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Effect of 30 per cent maternal nutrient restriction from 0.16 to 0.5 gestation on fetal baboon kidney gene expression

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Previous studies in rodents and sheep show that maternal nutrient restriction during pregnancy alters fetal renal development. To date, no studies using fetal baboon RNA with human Affymetrix gene chips have been published. In the present study we have (1) evaluated the specificity of the Affymetrix human gene array 'Laboratory on a Chip' system for use with fetal baboon mRNA and (2) investigated the effects of moderate maternal global nutrient restriction (NR; 70% of *ad libitum* animals) from early (30 days gestation (dG)) to mid-gestation (90 dG; term = 184 dG) on the fetal baboon kidney. Morphometric and blood measurements were made on 12 non-pregnant baboons before they were bred. All baboons were fed *ad libitum* until 30 days pregnant, at which time six control baboons continued to feed *ad libitum* (control – C) while six received 70% of the C diet on a weight adjusted basis. Fetal kidneys were collected following caesarean section at 90 dG, with samples flash frozen and fixed for histological assessment. Fetal hip circumference was decreased in the NR group (68 ± 2 *versus* 75 ± 2 mm), while fetal body weight and all other measurements of fetal size were not different between C and NR at 90 dG. Maternal body weight was decreased in the NR group (12.16 ± 0.34 *versus* 13.73 ± 0.55 kg). Having established the specificity of the Affymetrix system for fetal baboon mRNA, gene expression profiling of fetal kidneys in the context of our maternal nutrient restriction protocol shows that NR resulted in a down-regulation of genes in pathways related to RNA, DNA and protein biosynthesis, metabolism and catabolism. In contrast, genes in cell signal transduction, communication and transport pathways were up-regulated in the NR group. These changes indicate that even a moderate level of maternal global NR impacts fetal renal gene pathways. Our histological assessment of renal structure indicates decreased tubule density within the cortex of NR kidneys compared with controls. The number of glomerular cross-sections per unit area were unaffected by NR, suggesting that tubule tortuosity and/or tubule length was decreased in the NR kidney. Taken together the changes indicate that NR results in accelerated fetal renal differentiation. The negative impact of poor maternal nutrition on the fetal kidney may therefore be in part due to shortening of critical phases of renal growth resulting in decreased functional capacity in later life. These findings may have important implications for postnatal renal function, thereby contributing to the observed increased predisposition to hypertension and renal disease in the offspring of nutrient restricted mothers.

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A compelling set of animal research studies exist to demonstrate that maternal nutritional deficiencies, in global intake (Gilbert *et al.* 2005) as well as in altered intake of micro- and macronutrients (Galaverna *et al.* 1995; Langley-Evans, 1996; Welham *et al.* 2005), have adverse effects on fetal growth and development and offspring physiology. Growth of the fetal kidney has received considerable attention due to the vital role it plays in blood pressure and body fluid homeostasis during fetal life, in the transition to an independent postnatal existence

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and throughout postnatal life (Ingelfinger & Woods, 2002; Rasch *et al.* 2004).

The effects of differing degrees of restriction in maternal nutrient availability (from 30 to 90% restriction) on development of fetal organs including the kidney has been extensively studied in rodents and sheep (Langley-Evans *et al.* 1996; Ingelfinger & Woods, 2002). There are several developmental differences between commonly studied laboratory species (e.g. rats and mice), large animals such as sheep, and primates. For example, most rodents are polytocous and deliver immature young. Although ruminants deliver fewer and more mature offspring, ruminant species have a different placental structure from primates. Oxygen and glucose consumption of the ruminant placenta are higher on a weight adjusted basis than those of the human placenta (Hay, 1994).

The mounting evidence for critical effects of maternal nutrition on fetal development in non-primate species supports the need to examine the effects of suboptimal maternal nutrition in a non-human primate model. We have developed a non-human primate model, the baboon, through which we can provide key comparative primate data to guide extrapolation of rodent and ruminant data to human pregnancy. The baboon is the non-human primate species in which the greatest amount of experimental data is available on maternal and fetal function (Ducsay *et al.* 1991; Hennessy *et al.* 1994; Koenen *et al.* 2002; Antonow-Schlorke *et al.* 2003; Pepe *et al.* 2003). We have developed a group housing system, described in detail elsewhere (Schlabritz-Loutsevitch *et al.* 2004), which allows each animal to maintain its normal physical and social activity while at the same time enabling us to regulate each animal's food intake and monitor weight daily.

The purpose of this study was twofold, first to perform an initial evaluation of specificity of the Affymetrix human gene array U133A Plus 2.0 'Laboratory on a Chip' system for use with baboon mRNA, and second to utilize this system to determine the impact of moderate global maternal nutrient restriction (NR) from 30 days of gestation (dG) to 90 dG (term 184 dG) on genes involved in key pathways in the fetal baboon kidney. Gene expression profiling in kidneys of fetuses of NR mothers showed down-regulation of genes in pathways related to RNA, DNA and protein biosynthesis, metabolism and catabolism, actin cytoskeleton assembly, and apoptosis compared with kidneys of fetuses from *ad libitum* fed (control, C) mothers. In contrast, genes involved in cell signal transduction, communication and transport pathways were up-regulated. Expression profiles of apoptosis-related genes were confirmed by quantitative reverse transcription real time PCR (QRT-PCR). These changes indicate that even moderate maternal global nutrient restriction affects various fetal renal metabolic pathways differently. Histological analysis of 90 dG kidney sections showed decreased structure per unit area in the NR *versus* the C kidney. Taken together the changes indicate that 30% maternal NR accelerates fetal renal differentiation and inhibits kidney structure development. The negative impact of poor maternal nutrition on the fetal kidney may therefore be in part due to shortening of critical phases of growth.

Methods

Animal care and maintenance

All procedures were approved by the Southwest Foundation for Biomedical Research (SFBR) Institutional Animal Care and Use Committee and conducted in facilities approved by the Association for Assessment and Accreditation of Laboratory Animal Care. Six baboons from two independently housed groups each consisting of 16 females and one male were studied. Each group was housed in a cage that was 3.5 m high with a floor area of 37 m^2. Details of housing structure and environmental enrichment provided have been published elsewhere (Schlabritz-Loutsevitch *et al.* 2004). Maternal morphometric measurements were made prior to pregnancy to ensure homogeneity of females in the two groups.

System for controlling and recording individual feeding

Once a day prior to feeding, all baboons were run into individual feeding cages. Baboons passed along a chute, over a weighing scale and into one of the individual feeding cages. Once in the individual cages they were fed either between 07.00 h and 09.00 h or between 11.00 h and 13.00 h as previously described (Schlabritz-Loutsevitch *et al.* 2004). Food was provided as Purina Monkey Diet 5038, standard biscuits. Water was continuously available in the feeding cage through individual lixits and at several locations in the group housing.

Formation of stable grouping for the nutrient restriction study

Each group of 16 females was initially housed with a vasectomized male to establish a stable social group (Schlabritz-Loutsevitch *et al.* 2004). All female baboons were observed twice a day for well-being and three times a week for turgescence (sex skin swelling) and signs of vaginal bleeding to enable timing of pregnancy (Hendrickx, 2001). At the end of a 30-day period of adaptation to the feeding system, a fertile male was introduced into each breeding cage. Pregnancy was dated initially by following the changes in the swelling of the sex skin and confirmed at 30 dG by ultrasonography.

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Diet and food consumption

The Purina Monkey Diet 5038 fed is described by the vendor as 'a complete life-cycle diet for all Old World Primates.' The biscuit contains stabilized vitamin C as well as all other required vitamins. Its basic composition is crude protein not less than 15%, crude fat not less than 5%, crude fibre not more than 6%, ash not more than 5% and added minerals not more than 3% (Schlabritz-Loutsevitch *et al.* 2004). At the start of the feeding period, each baboon was given 60 biscuits in the feeding tray. At the end of the 2-h feeding period after the baboons had returned to the group cage, the biscuits remaining in the tray and on the floor of the cage and in the pan were counted. Food consumption of animals, their weights and health status were recorded daily. The weight of each baboon was obtained as she crossed the electronic scale system (GSE 665; GSE Scale Systems, MI, USA). A commercial software application designed to capture weight data was modified to permit the recording of 50 individual measurements over 3 s. If the standard deviation of the weight measurement was greater than 0.01 of the mean weight, the weight was automatically discarded and the weighing procedure begun again. All baboons were fed *ad libitum* until 30 dG when six control baboons continued to feed *ad libitum* and six were fed 70% of feed consumed by controls on a weight adjusted basis.

