Maternal nutrient restriction inhibits ureteric bud branching but does not affect the duration of nephrogenesis in rats

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BACKGROUND: Maternal nutrient restriction produces offspring with fewer nephrons. We studied whether the reduced nephron number is due to the inhibition of ureteric branching or early cessation of nephrogenesis in rats. Signaling pathways involved in kidney development were also examined.

METHODS: The offspring of dams given food *ad libitum* (control (CON)) and those subjected to 50% food restriction (nutrient restriceted (NR)) were examined.

RESULTS: At embryonic day 13 (E13), there was no difference between NR and CON in body weight or kidney size. Ureteric buds branched once in both NR and CON. At E14 and E15, body and kidney size were significantly reduced in NR. Ureteric bud tip numbers were also reduced to 50% of CON. On the other hand, the disappearance of nephrogenic zone and a nephron progenitor marker Cited1 was not different between CON and NR. The final glomerular number of NR was 80% of CON. Activated extracellular signal-regulated kinase (ERK), p38, PI3K, Akt, and mammallian target of rapamycin (mTOR), and protein expression of β-catenin were downregulated at E15.

CONCLUSION: Ureteric branching is inhibited and developmentally regulated signaling pathways are downregulated at an early stage by maternal nutrient restriction. These changes, not early cessation of nephrogenesis, may be a mechanism for the inhibited kidney growth and nephrogenesis.

The concept that disturbed intrauterine organogenesis affects the health and disease in the adulthood, termed developmental origins of health and disease, has been increasingly recognized. With respect to the kidney, low birth weight is associated with a risk for hypertension and renal disease, and reduced nephron number is considered to be the cause. In animals, maternal protein or global nutrient restriction, uterine artery ligation, hyperglycemia, and exposure to various agents such as glucocorticoids or alcohol produce offspring with fewer nephrons (1–6). Depletion of stem cells by increased apoptosis has been suggested to be responsible for the decreased nephron formation (1,3). On the other hand, ureteric bud branching is a fundamental step in determining nephron number. The mechanism of reduced nephron endowment in dexamethasone or ethanol exposure has been suggested to be inhibited branching morphogenesis in an organ culture study (5,7). Reduced

ureteric branching has recently been reported in a model of maternal hyperglycemia *in vivo* (8).

Another possible mechanism of reduced nephron number is early cessation of nephrogenesis. In human premature infants, nephrogenesis is reported to continue only up to 40 d after birth (9). These infants are under various stresses including hypoxia, infection, and medications adversely affecting nephrogenesis. It has been postulated that adverse conditions during development promote differentiation aiming at early maturation and survival at the expense of reduced organ growth. Newborn rats resemble human premature neonates in that nephrogenesis continues after birth. Early cessation of nephrogenesis, therefore, may be a mechanism for low nephron number by maternal nutrient restriction.

A variety of signaling pathways are involved in kidney development. Among these, extracellular signal-regulated kinase (ERK) and p38 mitogen-activated protein kinase (p38) are required for nephrogenesis, metanephros growth, and ureteric bud branching (10–12). On the other hand, phosphatidylinositol 3-kinase (PI3K) and Akt, downstream signaling molecules of Ret, the kinase subunit of the glial cell line-derived neurotrophic factor (GDNF) receptor, promote branching morphogenesis (13). Recent evidence suggests a role of PI3K for the maintenance of progenitor cells as well (14). The multifunctional protein β-catenin mediates canonical Wnt signaling, and is necessary for both nephrogenesis and ureteric branching (15,16).

In the present study, we investigated whether inhibited ureteric bud branching and or early cessation of nephrogenesis is a mechanism for the reduced nephron number by maternal nutrient restriction. We also studied whether signaling pathways important in kidney development are altered.

RESULTS

Litter Size
Control dams had an average of 12.1 ± 0.6 pups/litter (*n* = 15). Maternal NR did not affect litter size (12.3 ± 0.3, *n* = 16).

Fetal Body Weight and Kidney Size
At E13, there was no difference in either fetal weight or kidney surface area between the offspring of nutrient-restricted mothers (NR) and CON (**Table 1**). At E14 and E15, both parameters were significantly decreased in NR. At E18, and postnnatal day

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0 (P0), body weight, kidney weight, and the kidney weight to body weight ratio of NR were significantly lower than those of CON (**Table 2**). At P6, body weight and kidney weight were lower in NR, but the kidney weight to body weight ratio was not different. At P14, there was no difference in these parameters between the groups. The glomerular density of NR was decreased compared with CON at E18 (16 ± 3 vs. $30 \pm 4/mm^2$, $n = 6$ and 11, respectively, $P < 0.05$) (**Figure 1**). Differences in size and morphology became smaller as the kidney matured, and disappeared at P14.

