental factors can only explain some of the pathogenesis of CAKUT. A retrospective analysis in a large population has found some adverse outcome factors associated with CAKUT during pregnancy. Some adverse intrauterine conditions have been confirmed in animal models. For example, previous studies by our team demonstrated that the intrauterine low-protein isocaloric diet causes an increased incidence of CAKUT in offspring mice [5]. A variety of CAKUT phenotypes, dominated by duplex kidney, appear, accompanied by metanephric developmental abnormalities such as ectopic budding and abnormal expression of key signaling molecules in metanephric development. By the construction of an animal model of intrauterine hypoxia and gestational diabetes, it was also found that hypoxia and high-glucose environmental stimulations can increase the risk of CAKUT in offspring mice [6, 7]. In recent years, a number of pathogenic genes related to abnormal development of the urinary system have been identified by genetic screening for CAKUT population and animal model construction. The most common gene mutations in CAKUT, such as paired box gene 2 and hepatocyte nuclear factor 1 β , can only explain 14–20% of CAKUT [10, 11]. Copy number variation mutations can explain 16% of CAKUT [12]. Gene mutations are not even detected in approximately 50–60% of CAKUT, or the pathogenicity of some detected gene mutations is too weak to be confirmed as pathogenic genes of CAKUT [9]. CAKUT genetic heterogeneity causes many difficulties in the study of the abnormal development of the urinary system. In clinical practice, even with the same CAKUT pathogenic gene locus, the ages of disease onset, the CAKUT phenotypes, and the severity of CAKUT in different families or different members of the same family may be completely different. In addition, in gene-mutant animal models, the CAKUT phenotypes also vary widely, which further suggests that interactions between genes and environment and those between genes

and genes may explain part of the mechanism of CAKUT pathogenesis [13].

In the previous studies, our team preliminarily explored the interaction between genes and environment by constructing a *Robo2*-mutant mouse model in an adverse intrauterine environment and found that under the stimulation of a low-protein diet [14], the incidence of CAKUT in the offspring of *Robo2*-mutant mice was significantly increased, and the phenotype was dominated by a duplicated collecting system, accompanied by the duplicated protrusion of UBs at an early stage, lower UB protrusions, and fewer UB branches. These findings suggested that an intrauterine adverse environment could increase the impact of *Robo2* gene mutations to a certain extent, resulting in a significant increase in the risk of CAKUT in the offspring. To find out whether an adverse intrauterine environment increases the incidence of CAKUT in the offspring of mice with other gene mutations, this study constructed a *Gen1*-mutant mouse model in an adverse intrauterine environment. Under low-protein dietary stimulation, the proportion of CAKUT mice in the offspring of *Gen1*-mutant mice was increased, and the CAKUT phenotype was mainly dominated by duplex kidney, accompanied by duplicated protrusion of UBs at an early stage, lower UB protrusions, and fewer UB branches. These findings suggest that a low-protein diet could lead to an increased risk of CAKUT in the offspring of *Gen1*-mutated mice. These results further indicate that a low-protein diet, as an environmental stimulus, could significantly increase the risk of CAKUT in offspring of mice with different gene mutants.

The correct protrusion of UBs and the process of stepwise branching are regulated by a complex signaling network in which the glial cell-derived neurotrophic factor / rearranged during transfection (RET) signal is critical [22, 23]. RET is a transmembrane receptor tyrosine kinase. RET activation can participate in the regulation of the normal development of tissues and organs, and its abnormal expression can lead to the occurrence of many congenital diseases, such as CAKUT [24]. Akt, PLC γ , ERK1/2 mitogen-activated protein kinase, and SRC are the main signaling molecules in the cytoplasm that are activated after RET activation [24]. These molecules participate in the regulation of cell proliferation, apoptosis, differentiation, and migration and maintain normal tissue development. Abnormal proliferation and apoptosis can affect UB protrusion and cause different phenotypes of CAKUT [25]. A low-protein diet can cause abnormal expression of key molecules in kidney development in *Robo2*-mutant mice and mainly affect key steps in meta-

nephric development, such as UB protrusion, and mediate UB tissue apoptosis through Akt/Creb3 signaling [14]. How can low-protein diets as environmental stimuli significantly increase the risk of CAKUT in the offspring of *Gen1* mutated mice? Is the mechanism similar to that in *Robo2*-mutant mice? We measured the activation of the main signaling molecules in the cytoplasm downstream of RET-p-PLC γ , p-Akt, and p-ERK1/2 in UBs and the CND on E11.5, as well as the changes in tissue proliferation and apoptosis, in the LD + *Gen1*^{PB/+}, *Gen1*^{PB/+}, and LD groups. The results showed that compared with the *Gen1*^{PB/+} and LD groups, the LD + *Gen1*^{PB/+} group had lower p-PLC γ activity and increased apoptosis. During the development of the urinary system, different tyrosine docking sites of RET have different functions [26]. Mice with a mutation at RETY1015 (docking site for PLC γ) show duplex ureter, megaureter, and other CAKUT phenotypes [27], which are similar to the CAKUT phenotypes in offspring of the intrauterine low-protein diet fed *Gen1* mutation group in this study. These findings indicate that a low-protein diet, as an environmental stimulus, can mediate RET downstream signaling and aggravate CAKUT in *Gen1*-mutant mice by influencing tissue apoptosis.

In this study, we constructed a *Gen1*^{PB/+} mouse model with intrauterine growth retardation and found that compared with the *Gen1*^{PB/+} group and the LD group, the LD + *Gen1*^{PB/+} group more often had CAKUT phenotypes, mainly dominated by a duplicated collecting system. In addition, the LD + *Gen1*^{PB/+} group had more duplicated protrusions at E11.5 than the *Gen1*^{PB/+} and LD groups. The intrauterine low-protein diet and *Gen1* mutation might influence the apoptosis of UBs and the CND through the PLC γ signaling pathway, leading to the ab-

normal development of the urinary system in the offspring of the *Gen1*-mutant mice. We can speculate that a low-protein diet could synergize with *Gen1* mutation to increase the incidence of abnormal urinary system development in *Gen1*-mutant mice.

Statement of Ethics

The protocol was approved by the welfare and use of experimental animals issued by the Animal Care and Use Committee of the Institute of Developmental Biology and Molecular Medicine at Fudan University (Permit number: SYXK [hu]2020-0011).

Conflict of Interest Statement

The authors have no conflicts of interest to declare.

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Author Contributions

X.W., H.X., and Q.S. conceived and designed research; M.Y. and Y.L. performed experiments; M.Y. analyzed data; J.C., Y.Z., J.R., and X.F. interpreted results of experiments; M.Y., L.T., and Y.L. prepared figures; M.Y. drafted the manuscript; Q.S. edited and revised the manuscript; and Q.S. approved final version of the manuscript.

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