Caesarean sections were performed at 90 dG under isoflurane anaesthesia (2%, $2\,\mathrm{l\,min^{-1}}$) to obtain the fetus and placenta. Techniques used and postoperative maintenance have been previously described in detail (Schlabritz-Loutsevitch *et al.* 2004). Analgesia was provided with buprenorphine hydrochloride at $0.015\,\mathrm{mg\,kg^{-1}\,day^{-1}}$ during 3 postoperative days (Buprenex® Injectable, Reckitt Benckiser Health care (UK) Ltd, Hull, UK). Fetal morphometrics were obtained at the time of caesarean section. In addition, fetal kidneys were collected and cut in half longitudinally. One half was immediately snap frozen in liquid nitrogen and then stored at −80°C until used for RNA extractions. The other kidney half was fixed in formalin and embedded in paraffin for histological analyses.

RNA isolation from tissue

RNA was isolated from tissue using TRIzol Reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Briefly, approximately 100 mg section of frozen kidney was cut from one pole of a longitudinally sliced kidney half. The tissue was homogenized in 1 ml Trizol Reagent using a Power General Homogenizer (Omni International, Wilmington, DE, USA). Genomic DNA in the sample was sheared by passing the homogenate three times through a 22-gauge needle attached to a 1 ml syringe. The homogenized samples were incubated for 5 min at 25°C. Two hundred microlitres of chloroform was added to each sample, and the samples were shaken vigorously by hand for 15 s and incubated at 25°C for 3 min. Samples were then centrifuged at 4°C and 12 000 *g* for 15 min. The aqueous phase containing RNA was transferred to a fresh tube and the RNA precipitated by addition of 0.5 ml of isopropyl alcohol. Samples were incubated for 10 min at 25°C and then centrifuged at 4°C and 12 000 *g* for 10 min. The RNA precipitate was washed with 1 ml of 75% ethanol and centrifuged at 4°C and 7500 *g* for 5 min. The RNA was resuspended in 100 μl DEPC-treated water and stored at −80°C.

Preparation of cRNA probe for gene chip interrogation

Total RNA samples were shipped on dry ice to Genome Explorations, Inc. (Memphis, TN, USA) for RNA quality check, cRNA synthesis, and determination of gene expression profiles for each RNA sample by interrogation of the Affymetrix Human Genome U133 Plus 2.0 Array (Affymetrix, Santa Clara, CA, USA). RNA Quality was checked using an Agilent Bioanalyser 2100 'Laboratory on a Chip' system. RNA concentrations were confirmed by quantification using a dual beam spectrophotometer on approximately 200 ng of each RNA sample. Complementary RNA was synthesized and biotin labelled at Genome Explorations, Inc. using the MessageAmp™ aRNA Kit (catalogue no. 1750, Ambion, Austin, TX, USA) according to the manufacturer's instructions. Total RNA was used for first and second strand cDNA synthesis followed by an *in vitro* transcription step to synthesize biotin-labelled cRNA. The cRNA was quality checked and then hybridized to the Human Genome U133 Plus 2.0 Array (Affymetrix).

Gene chip data collection and analysis

Gene expression was detected using GCOS software (Affymetrix). Sequence data available for baboon genes show sequence differences in 3′UTRs compared with human genes. Since the majority of Affymetrix's probe sets are based on 3′UTR sequences and baboon RNA was used to screen human gene chips, we evaluated gene expression from unfiltered complete data sets in addition to the standard Affymetrix filtered data set. Analyses, including data normalization, transformation, *t* tests, and gene expression profile overlaying Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways, were carried out using GeneSifter software (GeneSifter.Net, VizX Laboratories, Seattle, WA, USA).

To assess the frequency of individual probe mismatches within a gene's probe set and determine the impact of

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mismatches on ‘gene quality’ called by the Affymetrix software, we evaluated individual probe hybridizations (11 probes per probe set) for 40 genes on the human Affymetrix gene chip that were probed with 12 different RNA pools (480 observations). For this analysis, kidney RNA was pooled from the same animals used for interrogation of Affymetrix arrays. RNA was also pooled for lung and placental tissues for the control and experimental groups and pooled for these tissues from 175 dG placentas and fetuses (authors’ unpublished data).

Statistical analysis

Fetal and maternal morphometric data analysis was performed using a one-sided Student’s *t* test. Array data were all-median normalized and $\log_2$ transformed using GeneSifter software (GeneSifter.Net). Statistical analyses of array data were performed by *t* test using GeneSifter software.

Rationale for array analysis methods

To assess the impact of Affymetrix’s filtering software on cross-species (baboon/human) probe-to-target hybridizations, we assessed the frequency of individual probe mismatches within a gene’s probe (set) for 40 genes on the human Affymetrix gene chip that were probed with 12 different RNA pools. We also determined the relationship between probe mismatches within a gene’s probe set on ‘gene quality’ called by the Affymetrix software.

Affymetrix filtering software indicates an ‘absent call’ for any gene with a detection P-value > 0.059 and assigns that gene a quality score of 0; genes with detection P-values < 0.05 are called ‘present’ and assigned quality scores of 1. We found that high intensity genes with as many as five probe mismatches in an 11 probe set for each gene had detection P-values < 0.05 and were called ‘present’, whereas less intense genes (70–410) with three mismatches had detection P-values > 0.05 and were called ‘absent’. Moderately intense genes (410–580) were called ‘present’ with zero, one, two, or three probe mismatches but were called ‘absent’ with four mismatches (< 410) or with 5 mismatches (< 580).

In the absence of baboon sequence data for these 40 genes, it is not possible to determine if absent calls are due to probe sequence mismatches for baboon RNA to human probe set hybridizations or due to cross-hybridization from gene family members. For genes with probe set mismatches of one or two probes, it is more likely that these mismatches are due to species sequence differences, whereas probe set mismatches of three or more probes could be argued for either case.

Based on the correlation between gene expression intensity and allowable probe mismatches within a gene’s probe set, we analysed the gene expression profiles using both unfiltered (no quality minimum required) and filtered gene lists (minimum quality for group averages $= 0.5$) with the rationale that low expression genes with large variations in expression will not be statistically significantly different between the two groups ($P < 0.05$). Although filtered *versus* unfiltered gene lists impacts inclusion of individual genes in the dataset, this did not alter KEGG pathways (see below) with significant z-scores (data not shown). The final data set presented and analysed in this study ($n = 685$) only includes genes with minimum quality scores of 0.5.

Pathway analysis

To perform pathway analyses, we first created a ‘custom baboon array’ using GeneSifter. z-Score calculations defining significant gene categories and pathways are based on the total number of genes on the array. Thus, to accurately calculate z-scores using GeneSifter software, the array of baboon genes for which expression was detected on the human gene chip had to be defined. To do so, we merged expression array data from five baboon tissues at three fetal time points and three adult baboon tissues. Any gene from any baboon RNA sample with a marginal or present call on the human genechip (Affymetrix U133A 2.0) was considered expressed and included in the ‘custom baboon array’. Using this method, 16 186 of the 22 227 genes on the genechip were detected using baboon RNA. Thus, these 16 186 genes comprise the ‘custom baboon array’ from which z-scores were calculated.

Array data for significantly differently expressed genes were overlaid onto Ontological pathways (http://www.geneontology.org/) (Ashburner *et al.* 2000) and KEGG pathways (www.genome.jp/kegg/) (Kanehisa *et al.* 2004) using GeneSifter software. The ontological and KEGG pathway analyses provide detailed data on individual genes in the context of that gene’s role in described biological/biochemical pathways. Pathways were considered significantly altered from the control gene expression profiles if the z-score for that pathway was less than -2 or greater than 2. z-Scores were calculated in GeneSifter using the following formula:

$$z\text{-Score} = [r - n(R/N)]/[v((n(R/N))(1 - R/N) \times (1 - ((n - 1)/(N - 1))))]$$

where R = total number of genes meeting selection criteria, N = total number of genes measured, r = number of genes meeting selection criteria with the specified GO term, and n = total number of genes measured with the specific GO term (Doniger *et al.* 2003).

