Maternal diet programs embryonic kidney gene expression

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Maternal diet programs embryonic kidney gene expression. Physiol Genomics 22: 48–56, 2005. First published April 12, 2005; 10.1152/physiogenomics.00167.2004.—Human epidemiological data associating birth weight with adult disease suggest that organogenesis is “programmed” by maternal diet. In rats, protein restriction in pregnancy produces offspring with fewer renal glomeruli and higher systemic blood pressures than controls. We tested the hypothesis that maternal diet alters gene expression in the metanephros, the precursor of the definitive mammalian kidney. We demonstrated that maternal low-protein diet initiated when pregnancy starts and maintained to embryonic day 13, when the metanephros consists of mesenchyme surrounding a once-branched ureteric bud, is sufficient to significantly reduce glomerular numbers in offspring by about 20%. As assessed by representation difference analyses and real-time quantitative polymerase chain reactions, low-protein diet modulated gene expression in embryonic day 13 metanephros. In particular, levels of prox-1, the ortholog of Drosophila transcription factor prospero, and cofilin-1, a regulator of the actin cytoskeleton, were reduced. During normal metanephrogenesis, prox-1 protein was first detected in mesenchymal cells around the ureteric tree and thereafter in nascent nephron epithelia, whereas cofilin-1 immunolocalized to bud derivatives and condensing mesenchyme. Previously, we reported that low-protein diets increased mesenchymal apoptosis cells when metanephrogenesis began and thereafter reduced numbers of precursor cells. Collectively, these studies prove that the maternal diet programs the embryonic kidney, altering cell turnover and gene expression at a time when nephrons and glomeruli have yet to form. The human implication is that the maternal diet ingested between conception and 5–6-wk gestation contributes to the variation in glomerular numbers that are known to occur between healthy and hypertensive populations.

THE PRECURSOR of the adult mammalian kidney is called the metanephros, and it appears around 5 wk of human gestation (7, 60). The equivalent morphological stage in the rat is embryonic day 12.5 (E12.5), when the organ simply consists of the ureteric bud epithelium, a branch of the mesonephric duct, enveloped by renal mesenchymal cells condensing from intermediate mesoderm. These tissues undergo reciprocal inductions to form collecting ducts and nephrons (i.e., glomeruli and proximal tubules), respectively. In humans, new layers of glomeruli are generated until 34 wk of gestation, whereas, in rats, nephrogenesis is prolonged through the first postnatal week. Human kidney and urinary tract malformations are common (60), and renal hypoplasia (too few glomeruli and nephron tubules), dysplasia (incomplete and metaplastic differentiation), and agenesis (failure of formation) account for most children who require chronic dialysis and renal transplantation. In addition, it has been suggested that individuals born with modest deficits in glomerular numbers (i.e., mild hypoplasia) are prone to systemic hypertension in later life (6), a contention supported by animal and human studies. For example, blood pressure correlates with glomerular numbers in mouse and rat strains (6, 13), and road traffic victims with a history of essential hypertension have, on average, half as many glomeruli per kidney as age-matched normotensive controls (26). Human studies also emphasize the wide range of glomerular numbers counted between healthy controls (24, 26).

What causes a person to be born with fewer nephrons than normal (renal hypoplasia)? There are probably at least three general causes: 1) mutations of nephrogenesis genes such as PAX2 and HNF1B (4; 51); 2) impairment of fetal urinary flow caused by physical obstruction in the lower urinary outflow tract, a phenotype that can be modeled in experimental animals (46, 59); and 3) teratogens (45). Recently, a fourth cause has been suggested, namely, that the maternal diet ingested during pregnancy modulates the development of the metanephros and other organs (40, 42). This idea is indirectly supported by human epidemiological data linking low birth weight with adult diseases such as hypertension as well as Type 2 diabetes mellitus and coronary heart disease (3, 61); such associations might be explained if fetal differentiation could be “programmed” by the maternal diet. Indeed, in rats, protein restriction in pregnancy produces offspring with fewer glomeruli and higher systemic blood pressures than controls (32, 33, 56). Furthermore, in humans, there is a positive correlation between birth weight and glomerular number (24).