DNA Content, Protein/DNA Ratio, and Cell Number of Fetal Kidney

We next assessed whether reduced kidney size in NR is due to reduced cell number or cell size. DNA content per organ, a parameter for total cell numbers, and protein/DNA ratio, an index of cell size, were numerically and significantly lower at E15 and E18, respectively, in NR than CON (**Table 3**). We counted metanephric cell number at E15, which was significantly fewer in NR ($314,000 \pm 24,052$) compared with CON ($455,000 \pm 30,537$). Thus, the decreased kidney size by maternal NR was due to reduced cell number at E15, and due to both cell number and cell size at E18.

Ureteric Bud Branching

Since ureteric bud branching is a major determinant of glomerular number, we counted the number of ureteric bud tips. At E13, when there was no difference in the kidney size between NR and CON, ureteric buds branched once in both NR and CON (**Figure 2a**). At E14 and E15, however, ureteric bud tip numbers were significantly reduced in NR to approximately half of CON in correlation with the kidney size reduction (**Figure 2a**,**b**). Of note, ureteric bud tip numbers increased from E14 to E15 in NR kidneys keeping the same ratio to CON.

Cessation of Nephrogenesis

The ureteric bud of NR kidneys continued to branch albeit fewer in number than CON (**Figure 2b**). If this process continues postnatally, nutrient restricted offspring may catch up in nephron number. Nephrogenic zone was seen in the outer cortex at P8 but was absent at P9 in CON (**Figure 3a**). This was also the case for NR kidneys. In mice, in which nephrogenesis ceases earlier than in rats, a nephron progenitor marker Cited1, which is expressed in cap mesenchyme, disappears at P3 as detected by immunohistochemistry (17). We examined Cited1 by immunoblot analysis. Cited1 expression decreased toward P12 similarly in CON and NR (**Figure 3b**). These results indicate that the cessation of nephrogenesis is not altered in NR kidneys.

Glomerular Number

Glomerular number, counted at 3 wk postnatally after the cessation of nephrogenesis was decreased in NR ($20,855 \pm 868/$ per kidney) by ~20% compared with CON ($26,251 \pm 876/$per kidney, $n = 7$, $P < 0.05$).

Table 1. Fetal body weight and kidney surface area

		Body weight (g) (*n*)	Kidney surface area (%) (*n*)
E13	CON	0.087 ± 0.003 (11)	100 ± 6 (6)
	NR	0.086 ± 0.004 (12)	97 ± 10 (6)
E14	CON	0.202 ± 0.003 (14)	100 ± 6 (8)
	NR	$0.187 \pm 0.003*$ (14)	$57 \pm 5*$ (6)
E15	CON	0.32 ± 0.01 (15)	100 ± 6 (15)
	NR	$0.26 \pm 0.01*$ (12)	$80 \pm 3*$ (14)

CON, control; NR, nutrient-restricted.
*$P < 0.05$ vs. CON. $n = 3$ litters.

Table 2. Fetal and neonatal body weight and kidney weight

		n	Body weight (g)	Kidney weight (mg)	Kidney weight/ body weight (mg/g)
E18	CON	12	1.62 ± 0.02	8.7 ± 0.3	5.4 ± 0.2
	NR	11	$1.52 \pm 0.04*$	$6.9 \pm 0.3*$	$4.5 \pm 0.2*$
P0	CON	6	6.34 ± 0.12	52.4 ± 1.0	8.3 ± 0.1
	NR	6	$5.45 \pm 0.13*$	$40.6 \pm 1.5*$	$7.5 \pm 0.2*$
P6	CON	13	14.0 ± 0.4	154 ± 5	11.0 ± 0.2
	NR	11	$12.2 \pm 0.4*$	$133 \pm 4*$	11.0 ± 0.3
P14	CON	7	28.2 ± 0.7	295 ± 9	10.4 ± 0.2
	NR	5	27.0 ± 2.5	270 ± 36	9.9 ± 0.4

CON, control; NR, nutrient-restricted.
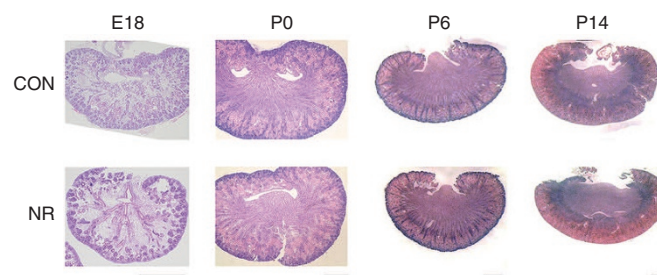*$P < 0.05$ vs. CON. $n =$ at least three litters.

Figure 1. The effect of maternal nutrient restriction on kidney size and morphology. (**a**) At embryonic day 18 (E18), Hematoxylin and Eosin staining indicates that the kidney of offspring from nutrient restricted mothers (NR) is smaller with decreased glomerular density compared with controls (CON) (×40). The difference between NR and CON becomes smaller after birth (**b**, postnatal day 0, P0, ×20; **c**, P6, ×16), and disappears by P14 (**d**, ×10). Scale bar, 1 mm.