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QRT-PCR quantification of target gene abundance

To measure mRNA levels of target genes, we used the Assays-on-Demand system (Applied Biosystems, Foster City, CA, USA). Although no baboon specific primers and probes are available, we have successfully used the Assays-on-Demand system for more than 40 different baboon genes. We quantified mRNA according to manufacturer's instructions (Applied Biosystems). In brief, total RNA (50 ng) was reverse transcribed in a 100-μl reaction using a High-Capacity cDNA Archive Kit (Applied Biosystems). Complimentary DNA synthesis was followed by real-time-PCR using gene specific primers provided by the manufacturer (CREBBP, Hs00231733_m1; BCL2L13, Hs00209787_m1; SGK, Hs00178612_m1; VEGF, Hs00900054_m1; RHOB, Hs00269660_s1) TaqMan Universal PCR master mix (Applied Biosystems) and the target cDNA. 18S rRNA was quantified as an endogenous control using the Applied Biosystems human Assay-on-Demand probe set. All samples were assayed in triplicate.

For relative quantification of gene expression, the comparative threshold cycle (Ct) method was employed (described in User Bulletin 2 for ABI PRISM® 7700 Sequence Detection Systems). The value obtained for Ct represents the PCR cycle at which an increase in reporter fluorescence above a background signal can first be detected (10 times the standard deviation of the baseline). Using this approach, the endogenous control Ct values were subtracted from the gene of interest Ct values to derive a ΔCt value. The relative expression of the gene of interest was then evaluated using the expression 2-$\Delta\Delta$Ct, where the value for $\Delta\Delta$Ct was obtained by subtracting the ΔCt of the calibrator from each ΔCt, using the mean of the negative control as the calibrator.

Kidney morphology

Formalin-fixed 90 dG kidneys from control and experimental animals were paraffin embedded and cut into 5 μm sections. Kidney sections were photographed at 20× magnification. Two 5 μm paraffin cross-sections (50 μm apart) from approximately halfway between one pole and the hilus were stained with H&E. Each section was assessed using a Nikon E600 microscope (Nikon Instruments Inc., Lewisville, TX, USA) and a SPOT RT Color CCD camera (Diagnostic Instruments Inc., Sterling Heights, MI, USA). Using 20× magnification, photographs were taken of adjacent, non-overlapping fields around the periphery of each section immediately below the nephrogenic zone. An average of 15 photographs per kidney were examined. Cross sections of convoluted tubules, ducts and glomeruli were counted and the area/field occupied by convoluted tubules was calculated using ImageJ (Abramoff *et al.* 2004) and its cell counter (De Vos, 2006) and multiple region of interest (Abramoff *et al.* 2004) plugins. Glomerulus cross-sections that extended beyond the periphery of the field were ignored. The area of the field occupied by tubules was expressed as a percentage of the total field. The area and cross-section counts from the two sections for each animal were averaged.

Results

Morphometric measurements of mother, fetus and fetal kidney

Morphometric measures were collected on the mothers before pregnancy (Table 1A). There were no differences between the two sets of animals that were used for the *ad libitum* and NR groups. Average maternal weight decreased 1.30 kg in the NR mothers at 90 dG compared with preconception average weight ($P < 0.05$). Measures were also collected on the 90 dG fetuses and placentas (Table 1B). Hip circumference was less in NR fetuses than control fetuses ($P < 0.05$).

Overview of gene expression differences between control and nutrient restricted 90 dG kidneys

A pair-wise comparison of gene expression was performed between control ($n = 6$) and maternal nutrient restricted ($n = 6$) 90 dG fetal kidney RNA samples. Data were all-median normalized, $\log_2$ transformed and analysed using the custom baboon array. Data were then analysed by comparing control and experimental group averages for fold differences in expression at 1.5×, 1.8× and 2.0×. A more rigorous approach using pair-wise comparisons was performed on the data using a *t* test to identify genes expressed significantly differently between groups. This dataset was then filtered using a minimum quality score of 0.5. A final sorting of genes was performed by increasing the minimum gene expression difference between groups to 2.0×.

Evaluation of gene expression based on gene product function and different selection criteria

Table 2 presents the overview of gene expression differences between C and NR fetal kidneys. Genes identified in Table 2, using increasingly rigorous selection criteria, were grouped by biological function from Gene Ontology terms (Ashburner *et al.* 2000). Table 3 presents the number of genes differentially expressed in each category listed in the table. *z*-Scores for each category are also presented with significant *z*-scores ($= -2$ or $= 2$) indicated with bolded font. The data presented in this table show that one category of gene products regardless of gene selection criteria, general signal transducer activity,

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Table 1. Morphometric characteristics of (*A*) non-pregnant baboons before pregnancy in *ad libitum* (*Ad lib*) fed ($n = 6$) and nutrient restricted (NR, $n = 6$) at 90 days' gestation and (*B*) their fetuses and placenta at 90 days' gestation

	Unit	*Ad lib* ($n = 6$)	NR ($n = 6$)
A. Maternal non-pregnant			
Weight at physical	kg	13.44 ± 0.708	13.46 ± 0.484†
Weight –4 weeks	kg	13.39 ± 0.647	13.02 ± 0.241†
Height	cm	87.63 ± 1.602	87.68 ± 0.764
Biparietal distance	cm	9.40 ± 0.732	9.72 ± 0.334
Abdominal distance	cm	9.88 ± 1.047	11.07 ± 0.392
Sterno-pubis distance	cm	38.05 ± 1.199	39.37 ± 1.314
Chest circumference	cm	54.62 ± 1.893	54.50 ± 1.065
Waist circumference	cm	42.96 ± 1.638	49.58 ± 2.031†
Hip circumference	cm	53.00 ± 3.329	52.83 ± 1.364
B. 90 dG			
Maternal and placenta			
Body weight last day	kg	13.73 ± 0.552	12.16 ± 0.339*
Body weight last week	kg	13.46 ± 0.540	12.10 ± 0.307
Placenta	g	73.08 ± 6.510	62.93 ± 1.485
Placenta diameter	cm	8.20 ± 0.339	7.25 ± 0.316
Fetal membranes	g	12.86 ± 3.842	7.52 ± 1.036
Umbilical cord length	cm	14.10 ± 0.775	11.83 ± 0.901
Fetal			
Weight	g	102.13 ± 4.278	95.43 ± 3.257
Length	cm	17.66 ± 0.333	17.58 ± 0.436
Weight/length	g cm^{-1}	5.77 ± 0.142	5.43 ± 0.145
BMI	kg m^{-2}	3.266 ± 0.048	3.10 ± 0.122
Biparietal distance	mm	33.50 ± 0.529	34.17 ± 0.703
Abdominal distance	mm	22.83 ± 3.198	29.17 ± 1.621
Femur length	mm	35.00 ± 1.506	32.50 ± 1.118
Chest circumference	mm	89.16 ± 3.005	87.92 ± 2.275
Waist circumference	mm	77.33 ± 2.667	75.83 ± 2.713
Hip circumference	mm	75.00 ± 1.826	68.33 ± 1.667*
Sterno-pubis	mm	66.66 ± 5.426	70.83 ± 0.833
Total kidney	g	0.85 ± 0.122	0.83 ± 0.127

Values are means ± S.E.M.; $^*P < 0.05$ compared to *ad lib* fed group: $†P < 0.05$ compared with 90 dG maternal weight.

Table 2. Summary of expression-based gene selection

Expressed genes	≥Fold change	$P \leq$	Quality
22277	—	—	0
2793	1.5	—	0
1205	1.8	—	0
742	2.0	—	0
1088	1.1	0.05	0
685	1.1	0.05	0.5
19	2.0	0.05	0.5

'Expressed genes' indicates the number of genes detected based on fold change between mean control and mean nutrient restricted RNA samples. 'Quality' indicates the Affymetrix assessment of probe to target hybridization quality where 0.5 quality indicates that at least one-half of all samples (control plus experimental, $n = 12$) were called 'present'. All data were all-median normalized and $\log_2$ transformed.

is significantly altered when comparing 90 dG kidney RNA samples from fetuses in the C and NR groups. The cellular processes category was significantly altered when the expressed genes were sorted according to fold change between C and NR but not when sorted according to *P*-values. This suggests that although more genes than expected by chance differ between C and NR samples in this category, and that the differences are as much as 2-fold, a number of these differentially expressed genes have large variations in gene expression within each sample group. An interesting note is that genes categorized as important for transcriptional regulatory activity only show a significant *z*-score by using a 1.8-fold minimum expression difference or the most rigorous selection method of statistical significance and quality score.