What biological events underlie embryonic kidney programming? During normal metanephric kidney development, subsets of cells are deleted by apoptosis (12, 18, 57), a mechanism that probably limits the number of nephrons generated and/or the number of cells in each nephron. We previously demonstrated that maternal low-protein diets (LPD; 9% and 6% protein vs. 18% in controls) fed to rats from the day of conception until birth, which occurs around day 21 of pregnancy, led to glomerular deficits of about 25% when measured 2 wk after birth (56). LPD were associated with increased apoptosis in E13.0 metanephric mesenchyme, as assessed by counting pyknotic and in situ end-labeled nuclei. At this stage, the ureteric bud has branched once, branch tips are surrounded by condensed mesenchyme, which will later undergo epithelial transformation into nephrons, and the ureteric stalk is surrounded by loose mesenchyme, called stroma. By contrast, there was no difference in apoptosis between dietary groups at E15.0, when several rounds of ureteric bud branching and formation of primitive nephrons (vesicles, comma- and S-shapes) has already occurred. The total number of metanephric cells was similar (about 2 × 10⁴) in all dietary groups at E13.0,
with a 100-fold increase in control metanephri from the next 48 h. At E15.0, however, total cell numbers were significantly reduced in metanephri exposed to maternal LPD. These observations support the hypothesis that LPD alters the trajectory of metanephric growth by modulating cell turnover at the inception of organogenesis.

In the present study, we addressed two questions: 1) does maternal LPD have detrimental effects on final glomerular numbers if experimental diets are instigated at the start of pregnancy and continued to just E13.0 rather than the end of gestation, and 2) does LPD modulate gene expression in the E13.0 metanephri? The results of these experiments demonstrate that the maternal diet programs transcription in the embryonic kidney at a time when nephrons and glomeruli have yet to form.

MATERIALS AND METHODS

Animal protocols were approved by the United Kingdom Home Office. All reagents were from Sigma Chemical (Dorset, UK) unless otherwise stated.

Dietary manipulations and glomerular counting. Female Wistar rats (Harlan; Bicester, UK) were time mated by exposure to males between 14.00 and 17.00 hours. At 9.00 hours the next day, rats with vaginal plugs were selected for further study, and this time was designated E0.0. Females were immediately randomly supplied one of three diets (Dyets; Bethlehem, PA) containing either control diet (18% protein by weight) or LPD (9% or 6% protein by weight). The constituents of the 18%, 9%, and 6% protein diets were as follows: casein (180, 90, and 60 g/kg), maize starch (425, 485, and 505 g/kg), and sucrose (213, 243, and 253 g/kg). All diets additionally contained methionine (5 g/kg), corn oil (100 g/kg), vitamin mix AIN-76 (5 g/kg), mineral mix (20 g/kg), choline chloride (2 g/kg), and cellulose (50 g/kg) as previously described (56); all diets had the same caloric, vitamin, and mineral contents. We used an identical protocol to that of Langley-Evans et al. (33); using the same rat strain, these investigators demonstrated that food intake was equal among the different diets. Rats were given free access to water through the study, although water intake was not formally quantified. All groups received standard chow from term until 2 wk after birth, with one of the following five variations before birth: 1) 18% protein throughout pregnancy; 2) 9% protein throughout pregnancy; 3) 6% protein throughout pregnancy; 4) 9% protein until E13.0, followed by 18% protein to term; and 5) 6% protein until E13.0, followed by 18% protein to term. Offspring were killed at 2 wk of age, 1 wk after the end of the nephrogenic period, and glomerular numbers were determined after acid digestion using the protocol of Welham et al. (56), modified to use 0.1 M HCl. A multilevel statistical model was used to analyze data to negate the possibility of pseudoreplication (19) as previously described (56).