Table 3. DNA content and protein/DNA ratio of fetal kidney

		n	DNA/kidney (µg/organ)	Protein/DNA (mg/mg)
E15	CON	5	3.72 ± 0.72	24.4 ± 4.0
	NR	5	3.27 ± 0.29	22.7 ± 3.0
E18	CON	10	19.80 ± 1.68	28.54 ± 2.3
	NR	10	$13.94 \pm 1.11*$	$18.04 \pm 0.78*$

CON, control; NR, nutrient-restricted.
*$P < 0.05$ vs. CON. $n = 3$ litters.

Developmentally Regulated Signaling Pathways

We next examined signaling pathways important in kidney development including ureteric branching. At E15, phosphorylated and activated forms of ERK, p38, PI3K, and Akt, and protein expression of β-catenin were reduced in NR compared with CON (**Figure 4**). In contrast, at E18, ERK, p38, PI3K, and Akt were more phosphorylated and β-catenin was upregulated in the NR kidney compared with CON (**Figure 5**). This was considered to be a compensatory response for the inhibited kidney development. We assessed phosphorylated mTOR as an indicator of nutrition status, which was downregulated at E15 and upregulated at E18 in NR in a similar manner to other signaling molecules.

Proliferation and Apoptosis

We examined whether proliferation and apoptosis were altered in NR. The expression of proliferating cell nuclear antigen (PCNA), a marker of proliferation, was not different between NR and CON at E15 (**Figure 4**). On the other hand, cleaved caspase-3, a marker of apoptosis, was dramatically decreased in NR (**Figure 4**). At E18, when signaling pathways were activated, PCNA was slightly decreased, and cleaved caspase-3 was again dramatically decreased in NR (**Figure 5**).

Immunohistochemically, Ki67 was stained in metanephric mesenchymal cells and occasional ureteric bud cells at E13 (not shown) and at E15 (**Figure 6a**). No difference was observed between NR and CON. At E18, Ki67-positive cells were detected abundantly in the nephrogenic zone, which was more intense in the NR kidney. Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL)-positive cells were rarely observed at E13 in both control and NR kidneys (not shown). At E15, a few mesenchymal and ureteric bud cells stained positive with no significant differences between CON and NR (**Figure 6b**). At E18, TUNEL-positive cells were found in the nephrogenic zone, with an increasing tendency in the NR kidney in a similar manner to Ki67 (**Figure 6b**). The expression of cleaved caspase-3 did not correlate with TUNEL staining, which was detected in mesenchymal cells with less intensity in the NR kidney.

DISCUSSION

We examined the effect of maternal nutrient restriction on ureteric branching and on the duration of nephrogenesis in rats. The most important finding of our study was that ureteric branching was decreased in NR, which has not been described previously. Since glomeruli are formed around the tip of ureteric buds, ureteric branching, to a large extent, determines nephron number. There is no developmental delay or suppression up to E13 in NR kidneys. At E14, however, ureteric bud tip numbers in NR kidneys were approximately half of CON. Decreased ureteric branching has been reported in metanephroi treated with dexamethasone, ethanol, and gentamicin in an organ culture (5,7,18).

In a similar manner to ureteric branching, body weight and metanephros size of NR fetuses were not different from controls at E13. At E14, weight and kidney size became smaller in NR fetuses. DNA content, an index of total cell numbers of the kidney (19), was numerically and metanephric cell number
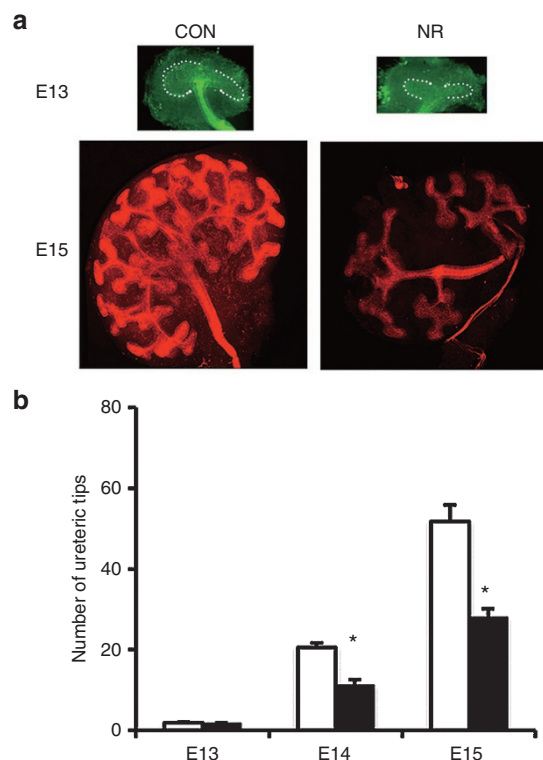