A general analysis of the genes ($n = 685$) selected based on *P*-values ($P = 0.05$) and probe set hybridization

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Table 3. Summary of gene expression by biological function category

		1.5× 2793		1.8× 1205		2.0× 742		1.1×, $P < 0.5$ 1088		1.1×, $P < 0.5$, $Q = 0.5685$	
Gene ontogeny categories	Gene total	No. of genes	*z*-score	No. of genes	*z*-score	No. of genes	*z*-score	No. of genes	*z*-score	No. of genes	*z*-score
Antioxidant activity	24	2	−1.7	1	−0.99	1	**2.69**	2	−0.89	2	0.63
Behaviour	55	14	0.83	7	1.34	5	1.14	7	0.72	4	0.68
Binding	5561	1315	1.61	588	1.87	367	1.82	542	−0.55	340	−1.73
Catalytic activity	3129	687	**−2.15**	290	−1.74	184	−1.04	301	−0.93	204	0.51
Cellular process	4110	1013	**3.18**	472	**2.72**	301	**2.58**	424	1.9	255	−0.56
Chaperone activity	1	0	−0.38	0	−0.26	0	−0.21	0	−0.6	0	−0.54
Chaperone regulator activity	5	1	−0.85	1	1.85	0	−0.21	0	−0.27	0	−0.24
Development	1130	303	**3.11**	129	1.04	87	1.77	110	−0.31	71	−0.52
Enzyme regulator activity	403	110	1.65	51	1.38	34	1.39	48	1.85	30	1.16
Motor activity	103	25	0.31	8	−1.04	5	−0.87	6	−1.15	5	−0.69
Nutrient reservoir activity	1	0	−0.38	0	−0.26	0	−1.85	0	−0.27	0	−0.24
Obsolete biological process	9	0	−1.14	0	−0.79	0	−0.63	0	−0.8	0	−0.72
Physiological process	6513	1506	0.35	664	−0.9	407	−1.66	655	1.05	425	1.21
Reg. of biological process	348	83	1.9	37	1.62	24	1.24	44	**2.15**	28	1.71
Signal transducer activity	1367	369	**4.36**	176	**3.01**	116	**2.9**	118	**−3.01**	64	**−3.42**
Structural molecule activity	476	105	−1.05	46	−0.41	25	−0.87	43	−0.83	28	−0.75
Transcription regulator act.	832	194	−1.53	72	**−2.38**	47	−1.37	87	0.55	55	2.16
Translation regulator act.	79	9	**−2.37**	4	−1.85	1	−0.74	5	−0.97	3	−0.88
Transporter activity	933	239	**2.67**	113	1.45	70	1.94	92	0.29	59	0.76
Viral life cycle	26	8	1.6	3	1.05	1	−0.77	2	−0.93	2	0.51

The top row of the table includes abbreviated gene sorting information from Table 2 and the second row indicates the number of differentially expressed genes from the indicated selection criteria. 'Gene total' indicates the total number of genes on the Affymetrix chip in the stated category; 'No. of genes' indicates the number of genes differentially expressed in that category. Categories in bold font show at least one sorted group with a significant *z*-score.

quality ($Q = 0.5$) show 96 genes significantly up- and 589 significantly down-regulated when comparing kidney RNA from C compared with NR fetuses. The graphical representation of this analysis, presented as a heat map in Fig. 1, provides an overall view of the variation within groups and between groups for this dataset. Array data from each kidney RNA sample are represented by one column in the heat map.

Evaluation of gene expression according to ontological categorizations

We performed further analysis of the 685-gene set by examining the changes in gene expression between kidney samples from fetuses of C and NR mothers based on biological classifications established by the Gene Ontology Consortium (Ashburner *et al.* 2000). Gene products have been categorized based on the descriptive terms included in gene databases such as GenBank and LocusLink. Gene ontologies, organized in a hierarchical structure, allow categorization of each gene product based on current detailed information; this also allows placement of genes into multiple categories based on known gene product function or conserved domain-based prediction of gene product function. The detailed gene ontology report provides an overview of all genes expressed on the array grouped by gene ontology. An abbreviated form of the gene ontology report, which includes categories containing four or more genes, is provided in Tables 4 and 5. Table 4 shows those specific gene ontologies that are differentially up-regulated, and Table 5 those that are down-regulated. Since each gene may be included in multiple ontologies, the total number of genes listed in Tables 4 and 5 exceeds the total number of differentially expressed genes in the data set. The classification of differentially expressed genes suggests that groups of genes involved with electron, Golgi vesicle and nucleocytoplasmic transport, carbohydrate, sterol and steroid metabolism, metal ion binding, protein transport and targeting, protein tyrosine phosphatase and heterodimerization activity, regulation of cyclin-dependent protein kinase activity, and voltage-gated potassium channel activity are up-regulated in 90 dG fetal kidneys as a result of maternal nutrient restriction. This classification approach also indicates that groups of genes involved with DNA and RNA biosynthesis, metabolism, processing and packaging, and nucleic acid component biosynthesis and metabolism are down-regulated. Also down-regulated are groups of genes associated with protein biosynthesis and metabolism,

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cell growth, communication, organization, biogenesis, and surface receptor signal transduction, cytoskeleton organization and cytoskeleton biogenesis. Furthermore, genes relevant to protein complex assembly, are also down-regulated. Narrower categories down-regulated due to NR included ubiquitin-mediated proteolysis and cell cycle. Genes specific to cell cycle regulation and ubiquitin-mediated proteolysis are shown in Table 6.

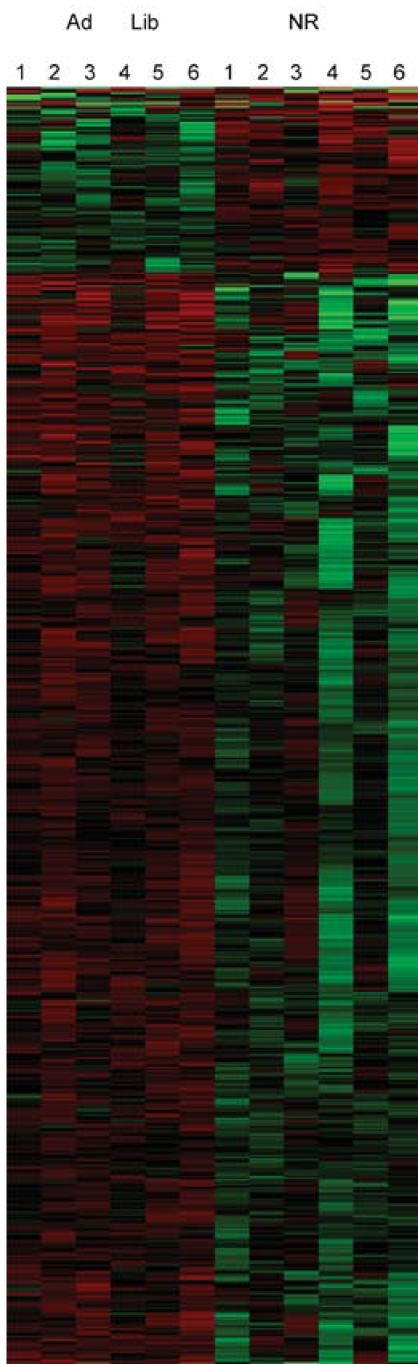

Figure 1. Heat Map of 685 significantly differently expressed genes comparing RNA samples from kidneys of 90 dG fetuses from control ($n = 6$) and nutrient restricted mothers ($n = 6$)
Control fetuses are represented in the 6 left columns (Ad Lib) and samples from fetuses of nutrient restricted mothers are represented in the 6 right columns (NR). Increased expression of experimental *versus* control samples is indicated in red and decreased expression of experimental *versus* control is represented in green.

Evaluation of gene expression based on KEGG pathways

We performed a more detailed analysis of the differently expressed genes to provide insight into cellular functions influenced by these changes in gene expression. Differentially expressed genes were overlaid onto KEGG Pathways to identify pathways impacted by changes in expression for these genes. From the KEGG pathway analysis of the 685 significantly differently expressed genes, we found a number of pathways with significant *z*-scores.

KEGG pathways in which all or most statistically different genes, based on *t* test analyses, were up-regulated in the NR kidneys include: C_{21}-steroid hormone metabolism (z-score = 3.47), complement and coagulation cascades (z-score = 2.88), cyanoamino acid metabolism (z-score = 2.82), D-glutamine and D-glutamate metabolism (z-score = 2.82), terpenoid biosynthesis (z-score = 2.45), and *O*-glycan biosynthesis (z-score = 2.19). One example, presented in Fig. 2, shows a graphical representation of the C_{21}-steroid hormone metabolism KEGG pathway, with genes encoding HSD11B1 (hydroxysteroid (11-β) dehydrogenase) and CYP17A1 (cytochrome P450, family 17, subfamily A, polypeptide 1) significantly up-regulated.