Dietary manipulations performed for molecular and histological analyses. Pregnant rats were supplied either 18%, 9%, or 6% protein diet from E0 until either E13.0 or E15.0. At E13.0 and E15.0, dams were killed, and embryos were removed and kept at 4°C. Metanephri were snap frozen in liquid nitrogen for biochemical/molecular analyses or fixed in 4% paraformaldehyde. Representational difference analyses were performed as previously described (23). In brief, total RNA pooled from E13.0 metanephri from dams fed either 18%, 9%, or 6% protein diets was converted into cDNA (here, for the 18% and 9% protein groups, we used contralateral organs to those used in the RDA above). For each diet, we pooled organs from three to four litters due to the small quantities of tissue available and the requirement to determine expression of a number of genes. GAPDH was used as a standard against which the expression of individual genes was factored to control for possible differences arising from unequal template concentrations. GAPDH standards were created forward and reverse primers designed to produce products of 139 bp (5′-GATCTTCCTCG-3′ and 5′-AGGCCACTGTGCTATCCGAGGGA-3′), and the final difference product from each hybridization was isolated. Each difference product was ligated into BamH1-digested K5+ Bluescript vectors and propagated in Maximum Efficiency cells (Invitrogen, Paisley, UK). Products were sequenced on an ABI automated sequencer (Applied Biosystems; Foster City, CA), and BLAST searches were carried out. Only sequences with more than 100 identified bases and with a sequence homology of 95% or greater with known sequences were selected as bona fide matches.

Real-time PCR. Because of the small quantities of RNA available from E13.0 metanephri (each metanephros contains about 2 × 10⁴ cells) (56), sequences identified by RDA as being either up- or downregulated by maternal LPD were validated using quantitative (real-time) PCR with SYBR green. Total RNA pooled from E13.0 metanephri from dams fed either 18%, 9%, or 6% protein diets was converted into cDNA (here, for the 18% and 9% protein groups, we used contralateral organs to those used in the RDA above). For each diet, we pooled organs from three to four litters due to the small quantities of tissue available and the requirement to determine expression of a number of genes. GAPDH was used as a standard against which the expression of individual genes was factored to control for possible differences arising from unequal template concentrations. GAPDH standards were created forward and reverse primers designed to produce products of 139 bp (5′-TGCCACTCAGAGACGTGG-G-3′ and 5′-GAGCTTCCTCGAGCAGTCTT-3′). Known concentrations of this product were diluted to produce a range of standards. For the genes that were apparently differentially expressed according to the RDA results, primers were designed to produce PCR products of between ~100 and 250 bp (primer sequences and relative nucleotide positions are shown Table 2). Individual PCR were performed using annealing temperatures appropriate for the primer sets. The relative transcript levels between different diets, which are quoted in RESULTS, represent the average measured ratios from three sets of real-time PCRs on each dietary sample. In addition to genes identified as differentially expressed by the RDA screen, both Bax and Bcl2 were also assayed by real-time PCR to assess the levels of expression of known apoptosis-related transcripts.

Western blot analysis and immunohistochemistry. Ten micrograms of protein from each dietary group, constituting pools from several litters in each group, were electrophoresed through a 12% SDS polyacrylamide gel and transferred onto nitrocellulose membranes, as previously described (56). Blots were probed using primary antibodies against prox-1 (ReliaTech; Braunschweig, Germany) and cofilin-1 (Chemicon; Temecula, CA) at a concentration of 1:500.

The cofilin-1 antibody used in Western blot analysis recognizes both the phosphorylated (inactive) and dephosphorylated (active) forms of cofilin-1. Five-micrometer sections of paraaffin-embedded E13.0 and E15.0 metanephri were dewaxed and probed with antibodies against prox-1 and cofilin-1 (in addition to the cofilin-1 antibody described earlier, we also used an antibody that exclusively recognized the phosphorylated form of cofilin-1; Chemicon) using previously published im-
mumon histochemical protocols (58). Sections were incubated in Retrieval buffer (BD; Oxford, UK) at 95°C for 1 h to unmask antigens, and endogenous peroxidase activity was quenched by incubating sections in 3% H2O2. Sections were blocked first with 10% FCS and then Serum-Free Protein Block (DAKO; Cambridgeshire, UK). Primary antibodies were diluted to concentrations of 1:50 to 1:200, and slides were incubated overnight at 4°C. Sections were then incubated with appropriate secondary antibodies, and signals were developed using a horseradish peroxidase-based system (anti-rabbit Envision kit, DAKO). Finally, slides were counterstained with hematoxylin.