Figure 2. Ureteric bud branching is inhibited by maternal undernutrition after E14. (**a**) Ureteric buds were visualized by staining with fluorescein-*Dolichos biflorus* agglutinin or antibody against pancytokeratin. (**b**) Quantitative analyses of ureteric tip numbers. E13–15, embryonic day 13–15; CON, controls, □; NR, offspring of nutrient restricted mothers, ■; *P <0.05 vs. CON. n = 3 litters.
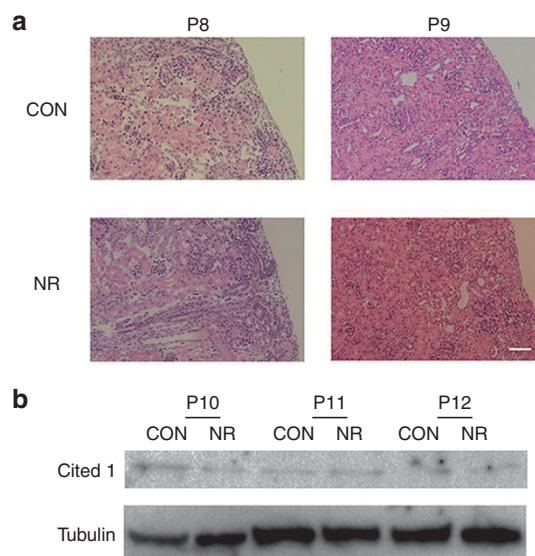

Figure 3. Cessation of nephrogenesis is not altered in kidneys of the offspring of nutrient restricted mothers. (**a**) Nephrogenic zone is observed at postnatal day 8 (P8) but no more at postnatal day 9 (P9) in controls (CON) and kidneys of the offspring of nutrient restricted mothers (NR) (×200). Scale bar, 0.1 mm. (**b**) The expression of Cited 1, a marker of nephron progenitors, decreases toward P12 in both CON and NR. Immunoblot with anti-Cited1 antibody. P10–12, postnatal day 10–12.
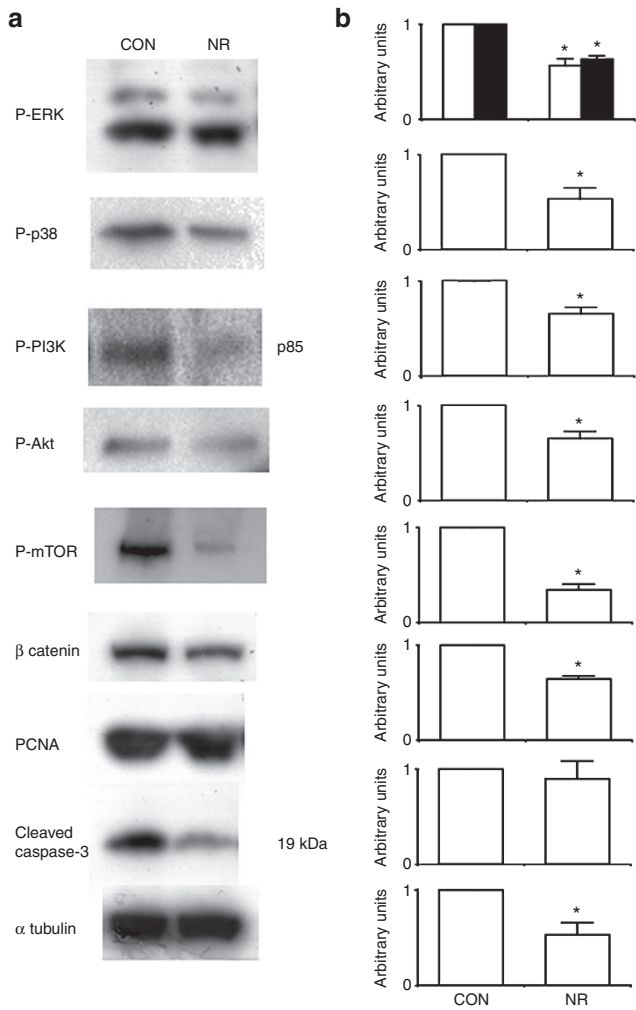
Figure 4. Developmentally regulated signaling pathways at embryonic day 15. (**a**) Immunoblot with antibodies against phospho-ERK (P-ERK), -p38 (P-p38), -Akt (P-Akt), -PI3K (P-PI3K), -mTOR (P-mTOR), β-catenin, PCNA, or cleaved caspase-3. (**b**) Quantitative analyses. Levels of the proteins were standardized against the tubulin. CON, controls; NR, offspring of nutrient restricted mothers. *$P < 0.05$ vs. CON. □, ERK1; ■, ERK2. Representative immunoblots. $n = 3–4$.