KEGG pathways in which all or most statistically different genes are down-regulated in the nutrient restricted kidneys include: Wnt signalling pathway (z-score = 4.03), peptidoglycan biosynthesis (z-score = 3.59), oxidative phosphorylation (z-score = 3.13), focal adhesion (z-score = 2.91), adherens junction (z-score = 2.66), neuroactive ligand–receptor interaction (z-score = −2.63), tight junction (z-score = 2.38), C5-branched dibasic acid metabolism (z-score = 2.34), ATP synthesis (z-score = 2.29), arginine and proline metabolism (z-score = 2.19), and regulation of actin cytoskeleton (z-score = 1.94).

Both ontological categorization and KEGG pathway analysis indicate down regulation of genes encoding actin cytoskeleton assembly and polymerization components in NR kidneys. Figure 3 provides a graphical representation of the KEGG pathway for genes in the actin cytoskeleton assembly and polymerization pathway. Of the 16 significantly differently expressed genes, 14 are down-regulated in the nutrient restricted group. Signal was not detected for 10 genes in this pathway (indicated by 'ND' in Fig. 3).

Some KEGG pathways and ontological groups showing significant *z*-scores include genes that are significantly up-regulated as well as genes that are significantly

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Table 4. Ontological categorization of up-regulated genes

Up-regulated biological pathways	Diff exp genes	Up-reg	Down-reg	Tot on array	*z*-Score up
Electron transport	19	6	13	236	2.61
Golgi vesicle transport	8	2	6	53	2.15
Lyase activity	8	3	5	102	2.08
Main pathways of carbohydrate metabolism	7	3	4	75	2.77
Metal ion binding	111	9	102	1927	−2.52
Nucleocytoplasmic transport	11	3	8	88	2.43
Protein heterodimerization activity	2	2	0	27	3.46
Protein targeting	8	3	5	107	2.02
Protein transport	36	8	28	384	2.4
Protein tyrosine phosphatase activity	3	2	1	47	2.34
Regulation of cyclin dependent protein kinase activity	3	2	1	30	3.27
Steroid metabolism	10	3	7	104	2.08
Sterol metabolism	7	2	5	55	2.09
Transition metal ion binding	58	3	55	1042	−2.37
Voltage-gated potassium channel activity	2	2	0	50	2.22
Zinc ion binding	42	0	42	790	−2.9

'Diff exp genes' indicates the total number of genes differentially expressed on the array in that category; 'Up-reg' indicates the total number of up-regulated genes; 'Down-reg' indicates the total number of down-regulated genes; 'Tot on array' indicates the total number of genes on the array in that ontological category; and '*z*-Score up' indicates the *z*-score for that category.

Table 5. Ontological categorization of down-regulated genes

Down regulated biological pathways	Diff exp genes	Up-reg	Down-reg	Tot on array	*z*-Score down
Actin binding	21	3	18	160	3.27
Actin polymerization and/or depolymerization	4	0	4	21	2.74
Amine biosynthesis	7	0	7	50	2.66
Amino acid derivative biosynthesis	3	0	3	15	2.48
Amino acid derivative metabolism	5	0	5	35	2.3
Biogenic amine biosynthesis	3	0	3	12	2.98
Biogenic amine metabolism	5	0	5	29	2.79
Carbon-carbon lyase activity	5	1	4	26	2.24
Carboxy-lyase activity	4	0	4	20	2.87
Cation channel activity	4	3	1	129	−2.36
Coenzyme binding	3	0	3	16	2.35
Cytochrome-c oxidase activity	4	0	4	17	3.29
Cytoskeletal protein binding	24	3	21	221	2.69
Defense response	17	3	14	510	−2.8
Endosome to lysosome transport	3	0	3	5	5.37
Endosome transport	3	0	3	16	2.34
Extracellular matrix organization and biogenesis	4	0	4	21	2.74
Extracellular matrix structural constituent	8	0	8	59	2.76
Extracellular structure organization and biogenesis	4	0	4	21	2.74
G2/M transition of mitotic cell cycle	2	0	2	9	2.21
Glutathione transferase activity	4	0	4	15	3.62
Glycerophospholipid biosynthesis	4	0	4	20	2.86
Glycerophospholipid metabolism	4	0	4	25	2.32
G-protein coupled receptor activity	6	2	4	207	−2.26
Haeme-copper terminal oxidase activity	4	0	4	17	3.29
Hexose transport	3	0	3	13	2.8
Hydrogen ion transporter activity	14	1	13	107	3.08
Hydrolase activity	62	13	49	1187	−2.17

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Table 5. Continued

Down regulated biological pathways	Diff exp genes	Up-reg	Down-reg	Tot on array	*z*-Score down
Immune response	16	3	13	455	−2.52
Insulin-like growth factor binding	4	0	4	15	3.62
Lipid transport	6	0	6	48	2.15
Lipoprotein biosynthesis	3	0	3	15	2.48
Lysosomal transport	3	0	3	6	4.8
Lysosome organization and biogenesis	4	0	4	9	5.15
Membrane lipid metabolism	10	0	10	84	2.61
Metabolism	318	44	274	4632	2.13
Monosaccharide transport	3	0	3	13	2.8
Monovalent inorganic cation transporter activity	14	1	13	115	2.79
Mrna metabolism	16	1	15	157	2.28
Negative regulation of cell proliferation	12	1	11	104	2.31
Negative regulation of microtubule depolymerization	2	0	2	6	3
Nucleotide metabolism	13	1	12	125	2.05
Oxidoreductase activity	4	0	4	17	3.29
Phosphatase regulator activity	6	1	5	34	2.38
Phosphoinositide biosynthesis	3	0	3	15	2.48
Phosphoinositide metabolism	3	0	3	16	2.34
Polyamine biosynthesis	3	0	3	6	4.8
Polyamine metabolism	4	0	4	8	5.54
Primary active transporter activity	15	1	14	131	2.66
Protein complex assembly	11	0	11	93	2.72
Protein lipidation	3	0	3	15	2.48
Protein phosphatase type 2A regulator activity	3	0	3	15	2.49
Purine nucleotide biosynthesis	8	0	8	66	2.39
Purine nucleotide metabolism	8	0	8	69	2.25
Purine ribonucleotide biosynthesis	8	0	8	63	2.54
Purine ribonucleotide metabolism	8	0	8	66	2.39
Receptor activity	38	12	26	802	−2.9
Regulation of actin polymerization	2	0	2	10	2.02
Regulation of cell migration	2	0	2	9	2.21
Response to biotic stimulus	19	3	16	608	−3.2
Response to external stimulus	30	5	25	865	−3.54
Response to hypoxia	3	0	3	6	4.8
Response to stress	26	4	22	631	−2.28
Ribonucleotide biosynthesis	9	0	9	69	2.78
Ribonucleotide metabolism	9	0	9	73	2.59
RNA splicing factor activity	3	0	3	16	2.35
Secondary metabolism	4	0	4	8	5.54
Serine family amino acid biosynthesis	3	0	3	12	2.98
Signal transducer activity	65	16	49	1367	−3.33
Sphingoid metabolism	3	0	3	14	2.63
Sphingolipid metabolism	6	0	6	30	3.51
Sulphur amino acid metabolism	4	0	4	21	2.74
Transcriptional repressor activity	6	0	6	48	2.16
Transferase activity	8	1	7	33	4
Transmembrane receptor activity	21	8	13	487	−2.79
Vesicle-mediated transport	24	4	20	224	2.31
Wnt receptor signalling pathway	11	2	9	66	2.93

'Diff exp genes' indicates the total number of genes differentially expressed on the array in that category; 'Up-reg' indicates the total number of up-regulated genes; 'Down-reg' Indicates the total number of down-regulated genes; 'Tot on array' indicates the total number of genes on the array in that ontological category; and '*z*-Score down' indicates the *z*-score for that category.