RESULTS

Maternal diets and final glomerular number. To determine the effects of maternal LPD on final nephron number in offspring, we first examined protocols in which dietary protein content was controlled throughout pregnancy (Fig. 1). Offspring of dams fed 18% protein from E0.0 to term possessed $3.1 \times 10^5 \pm 0.7 \times 10^5$ (means $\pm$ SE) glomeruli/kidney; offspring exposed to a 9% LPD throughout gestation possessed $6.3 \times 10^4 \pm 1.7 \times 10^4$ fewer glomeruli/kidney than controls (about 20% fewer, $P < 0.001$), and those exposed to maternal 6% LPD throughout pregnancy possessed $7.1 \times 10^3 \pm 1.6 \times 10^3$ glomeruli/kidney fewer than controls (about 23% fewer, $P < 0.001$). There was no significant difference between the number of glomeruli in kidneys of 9% versus 6% LPD offspring ($P = 0.716$). The relationships between dietary groups are similar to our previously published data (56), although the absolute numbers in this study are somewhat higher, probably explained by the more gentle dissociation method used here. Strikingly, when glomerular numbers were quantified in offspring from dams exposed to LPD restricted to the period between E0.0 and E13.0, we observed glomerular deficits of similar magnitude compared with the offspring of dams in which LPD was continued beyond E13.0 to the end of pregnancy. For example, offspring of the group exposed to 9% LPD until E13.0 only possessed $5.8 \times 10^5 \pm 1.4 \times 10^5$ glomeruli/kidney fewer than controls (about 19% fewer, $P < 0.001$), and those from the similarly timed 6% LPD group possessed $7.5 \times 10^3 \pm 1.3 \times 10^3$ glomeruli/kidney fewer than controls (about 24% fewer, $P < 0.001$).

Effects of LPD on metanephric gene expression assessed by RDA. The above results suggest that LPD has somehow altered the trajectory of rat nephrogenesis by the day that the metanephros forms. Consistent with this, we (56) previously documented a increased metanephric mesenchymal apoptosis at E13.0 in embryos of mothers exposed to LPD initiated at the start of pregnancy. We reasoned that this programming effect might be correlated with alternations of metanephric gene expression and therefore performed an RDA screen comparing E13.0 metanephroi between control and LPD-exposed animals. By sequencing 31 colonies with inserts in the 18T versus 9D hybridization and 18 colonies with inserts in the 9T versus 18D hybridization and excluding “spurious” products appearing in both sets, several transcripts were isolated by this screen as being either up- or downregulated by maternal LPD (Table 1) (9, 21, 25, 28, 29, 34, 39, 47, 52, 55, 62), with some appearing a number of times.

Effects of LPD on metanephric gene expression assessed by real-time PCR. Several of these genes isolated in the RDA screens code for proteins that fulfill the criteria of being potentially implicated in differentiation and organogenesis (e.g., transcription factors, adhesion molecules, proteins that affect the cytoskeleton, etc). These were selected for measurement in E13.0 metanephroi using real-time PCR in both 9% and also 6% LPD versus control diets. The results are shown in Table 2.

Immunohistochemistry. From genes found to be differentially expressed, we focused on prox-1, which codes for a homeobox transcription factor (47), and cofilin-1, which codes for an actin depolymerizing protein (55). With the use of E13.0 metanephrons from control animals, immunoprobing for prox-1 demonstrated a signal in stromal cells around the stalk of the ureteric bud (Fig. 2, A–C). As expected for a transcription factor protein, the signal was nuclear. A very faint signal was also seen in a few nuclei in ureteric bud branches and condensing mesenchyme. At E15.0, prox-1 immunoreactivity was detected in the nephrogenic cortex where subsets of nuclei were positive both in mesenchyme and also in epithelia of ureteric bud branches and nephron precursors such as vesicles (Fig. 2, D and E). We immunolocalized cofilin-1 using antibodies that recognize either both the phosphorylated (inactive) and dephosphorylated (active) forms and an antibody that recognizes only the phosphorylated form. With the use of the former antibody in sections of control E13.0 metanephros (Fig. 3, A and B), cytoplasmic immunoreactivity was detected in condensing mesenchyme. Cofilin-1 protein was also detected in the ureteric stalk and its first branches; in these epithelia, cofilin-1 immunoreactivity was prominent in the zone between the nucleus and the apical (lumenal) surface.
significant signal in stromal cells, the compartment shown to express prox-1 at this stage. With the use of the antibody specific for the phosphorylated form of cofilin-1, a similar pattern was observed (Fig. 3C). At E15.0, cofilin-1 was immunolocalized in deeper sections of the ureteric tree (i.e., mature collecting ducts), where there was a subapical accentuation of the signal (Fig. 3D). In the nephrogenic cortex, there was little signal above background (data not shown). Western blot analysis confirmed the expression of prox-1 (83 kDa) and cofilin-1 (18 kDa) in lysates of pools of E13.0 and E15.0 metanephroi (data not shown); formal quantification of proteins was not performed in this study.