Figure 5. Developmentally regulated signaling pathways at embryonic day 18. (**a**) Immunoblot with antibodies against phospho-ERK (P-ERK), -p38 (P-p38), -Akt (P-Akt), -PI3K (P-PI3K), -mTOR (P-mTOR), β-catenin, PCNA, or cleaved caspase-3. Representative immunoblots. (**b**) Quantitative analyses. Levels of the proteins were standardized against the tubulin. CON, controls; NR, offspring of nutrient restricted mothers. *$P < 0.05$ vs. CON. $n = 3–4$.

was significantly smaller in NR kidneys at E15. At E18, DNA content was significantly smaller in NR. Our results were in accord with the previous report by Welham *et al.* (1) that examined the effect of maternal low protein diet (LPD) on kidney development. They speculated that depletion of nephron precursors or supportive interstitial precursors is responsible for the nephron reduction. Very recently, Cebrian *et al.* (20) have published a paper that genetic deletion of fetal nephron progenitor cells decreases nephron number by reducing ureteric branching. Their study integrates our observation with that of Welham's. Thus, Cebrian *et al.* found that progenitor cell depletion delayed ureteric branching, and speculated that a decrease in progenitor cells results in a lower level of signaling to the ureteric bud tip cells thus reducing branching.

In our model of global nutrient restriction, unlike that by Welham *et al.*, apoptotic cell number was few and similar between NR and control kidneys at both E13 and E15. The
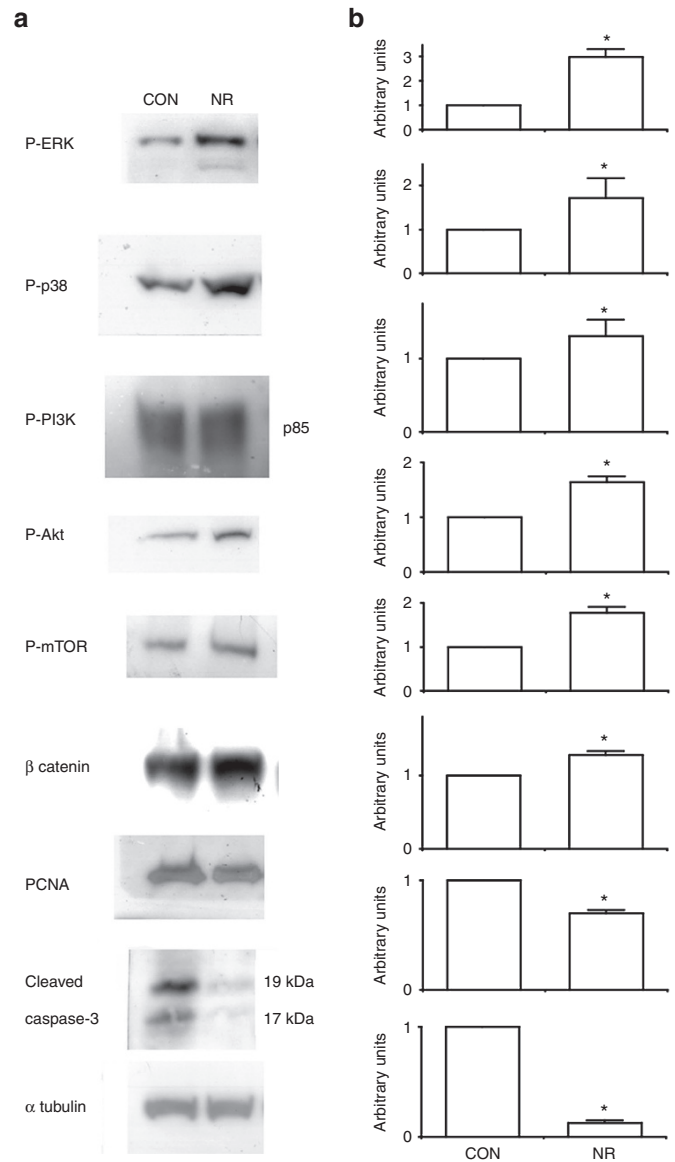
expression of Ki67 and PCNA was also similar. There are two possibilities for this seemingly illogical observation, i.e., unaltered numbers of TUNEL- and Ki67-positive cells in the face of decreased total cell numbers in the NR kidney. Firstly, TUNEL staining may not reflect the apoptotic event since the clearance rate of apoptotic cells may be different between NR and CON. Secondly, cell cycle may be prolonged in NR. A prolonged G1 phase has been reported in the maturing pancreas exposed to maternal LPD (21). A recent study, using two established models of maternal protein and iron restriction, suggests that cell cycle regulation is a common mechanism for the nutritional programming (22).
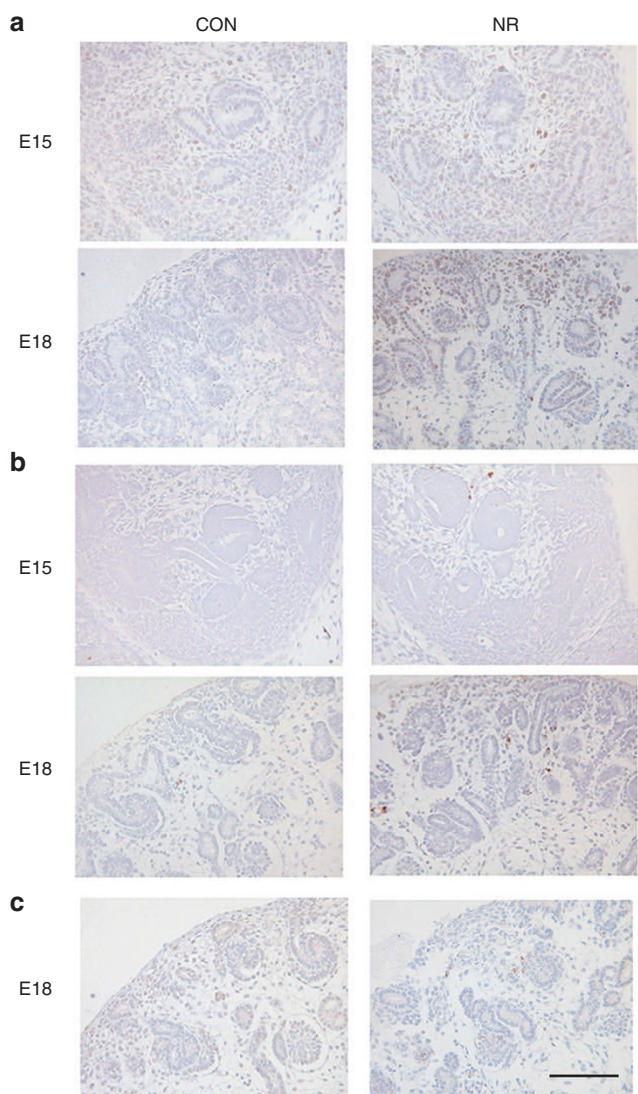
Figure 6. Proliferation, apoptosis, and the expression of cleaved caspase-3. Immunohistochemistry with Ki67 (**a**) and cleaved caspase-3 (**c**), and TUNEL staining (**b**), at embryonic day 15, (E15) and 18 (E18) (×200). CON, controls; NR, offspring of nutrient restricted mothers. Scale bar, 0.1 mm.