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Table 6. Expression profiles of genes encoding ubiquitin-mediated proteolysis and cell cycle genes

Gene name	Gene ID	Fold change	Direction of change
Beta-transducin repeat containing	BTRC	1.41	Up
Cyclin B1	CCNB1	1.35	Down
Cyclin G2	CCNG2	1.55	Down
Cyclin-dependent kinase inhibitor 3	CDKN3	1.31	Up
Chromodomain helicase DNA BP3	CHD3	1.54	Down
Connective tissue growth factor	CTGF	1.63	Down
Cullin 4B	CUL4B	1.61	Up
Cullin 4B	CUL4B	1.61	Up
Dynactin 1	DCTN1	1.28	Down
Damage-specific DNA BP1	DDB1	1.24	Up
E1A binding protein p300	EP300	1.26	Down
Exostoses 2	EXT2	1.38	Down
F-box protein 21	FBXO21	1.19	Down
F-box protein 38	FBXO38	1.47	Down
F-box protein 42	FBXO42	1.26	Down
F-box protein 7	FBXO7	1.22	Down
F-box and WD-40 domain protein 11	FBXW11	1.72	Up
Growth arrest-specific 1	GAS1	1.35	Down
HECT, C2 and WW domain E3 ubiquitin pro lig 1	HECW1	1.47	Up
Katanin subunit A 1	KATNA1	1.26	Down
KH domain containing, RNA bind, sig *trans* 1	KHDRBS1	1.49	Down
Karyopherin alpha 2	KPNA2	1.46	Down
Ring finger protein 187	LOC149603	1.49	Down
Mitogen-activated protein kinase 7	MAPK7	1.59	Down
Membrane-associated ring finger 6	MARCH-VI	1.47	Down
Minichromosome maintenance deficient 6	MCM6	1.18	Down
Makorin, ring finger protein, 1	MKRN1	1.21	Down
Nuclear protein localization 4	NPL4	1.71	Down
Nuclear distribution gene C	NUDC	1.35	Down
Polymerase epsilon 3	POLE3	1.44	Down
Protein phosphatase 1a	PPP1CA	1.51	Down
Protein phosphatase 1b	PPP1CB	1.41	Down
Protein phosphatase 1 g	PPP1CC	1.26	Down
PRP19/PSO4 premRNA processing factor 19	PRP19	1.29	Down
Protein tyrosine phosphatase IVA1	PTP4A1	1.42	Up
RNA binding motif protein 5	RBM5	1.59	Down
Ring-box 1	RBX1	1.44	Down
Ras homologue gene family, member B	RHOB	1.50	Down
Ring finger protein 103	RNF103	1.33	Down
Ring finger protein 111	RNF111	1.41	Down
Ring finger protein 34	RNF34	1.19	Down
Ring finger protein 4	RNF4	1.65	Down
SET translocation (myeloid leukaemia-associated)	SET	1.45	Down
Seven in absentia homologue 1 (*Drosophila*)	SIAH1	1.30	Down
Structural maintenance chromosomes 4L1	SMC4L1	1.77	Up
Stromal antigen 2	STAG2	1.82	Up
STIP1 homology, U-box containing protein 1	STUB1	1.29	Down
Suppressor of mif two 3 homologue 2	SUMO2	1.33	Down
Transcription elongation factor B1	TCEB1	1.56	Down
Tousled-like kinase 1	TLK1	1.35	Down
Tumor suppressor candidate 4	TUSC4	1.69	Down
Ubiquitin-conjugating enzyme E2E3-UBC4/5 homologue	UBE2E3	1.49	Down
Ubiquitin specific protease 4	USP4	1.22	Down

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Table 6. Continued

Gene name	Gene ID	Fold change	Direction of change
Ubiquitin specific protease 46	USP46	1.34	Down
Wilms tumour 1	WT1	2.01	Down
Kinetochore associated homologue	ZW10	1.22	Down

Genes previously identified that encode components of ubiquitin-mediated proteolysis and cell cycle pathways and shown to be differentially expressed in 90 dG kidneys from fetuses of nutrient restricted mothers *versus* controls are listed. 'Gene ID' indicates the assigned gene identification, 'Fold change' indicates the fold change between fetal kidneys from C and NR mothers, and 'Direction of change' indicates the direction of change for NR kidney RNA compared with C kidney RNA.

down-regulated. For example, the dephoshorylation gene cascade is another example of a pathway in which genes were both up- and down-regulated in the kidneys of fetuses from nutrient restricted mothers. Of the five genes, three were significantly up-regulated (cyclin-dependent kinase inhibitor 3, protein tyrosine phosphatase type IVA1, and protein tyrosine phosphatase, receptor type E) and two were down-regulated (protein phosphatase

Figure 2. Differential expression of genes encoding factors in the C_{21}-steroid hormone metabolism pathway
Both significantly differentially expressed genes in the C_{21}-steroid hormone metabolism pathway are up-regulated. *Homo sapiens* specific genes in the pathway are indicated by black font in green boxes. Differentially expressed genes are indicated by red font in a green box. The fold change in expression is indicated numerically with the direction of change indicated by 'U' for up-regulated genes. 1.1.1.146 (EC number) denotes HSD11B (gene ID), hydroxysteroid (11-β) dehydrogenase and 1 and 1.14.99.9 denotes CYP17A1, cytochrome P450, family 17, subfamily A, polypeptide 1.

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1D magnesium-dependent, delta isoform and protein phosphatase 2A, regulatory subunit B′ (PR 53)).

Evaluation of expression for kidney development-related genes

Twelve genes that play a role in rodent kidney development (Gazit *et al.* 1999; Latres *et al.* 1999; Farrell & Munsterberg, 2000; Stuart *et al.* 2001; Gasteiger *et al.* 2003; Sampogna & Nigam, 2004) are significantly differently expressed in the fetal kidneys from NR mothers compared to C. The name, gene ID, role in kidney development, and expression profile of developmental kidney genes from this study are presented in Table 7.

Evaluation of apoptosis-related genes and gene expression profile validation

Apoptosis-related differentially expressed genes from array interrogation were quantified by QRT-PCR. Thirty-nine