Expression of apoptosis-related genes Bax and Bcl-2. Expressions of the proapoptotic gene Bax and the antiapoptotic gene Bcl-2 were measured in the various dietary groups. With the use of real-time PCR of E13.0 metanephroi, there was a step-wise increase in the expression of both genes with 9% and 6% LPD (Table 3); the proportional increase was greater for the proapoptotic gene Bax versus the antiapoptotic gene Bcl-2. The increase in Bax expression relative to controls was 560% for 9% LPD and 1,269% for 6% LPD. A similar pattern was observed for Bcl-2, where 9% LPD showed an increase of 286% and 6% LPD showed an increase of 527% compared with controls. Comparison of the ratio of Bax to Bcl-2 demonstrated that this was roughly doubled versus controls in 9% LPD (97% increase) and 6% LPD (141% increase) metanephroi.

**DISCUSSION**

The phenomenon of metabolic programming has received considerable interest over the past few years. Studies examining the permanent effects of intrauterine exposure to a maternal LPD indicate that many physiological functions may be altered including immune function (30), glucose homeostasis (48), and, in particular, blood pressure, as alluded to in the Introduction. Previously, we (56) noted that rat E13.0 body and placental weights and also total numbers of cells within E13.0 metanephroi were unaffected by maternal LPD. We consider the present finding, namely, that maternal LPD alters meta-