The reduction in the organ size induced by an adverse environment was generally due to the reduction in cell number since adverse conditions promote progenitor cell differentiation at the expense of proliferation. Protein/DNA ratio, an index of cell size, was deceased by maternal NR at E18, and the decreased kidney size is due to reduced cell size as well. The response seems to vary depending on the organ and the insult.

The second new finding of our study is that nephrogenesis ceases normally in the NR kidney. Nephrogenic zone disappeared simultaneously in CON and NR. Also, the expression of Cited1 decreased toward P12 in both CON and NR kidneys. A previous study examining the effect of prenatal unilateral nephrectomy reported extended nephrogenesis in the contralateral kidney (23), but this was not the case in the present study. Our observation agrees with the study of Cebrian *et al.* (20) using progenitor cell depletion model. In their study also,

nephrogenesis was not prolonged resulting in a permanent deficit in nephron number despite compensatory renal growth.

The third major finding of the present study is that signaling pathways important in kidney development are altered by maternal nutrient restriction in a biphasic manner. At E15, phosphorylated ERK, p38, PI3K, and Akt and protein expression of β-catenin were decreased in NR. These molecules are important in ureteric branching. ERK, p38, and β-catenin are also necessary for nephrogenesis. In contrast to E15, phosphorylated ERK, p38, PI3K, Akt, and protein expression of β-catenin were upregulated at E18. This is thought to be a compensatory response to the disturbed kidney development at an earlier stage. In support of this hypothesis, Ki67 expression and TUNEL staining were more intense in the nephrogenic zone of NR kidneys than that in controls at E18. Similar compensatory response has been reported in dexamethasone and maternal LPD models (1,5,24). In maternal LPD, glomerular number of kidneys was even higher than controls at E20 (24). Of note, each ureteric tip induces the formation of one nephron during the fetal stage and up to five nephrons per ureteric tip after postnatal day 2 (25). The total glomerular number of NR at E18 was estimated to be approximately half of CON from the glomerular density and the kidney weight. Thus, the catch-up in glomerular number in NR may be due to increased ureteric branching after E18. Alternatively, not mutually exclusive to the above, the postnatal nephrogenesis may be facilitated under unrestricted food intake.