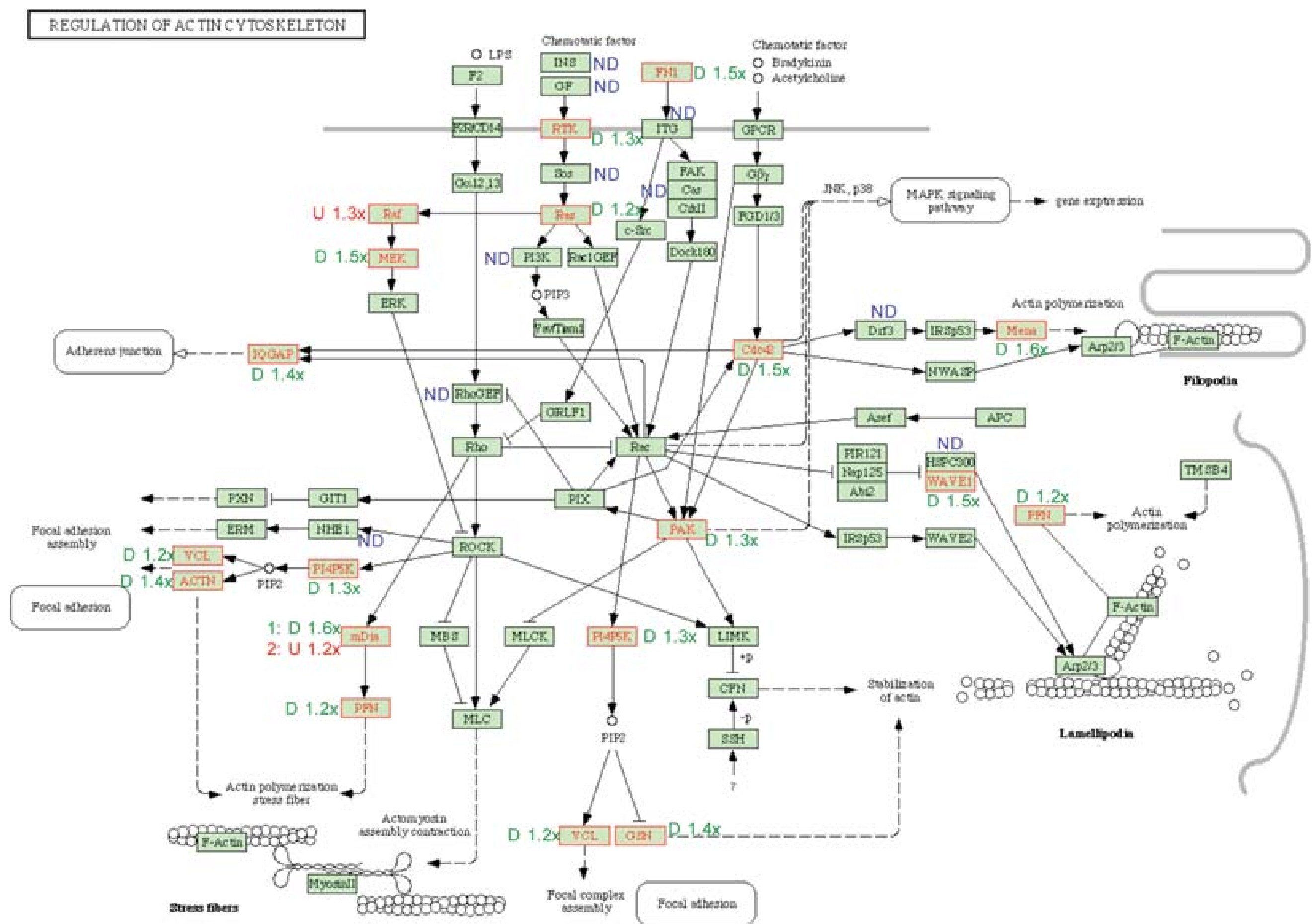

Figure 3. Differential expression of genes encoding actin cytoskeleton assembly
Fourteen of 16 differentially expressed genes are down-regulated. One gene ‘mDia’ has one family member up-regulated (DIAPH1) and one family member up-regulated (DIAPH2). *Homo sapiens* specific genes in the pathway are indicated by black font in green boxes. Genes that showed expression on the array are indicated by black font in blue boxes. Differentially expressed genes are indicated by red font in a green box. The fold change in expression is indicated numerically with the direction of change indicated by ‘D’ for down-regulated genes, ‘U’ for up-regulated genes, and ‘ND’ for genes where no signal was detected. Gene names, gene identifications in parentheses, and KEGG gene abbreviations for differentially expressed genes in this pathway are: actinin, α1 (ACTN1), ACTN; cell division cycle 42 (CDC42), CDC42; diaphanous homologue 1 (DIAPH1), mDia; diaphanous homologue 1 (DIAPH1), Mena; early lymphoid activating protein (DIAPH2), mDia; fibronectin 1 (FN1), FN1; gelsolin (GSN), GSN; IQ motif containing GTPase activating protein 1 (IQGAP1), IQGAP; mitogen-activated protein kinase kinase 1 (MAP2K1), MEK; P21(CDKN1A)-activated kinase 4 (PAK4), PAK; phosphatidylinositol-4-phosphate 5-kinase I gamma (PIP5K1C), PI4P5K; platelet-derived growth factor receptor, α polypeptide (PDGFRA), RTK; profilin 2 (PFN2), PFN; vinculin (VCL), VCL; V-Ki-ras2 Kirsten rat sarcoma viral oncogene (KRAS2), RAS; V-raf murine sarcoma viral oncogene homologue B1 (BRAF), RAF; WAS protein family, member 1 (WASF1), WAVE1.

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Table 7. Expression profiles of genes that control branching morphogenesis

Gene	Gene ID	Role	Fold change	Direction of change
Beta-transducin repeat containing	BTRC	Component of the SCF ubiquitin ligase complex in Wnt signalling (Latres *et al.* 1999)	1.41	Up
Frizzled homologue 1 (*Drosophila*)	FZD1	Wnt protein receptor (Gazit *et al.* 1999)	1.41	Down
Frizzled homologue 7 (*Drosophila*)	FZD7	Metanephric Kidney Formation (candidate) (Farrell & Munsterberg, 2000)	1.46	Down
Glypican-1	GPC3	Promotes UB branching and patterning (Sampogna & Nigam, 2004)	2.46	Down
Homeo box A11	HOXA11	Cell Proliferation in early metanephric development (Stuart *et al.* 2001)	1.51	Down
Integrin-aL	ITGA6	Kidney tubule formation (Sampogna & Nigam, 2004)	2.70	up
Matrix metalloproteinase 2	MMP2	Promotes branching and remodeling (Sampogna & Nigam, 2004)	1.35	Down
Sal-like 2	SALL2	Promotes UB Growth (SaLL1) (Sampogna & Nigam, 2004)	1.3	Down
Secreted frizzled-related protein 1	SFRP1	Inhibits UB branching and tubule formation (Gasteiger *et al.* 2003)	1.5	Down
Tissue inhibitor of metalloproteinases 1	TIMP1	Inhibits UB branching (Sampogna & Nigam, 2004)	2.98	Down
Wilms tumour 1	WT1	Promotes UB outgrowth (Sampogna & Nigam, 2004)	2.00	Down
Wingless-type MMTV integration site 5 A	WNT5A	Wnt's promote UB branching (Sampogna & Nigam, 2004)	1.53	Down

Genes previously identified that control branching morphogenesis and shown to be differentially expressed in 90 dG kidneys from fetuses of nutrient restricted mothers *versus* controls are listed. 'Gene ID' indicates the assigned gene identification, 'Role' is the gene function defined from previous work (Gazit *et al.* 1999; Latres *et al.* 1999; Farrell & Munsterberg, 2000; Stuart *et al.* 2001; Gasteiger *et al.* 2003; Sampogna & Nigam, 2004), 'Fold change' indicates the fold change between fetal kidneys from C and NR mothers, and 'Direction of change' indicates the direction of change between kidneys from NR and C mothers.

genes related to apoptosis were significantly differently expressed between C and NR kidney RNA samples: 11 genes were up-regulated and 29 were down-regulated. Five of these genes, BCL2L13, CREBBP, RHOB, SGK, VEGF, were selected for QRT-PCR validation. Gene expression values from arrays and QRT-PCR are presented in Table 8.

Kidney morphology

Histological sections of C and NR kidneys are presented in Fig. 4. Mean counts of the number of tubule, duct and glomerular cross-sections, and the percentage area occupied by tubules, in tissue sections from C and NR kidneys, are presented in Table 9. No differences in cross-section counts between groups were found; the area occupied by tubules as indicated in Fig. 4 was significantly decreased in NR kidneys compared to C ($31.7 \pm 2.0\%$ *versus* $40.5 \pm 2.2\%$, $P = 0.015$).

Discussion

The present study reports several important findings. Foremost, we present evidence of gene expression changes in the developing fetal primate kidney that result from moderate maternal NR. In addition, we have used the power of ontological and pathway analysis to demonstrate changes in biological and biochemical pathways impacted by maternal NR. Screening six individual RNA samples in each group allowed us to evaluate differential expression based on statistical significance in addition to the more commonly used 2-fold expression differences for pooled samples (Quackenbush, 2002). Selection of genes using differential expression and statistical criteria demonstrated that inclusion of genes with small but statistically significant expression differences highlights ontological pathways significantly altered by nutrient restriction that might be overlooked using a minimum twofold expression difference approach.

Further evidence that maternal NR impacts kidney development is provided by placing our expression data

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Table 8. Gene expression profiling data for apoptosis-related genes

	Array data (intensity)			QRT-PCR data (ΔCt)		
Gene ID	Control	Nutrient restricted	*P*-value	Control	Nutrient restricted	*P*-value
BCL2L13	1.0754 ± 0.0973	0.4951 ± 0.1718	0.0148	19.0452 ± 0.0398	18.7380 ± 0.0441	0.0003
CREBBP	2.0744 ± 0.0742	1.3647 ± 0.2566	0.0240	20.0298 ± 0.0242	19.6819 ± 0.0546	0.0001
RHOB	3.1924 ± 0.1216	2.6060 ± 0.0756	0.0022	23.1803 ± 0.1422	23.6845 ± 0.2301	0.0804
SGK	1.1186 ± 0.0986	0.6988 ± 0.0989	0.0132	19.1889 ± 0.0804	18.6509 ± 0.1185	0.0027
VEGF	4.4561 ± 0.0699	4.0797 ± 0.1253	0.0254	13.2016 ± 0.0398	12.6847 ± 0.0515	0.0294

Data are means ± S.E.M. Array Data were all-median normalized, $\log_2$ transformed and are presented as intensity values. QRT-PCR data were normalized against 18 s rRNA and are presented as ΔCt values. See QRT-PCR quantification of target gene abundance in Methods for a detailed description of ΔCt value calculations. Gene identification abbreviations are: BCL2L13 – BCL2-like 13 (apoptosis facilitator); CREBBP – CREB binding protein (Rubinstein–Taybi syndrome), RHOB – Ras homologue gene family, member B, SGK – serum/glucocorticoid regulated kinase, VEGF – vascular endothelial growth factor.

in the context of ontological categories and KEGG pathways. The pathway analysis data strongly suggest that NR impacts specific cellular pathways even for genes with small changes in expression. For example, in the oxidative phosphorylation KEGG pathway analysis, 15 of 16 genes that are differently expressed are down-regulated in the NR fetal kidney at 90 dG. The difference in expression for 13 of these genes ranges from 1.1× to 1.4×. Evaluating these expression patterns in the context of oxidative phosphorylation proteins suggests that protein modification is down-regulated in the NR kidneys resulting in decreased phosphorylation events in phosphorylation-dependent cascades and pathways. It is important to note that although 589 of the 685 differently expressed genes are down-regulated, pathways such as C21 steroid metabolism are up-regulated suggesting that NR effects are more complex than global down-regulation of all systems in the developing kidney.