### Table 2. Real-time PCR

<table>
<thead>
<tr>
<th>Gene</th>
<th>Accession No.</th>
<th>Function</th>
<th>Primer Pairs</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>α-Tubulin</td>
<td>NM_022298</td>
<td>Cytoskeletal component</td>
<td>5′- TTCAGTGAGACAGGAGCTGG-3′&lt;sup&gt;324&lt;/sup&gt;</td>
<td>34</td>
</tr>
<tr>
<td>Calmodulin-1</td>
<td>NM_031969</td>
<td>Intracellular calcium sensor</td>
<td>5′- CGATTGACTGGCTTCTTGGG-3′&lt;sup&gt;324&lt;/sup&gt;</td>
<td>28</td>
</tr>
<tr>
<td>Cofilin-1</td>
<td>NM_001714</td>
<td>Actin depolymerization enzyme</td>
<td>5′- TTTCTGGTAGGAGATGTGGGG-3′&lt;sup&gt;3264&lt;/sup&gt;</td>
<td>55</td>
</tr>
<tr>
<td>Kinectin-1</td>
<td>NM_00847*</td>
<td>Present on cytoplasmic face of membranous vesicles; function unclear</td>
<td>5′- ACCAGGTTTTCAGGTTACCC-3′&lt;sup&gt;324&lt;/sup&gt;</td>
<td>62</td>
</tr>
<tr>
<td>Lactate dehydrogenase A</td>
<td>NM_017025</td>
<td>Catalyzes conversion of lactate to pyruvate</td>
<td>5′- CATCCAAGTTGATCAGTGGC-3′&lt;sup&gt;31,860&lt;/sup&gt;</td>
<td>29</td>
</tr>
<tr>
<td>Nap1L1 (nucleosome assembly protein 1-like 1)</td>
<td>NM_053561</td>
<td>Putative participant in DNA replication</td>
<td>5′- CGATTGACTGGCTTCTTGGG-3′&lt;sup&gt;324&lt;/sup&gt;</td>
<td>52</td>
</tr>
<tr>
<td>Prox-1</td>
<td>XM_223067</td>
<td>Homeobox transcription factor</td>
<td>5′- TTTCTGGTAGGAGATGTGGGG-3′&lt;sup&gt;3264&lt;/sup&gt;</td>
<td>47</td>
</tr>
<tr>
<td>Translation initiation factor-4, γ-2</td>
<td>XM_341906</td>
<td>Translation repressor</td>
<td>5′- ACCAGGTTTTCAGGTTACCC-3′&lt;sup&gt;324&lt;/sup&gt;</td>
<td>25</td>
</tr>
<tr>
<td>Tyrosine 3-monooxygenase/tryptophan</td>
<td>XM_013053</td>
<td>Activation protein of tyrosine 3-monooxygenase, which catalyzes the conversion of L-tyrosine to L-dihydroxy-phenylalanine; this conversion is lacking in albinism</td>
<td>5′- TGGCTAGGAGCTGGCTTCTTGGG-3′&lt;sup&gt;324&lt;/sup&gt;</td>
<td>21</td>
</tr>
<tr>
<td>5-monooxygenase activation protein, θ-polypeptide</td>
<td>XM_341639</td>
<td>Glycoprotein mediating Ca&lt;sup&gt;2+&lt;/sup&gt;-dependent cell-cell adhesion</td>
<td>5′- GTAGCCATTGCCATCCTTGT-3′&lt;sup&gt;31,283&lt;/sup&gt;</td>
<td>9</td>
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<tr>
<td>CADHERIN-1</td>
<td>XM_344630</td>
<td>Homolog of yeast SMIF3 gene, which suppresses mutations in MIF2, a centromeric protein</td>
<td>5′- CATCCAAGTTGATCAGTGGC-3′&lt;sup&gt;31,860&lt;/sup&gt;</td>
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<tr>
<td>SMT3h2</td>
<td>XM_133594</td>
<td>Cytoskeletal component</td>
<td>5′- CATCCAAGTTGATCAGTGGC-3′&lt;sup&gt;31,860&lt;/sup&gt;</td>
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<tr>
<td>Topoisomerase (DNA) II binding protein</td>
<td>XM_343460</td>
<td>Negative regulator of transcription factor Miz1. Transactivation of Miz1 allows upregulation of p21Cip1 upon UV irradiation</td>
<td>5′- TGGCTAGGAGCTGGCTTCTTGGG-3′&lt;sup&gt;324&lt;/sup&gt;</td>
<td>21</td>
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Transcripts are classified as either down- or upregulated by maternal 9% low-protein diet (LPD). RDA, representational difference analysis. *Transcript for which there is currently no known rat sequence and thus refers to the mouse transcript.
nephric gene expression at E13.0, especially interesting. In
addition, we (56) have reported that maternal LPD increased
apoptosis at the start of metanephrogenesis and thereafter was
associated with a reduction in numbers of precursor cells.
Collectively, the experimental animal data prove that the ma-
ternal diet programs the embryonic kidney, altering metaneph-
pheric cell turnover and gene expression at a time when nephrons
and glomeruli have yet to form and at a time point when overall
“growth” of the conceptus is overtly normal. The human
implication is that the maternal diet ingested between concep-
tion and 5- to 6-wk gestation contributes to the variation in
glomerular numbers that are known to occur between and in
healthy and hypertensive populations.

In the present study, we used a dietary protocol that varied
the protein content of the synthetic diet supplied to pregnant
rats. If this was to have been the sole manipulation, then the
total energy content of the diet would be diminished in the LPD
versus normal diets. To enable conclusions to be drawn regard-
ing the consequences of a dietary protein restriction as opposed
to a global energy reduction, the energy content of the diets had
to be restored; to this end, we increased the quantity of
carbohydrates in the diet. The increase in carbohydrates was
around 15% in the LPD, whereas the decrease in protein was
50% or 66% for the 9% and 6% LPD, respectively. The
increase in carbohydrate content in the LPD was therefore
rather modest, and we consider it to be an unlikely cause of the
phenomena we reported in this study; although a confounding
effect cannot be excluded, we know of no independent pub-
lished data that prove that such a modest increase of carbohy-
drates perturbs embryonic growth.