Of note, a previous study by Henry *et al.* (26) reported decreased GDNF and ERK signaling in the kidney by maternal NR at E20. The disagreement with the present study may be explained by a different protocol. They started nutrient restriction at E10 just before the formation of metanephros, whereas we started nutrient restriction at E1.

Both maternal nutrient restriction and dexamethasone treatment have been reported to decrease the expression of GDNF (5,27). GDNF activates ERK, p38, PI3K, and Akt through binding to its receptor Ret (13,28,29), Therefore, reduced activity of ERK may be partly due to the decrease in GDNF. However, Ret is expressed only in the tips of ureteric buds. Furthermore, β-catenin and mTOR are not the downstream molecules of GDNF, and there probably exist a more general mediator of maternal nutrient restriction. In support of this hypothesis, the reduction in nephron number by genetic deletion of nephron progenitor cells was not rescued by heterozygosity for sprouty 1, a negative regulator of GDNF (20). In view of this study, markers of nephron progenitor cells such as OSR1, Sall1, and Six2 need to be examined in fetal NR kidney (30).

mTOR is considered to be a central molecule in sensing and responding to intracellular nutrient availability and has been shown to be altered by maternal nutrient restriction in various tissues (31–33). Nijland *et al.* (34) demonstrated, using pathway analysis of whole-genome expression profiles, that the mTOR signaling pathway is central to the decreased nephron number in the fetal baboon kidney from nutrient restricted mothers. Phosphorylated mTOR behaved in a similar manner to other signaling molecules in our study. The mechanism of

the upregulation of phosphorylated mTOR at E18 despite continued undernutrition remains to be clarified, which may be related to the compensatory response. Our preliminary study showed that L-type amino acid transporter 1, closely linked to mTOR, is downregulated and upregulated at E15 and E18, respectively.

Cleaved caspase-3 was dramatically decreased in the NR kidney at both E15 and E18. We examined cleaved caspase-3 as a marker for apoptosis, but it did not correlate with TUNEL staining. Thus, there was no difference in TUNEL positivity between NR and CON kidneys. Caspase-3 has previously been shown to be necessary for kidney development in an organ culture study (35). The kidney grown under caspase-3 inhibitor was decreased in size and branching in close resemblance to the NR kidney. It is therefore possible that reduced caspase-3 activity by maternal NR may contribute to the reduced ureteric branching and nephrogenesis. While apoptotic process may be necessary for development, recent evidence suggests that caspases mediate various cell processes such as motility, differentiation, and proliferation (36). Further study is underway to investigate the role of caspase-3 in renal development.

In conclusion, maternal nutrient restriction inhibits ureteric bud branching but does not affect the duration of nephrogenesis. Developmentally regulated signaling pathways are initially decreased and thought to mediate the suppressed branching morphogenesis, nephrogenesis, and kidney growth. These findings open up a possibility of developing intervention strategies for reduced nephron endowment, a risk factor for chronic kidney disease.

METHODS

Experimental Animals
Approval was obtained from the Animal Experiment Committee of Keio University. Female Sparague Dawley rats at embryonic day 1 (E1) were purchased from Sankyo Labo Service corporation (Tokyo, Japan). The offspring of dams given food *ad libitum* (CON, $n = 25$) and those subjected to nutrient restriction throughout pregnancy (NR, $n = 25$) were examined. Nutrient-restricted mothers were given half amount of chow consumed by control rats on the previous day. After delivery, food was given *ad libitum*. Kidneys from fetuses or neonatal rats were fixed in cold methanol for whole mount staining, lysed for immunoblotting, or fixed in 4% paraformaldehyde for Hematoxylin and Eosin staining or immunohistochemistry. Embryonic day 13 and 15 whole embryo were fixed in 4% paraformaldehyde and embedded in paraffin for immunohistochemistry.

Reagents
The following primary antibodies were purchased: phospho-ERK, -p38, -PI3K p85 (Tyr458)/p55 (Tyr199), -Akt (Ser473), -mTOR (Ser2448), cleaved caspase-3 (Asp175) (Cell Signaling, Danvers, MA), β-catenin, human Ki-67 (BD Biosciences, San Jose, CA), PCNA (DAKO, Carpinteria, CA), and Cited1, α-tubulin, pancytokeratin (C2562) (Sigma, St. Louis, MO). ApopTag Peroxydase In Situ Apoptosis Detection Kit was from Intergen Company (Purchase, NY). Fluorescein-*Dolichos biflorus* agglutinin was from Vector Laboratories (Burlingame, CA). Cy3-conjugated rabbit anti-mouse antibody was from Chemicon (Temecula, CA). SlowFade Antifade Kit was from Molecular Probes (Eugene, OR).

Measurement of Protein and DNA
DNA was measured using the fluorescent compound bisbenzimide H-33258 fluorochrome. Protein was measured with a DC protein assay (Bio-Rad Laboratories, Tokyo, Japan).