The current study also suggests that several molecules, some
not previously reported to be expressed in the metanephros,
might be important in kidney development.

In the present analyses of 9% LPD versus the control diet,
data from the quantitative PCR analyses confirmed the up- and
downregulation of genes identified as differentially expressed

Fig. 2. Prox-1 immunohistochemistry. Prox-1
immunolocalization in control metanephoi
is shown. A: E13.0 metanephoi at low
power showing immunoreactive signal
(brown) in stroma (s) around ureteric bud
(u); condensing mesenchyme (m) was gen-
erally negative. B: higher power image of A.
C: similar section to B, probed with isotype
control immunoglobulin. D: E15.0 metane-
phros at high power showing widespread
expression of prox-1 in mesenchyme, and
subsets of nuclei in ureteric bud branches
and nephron vesicles (v). Bar = 100 μm in
A and 25 μm in B–D. E: similar section to
D, probed with isotype control immuno-
globulin.

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in the RDA screen; furthermore, quantitative PCR analyses of a more severe diet restriction, i.e., 6% LPD, showed similar up- or downregulation of the same set of genes. With respect to these studies, it should be noted that mRNA used for the RDA and quantitative PCR analyses were derived from separate pools of embryonic organs for each diet.

Metanephric prox-1 transcripts were downregulated by LPD. Prox-1 is the mammalian homolog of prospero, a Drosophila homeobox transcription factor (35, 47). In fly neuroblast development, prospero localizes in nuclei of ganglion mother cells, causing them to exit the cell cycle and terminally differentiate. In murine development, prox-1 plays a similar role in retinal progenitor cells (17). Prox-1 is also expressed outside the nervous system. In embryonic mice, prox-1 is expressed in endoderm, which will give rise to the liver and pancreas, and null mutant mice have defective hepatocyte migration into the septum transversum and small livers (54). In mice, the gene also has a key role in inducing embryonic vasculature to form lymphatic channels (22), and prox-1 is also expressed in epithelia during taste bud differentiation (41). Recently, a role for the zebrafish homolog in differentiation of the intrarenal (adrenal) primordium has been demonstrated (37). Prox-1-null mutant mice die around E14, but the state of the metanephros, which begins to form at E11.0, has not been reported. Apart from a study (63) showing, as assessed by Northern blot analysis, that prox-1 is expressed in the human fetal kidney, ours is the first report that this gene is expressed in the metanephros. We detected prox-1 in several populations of rat kidney cells. At E13.0, the protein was prominent in “stromal” cells around the ureteric bud stalk; this is likely to be a heterogeneous population of loose mesenchymal cells comprising interstitial and vascular cell precursors. Two days later, at E15.0, a different picture emerged, with subsets of prox-1-positive nuclei in mesenchyme as well as epithelia of ureteric bud branches and nephron precursors. These patterns allow one to speculate that, during metanephrogenesis, prox-1 might enhance the growth of both the interstitial compartment and also epithelial cells. With regard to the former hypothesis, it is well established that metanephric stroma actively supports the growth of adjacent tubules (20). Apoptosis is prominent in this compartment during normal development and is upregulated by LPD (56), and it is possible that prox-1 may protect these cells from dying. Proliferation is downregulated as renal mesenchyme forms nephrons (58), and, with regard to these nascent epithelia, we speculate that prox-1 downregulates proliferation and induces differentiation, analogous to its role in the nervous system.

Metanephric cofilin-1 transcripts were downregulated by LPD. Cofilin-1 is a member of a small family of proteins (cofilin-1, cofilin-2, and ADF) that regulate actin filament turnover (2, 55). The active form, which is dephosphorylated,
binds to F-actin and enhances the rate of actin subunit disso-
cliation, and this form is found in the lamellipodia of migrating
cells (15). LIM kinase inactivates cofilin-1 by phosphorylating
the protein on serine 3 (2); of note, the human LIM gene is
deleted in some individuals with Williams syndrome (2), and
this disorder is associated with diverse urinary tract malforma-
tions including renal hypoplasia (49). In the adult kidney,
cofilin has been detected in cultured human glomerular mes-
angial/pericyte cells (16), and, in vivo, ischemic renal injury in
rats correlates with destabilization of the cortical actin network
within proximal tubule cells together with ADF activation and
relocation to the apical domain (1). Both cofilin-1 and ADF
have been reported to be expressed in the E14 mouse embry-
onic kidney, as assessed by in situ hybridization (55), although
the specific cell types expressing the gene were not detailed. In
the present rat study, at E13.0, we found that cofilin-1 was
immunolocalized in condensing renal mesenchymal cells and
also found in the apical domain of ureteric bud branch tips.
Both of these tissues are undergoing profound cell reorganiza-
tion (i.e., condensation and lumen formation in the mesen-
chyme/nephron lineage and branching in the ureteric bud
lineage). Given its role in actin-based cell motility, cofilin may
certainly play a role in these cell types. Recently, Chua et al.
(11) provided data to show that cofilin is involved in apoptosis
induction; however, at least in E13.0 rat metanephros, cofilin
protein did not appear to localize to loose stroma/loose mes-
enchyme, which is a “hotspot” for LPD-upregulated apoptosis
(56). To test the hypotheses that cofilin-1 or prox-1 might play
active roles in renal organogenesis, functional experiments will
be needed, probably using siRNA to reduce levels of these
proteins in organ cultures of wild-type metanephroi (14).
Furthermore, biochemical experimental analysis is required to assess
how prox-1 might fit into the cascade of transcription factors
that control nephrogenesis (7).

The present RDA screen identified several other genes that
were up- or downregulated by maternal LPD. While space does not
allow a detailed description of all of them, it is notable that lactate
dehydrogenase enzymes have been detected in murine
metanephros (29) and cadherin-11 has been reported to be
expressed within mesenchyme of renal and other organ rudi-
ments (10); of note, using a cDNA microarray analysis, cad-
herin-11 was identified as a gene upregulated in uninduced
mouse renal mesenchyme (9). We found that calmodulin was
downregulated by maternal LPD, and others have reported that
Calmodulin-binding protein-1 has been detected in forming
nephrons (38). The downregulation of α-tubulin by LPD is also
of note because this molecule is a major component of the
cytoskeleton and critical for the maintainance of renal epithelial
polarity and the differentiated state by its presence in
microtubules terminating in the subapical web and basal bodies
(5, 43).

Given that our previous LPD study (56) linked upregulated
metanephric apoptosis to maternal LPD, it was notable that the
RDA screen performed in the present study did not identify
altered expression of genes that are “classically” associated
with apoptosis. Bax and Bcl-2 are known to be expressed in
normal kidney development, and their expression is known to
be deregulated in certain conditions associated with perturbed
nephrogenesis and apoptosis, e.g., blockage of fetal urine flow
(53, 36). Using real-time PCR of E13.0 metanephroi, we
observed a step-wise increase in the expression of both genes
with 9% and 6% LPD; interestingly, the proportional increase
was greater for the proapoptotic gene Bax versus the antiapop-
totic gene Bcl-2, perhaps suggesting that LPD shifts the bal-
ance of expression to upregulate the death of metanephric
precursor cells. The fact that the RDA did not identify these
changes underlines the fact that this technique, while useful for
detecting some alterations in gene expression, is by no means
exhaustive.

How might gene expressions be altered by maternal diet?
Glucocorticoids mediate embryonic programming because
dexamethasone administration in pregnant rats causes renal
hypoplasia and hypertension in offspring (8), and LPD reduces
placental 11β-hydroxysteroid dehydrogenase, an enzyme
protecting the conceptus from maternal glucocorticoids, and up-
regulate fetal biochemical markers of glucocorticoid exposure
(31). The developing urinary tract expresses glucocorticoid
receptors (27), and it would be informative to study the effects
of glucocorticoids on the expression of the genes identified in
the present RDA screen. Experimental intrauterine growth
retardation is another scenario associated with increased renal
apoptosis and glucermeral deficits (50); interestingly, the same
study reported that p53 gene methylation status was altered in
these experimental animals. Intriguingly, altered methylation
of the human prox-1 gene occurs in some hematological
malignancies (44). Taken together, it would be interesting to
perform biochemical experiments to ascertain whether the
maternal LPD alters the methylation status and transcriptional
rate of genes such as prox-1 and cofilin-1.

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