Metanephric Cell Number
Metanephric cell number was counted at E15. Metanephroi were incubated in 60 μl of 0.05% trypsin/0.02% ethylenediaminetetraacetic acid (EDTA) for 30 min at 37 °C. Tissues were then homogenized using a 25 gauge needle. The number of cells in the suspension was determined using a hemocytometer (1).

Whole Mount Staining
For detection of ureteric buds, metanephroi were fixed with cold methanol for 10 min, washed three times with phosphate-buffered saline/0.1% Tween 20 (PBT), and incubated with *Dolichos biflorus* agglutinin (dilution 1:50) for 2 h at room temperature. Alternatively, metanephroi were blocked in 1% skim milk in PBT and incubated with anti-pancytokeratin antibody (dilution 1:400) for 2 h followed by incubation with Cy3-conjugated anti-mouse antibody (dilution 1:400) for 1 h. Samples were washed three times with PBT, mounted in SlowFade, and viewed under an OLYMPUS BX-50 (Olympus Optical, Tokyo, Japan) or a confocal imaging system (Leica TCS-SP5, Leica Microsystems, Tokyo, Japan). Kidney size was assessed by measuring planar surface area using ImageJ software (National Institutes of Health, Bethesda, MD). Embryonic kidney surface has been shown to correlate with volume (37).

Immunohistochemistry
Immunohistochemical staining was performed on serial sections 4-μm thick using enzyme-labeled antibody method. Paraffin sections were deparaffinized and rehydrated. Endogenous peroxide activity was quenched by incubating sections in 0.3% H_2O_2/methanol for 30 min. To unmask antigens, slides were boiled at 100 °C for 10 min in 10% citrate buffer (pH 6.0)/methanol or 40 min in 1 mmol/l EDTA (pH 8.0). Sections were incubated with antibodies against Ki-67 (10 μg/ml) or cleaved-caspase-3 (0.4 μg/ml) at 4 °C for overnight. After incubating with secondary antibody at a concentration of 1:100, immunoreaction products were developed using 3,3′-diaminobenzidine as the chromogen, with standardized development times. Sections were then counterstained with methyl green. The primary antibody was omitted from the staining procedure in negative controls.

Detection of Apoptosis
Paraffin-embedded sections were deparaffinized, and TUNEL staining was performed using the ApopTag kit. Sections were counterstained with methyl green. TdT was omitted from the staining procedure in negative controls.

Immunoblot Analysis
Metanephroi were pooled and lysed in solubilization buffer containing 20 mmol/l HEPES (pH 7.2), 1% Triton X-100, 10% glycerol, 20 mmol/l sodium fluoride, 1 mmol/l sodium orthovanadate, 1 mmol/l phenylmethanesulfonyl fluoride, 10 μg/ml aprotinin, and 10 μg/ml leupeptin. For the analysis at E15, metanephroi of embryos from one litter were pooled ($n = 10$ litters). Metanephroi from 6 to 12 fetuses were used for the analysis at E18 ($n = 4$ litters). Insoluble material was removed by centrifugation (10,500 g, 10 min). Lysates were resolved by SDS-PAGE and transferred to poly (vinylidene fluoride) membranes (Immobilon, Millipore, Bedford, MA). Nonspecific binding sites were blocked in Tris-buffered saline (10 mmol/l Tris HCl, pH 7.4, 0.15 mol/l NaCl) containing 0.1% Tween 20 and 5% skim milk or bovine serum albumin (BSA) overnight at 4 °C or for 1 h at 25 °C. Antibodies were added to Tris-buffered saline containing 0.1% Tween 20 with 5% BSA in saturating titers and incubated with mixing for 24 h at 4 °C. Bound antibodies were detected using the enhanced chemiluminescence Western blotting system (Amersham, Arlington Heights, IL). All experiments were repeated at least three times. Blots were quantitatively analyzed by ImageJ software.

Glomerular Number
Kidneys were incubated in 1 mol/l hydrochloric acid for 30 min at 37 °C. Acid was removed and replaced with 0.5 ml phosphate-buffered saline. The tissue was homogenized manually with pestles. A 20-μl sample was taken and placed on a slide and overlaid with a cover slip. Using a 10 objective lens, the number of glomeruli in the aliquot was counted in a blind fashion. This was carried out at least five times for

each sample (1). The results were averaged and used to determine the total number of glomeruli in the kidney.

Glomerular Density

Glomeruli from the S-shaped body stage onward were counted on Hematoxylin and Eosin section at E18 and expressed as glomerular number per mm². Glomerular density was determined using three kidney sections from each animal.

Statistical Analysis

The results are expressed as means ± SE. Statistical analysis was performed with Student's unpaired *t*-test. Statistical significance was determined as $P < 0.05$.

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