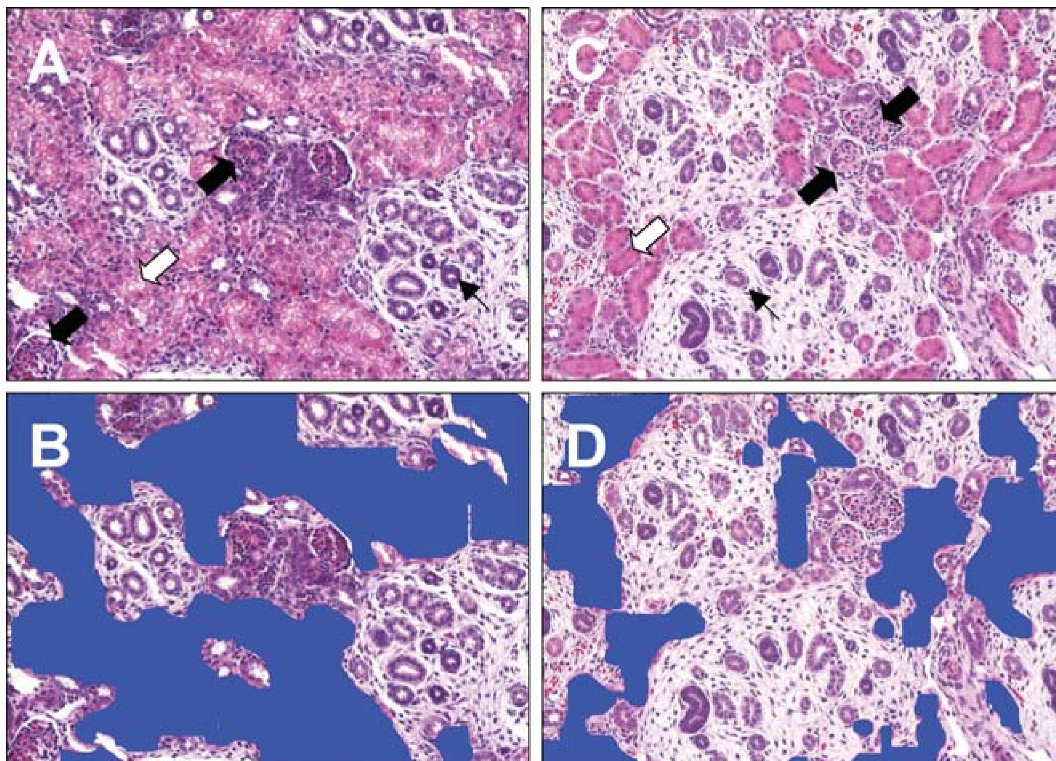

Figure 4. Histological analysis of 90 dG C and NR kidney sections
Representative photomicrographs (20× magnification) of 5 μm kidney sections from *ad libitum* fed controls (*A*) and 30% nutrient restricted (*C*) baboon fetuses at 90 days of gestation. The area occupied by tubules is represented in blue in the same photomicrographs in control (*B*) and nutrient restricted (*D*) animals.

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Table 9. Morphometric analysis of 90 dG kidney sections

	Control fed	Nutrient restricted	*P*
Glomeruli (per mm^2)	0.09 ± 0.01	0.10 ± 0.00	0.166
Convoluted tubules (cross-sections/mm^2)	0.67 ± 0.03	0.57 ± 0.04	0.115
Ducts (cross-sections/mm^2)	0.38 ± 0.08	0.30 ± 0.03	0.331
Convoluted tubule density (% field of view)	40.5 ± 2.2%	31.7 ± 2.0%	0.015

Nucleic Acids, ubiquitin mediated proteolysis and cell cycle regulation

The ontological analysis of gene expression indicates that pathways relevant to nucleic acid component biosynthesis and metabolism, modification and packaging, RNA translation, protein biosynthesis and metabolism, and ubiquitin-dependent protein catabolism contain significant numbers of pathway genes that are down-regulated in the fetal kidneys of NR mothers.

Ubiquitin-mediated proteolysis, through various renal specific complexes, is thought to target several cell-cycle regulators that inhibit G_1 to S progression for proteasomal degradation during renal growth (Franch, 2002). In the NR kidney RNA samples, ubiquitin-mediated proteolysis components such as ring-box 1, ubiquitin-conjugating enzyme E2E3, and ubiquitin-conjugating enzyme E2D are all down-regulated. Furthermore, cell cycle inhibitors such as cyclin-dependent kinase inhibitor 3 is up-regulated, whereas cell cycle progression factors such as cyclin B1 and cyclin G2 are down-regulated. Evaluating gene expression profiles in the context of KEGG pathways reveals a consistent pattern where cell cycle progression is inhibited and the availability of cellular components necessary for cell division, such as DNA, is reduced.

As shown in Table 4, a number of Gene Ontology pathways contain significantly up-regulated genes. Up-regulated pathways include: electron, Golgi vesicle and nucleocytoplasmic transport, carbohydrate, sterol and steroid metabolism, metal ion binding, protein transport and targeting, protein tyrosine phosphatase and heterodimerization activity, regulation of cyclin-dependent protein kinase activity, and voltage-gated potassium channel activity. These data suggest, consistent with the cell cycle inhibition evidence, that the NR fetal kidneys are more differentiated than those from C fetuses. It is important to note that the array data do not present a clear picture regarding cellular activity. For example, analysis of the KEGG mitogen activated protein kinase (MAPK) pathway shows large changes in gene expression; however, the direction of change for each gene in the pathway is not consistent with an increased rate of MAPK activity in the NR fetal kidneys. Our data show that adrenomedullin is up-regulated in the kidneys of fetuses from NR mothers, suggesting that nutrient restriction inhibits cell proliferation. Adrenomedullin inhibits proliferation and enhances apoptosis of kidney mesangial cells, through the modulation of MAPK cascades (Belloni *et al.* 2001). However at this time, the impact of these gene expression changes in the apoptosis and MAPK signalling pathways on cell proliferation and differentiation in the developing primate kidney is not clear. Further experiments will be necessary to ascertain the cellular activities of NR fetal kidneys compared with C kidneys. It will be of interest to see if the inconsistent gene expression profiles are due to differences between gene expression and gene product activity or if these factors play different roles in developing primate kidneys than the functions elucidated in other systems.

Expression of kidney development-related genes

Several genes have been identified that control different aspects of branching morphogenesis in the developing kidney. At least 30 genes have been described that play specific roles in rodent kidney development (Gazit *et al.* 1999; Latres *et al.* 1999; Farrell & Munsterberg, 2000; Stuart *et al.* 2001; Gasteiger *et al.* 2003; Sampogna & Nigam, 2004). Of these 30 genes, 12 are significantly differently expressed in the baboon fetal kidneys from nutrient restricted mothers *versus* controls. Beta-transducin repeat containing gene (BTRC), a component of the ubiquitin ligase complex and a factor in promotion of branching through Wnt signalling (Latres *et al.* 1999), is up-regulated (1.4×) in NR fetal kidney. Secreted frizzled-related protein 1 (SFRP1) and tissue inhibitor of metalloproteinases-1 (TIMP1), inhibitors of ureteric bud branching (Gasteiger *et al.* 2003; Sampogna & Nigam, 2004), are both down-regulated (1.5× and 3.0×, respectively) in the NR fetuses. The changes in expression of these genes suggest that branching activity is increased following maternal NR. Conversely, wingless-type MMTV integration site 5A (WNT5A), a member of the Wnt gene family known to promote ureteric bud branching (Sampogna & Nigam, 2004), is down-regulated (1.5×). Frizzled homologue 1 (FZD1), a Wnt protein receptor that promotes branching (Gazit *et al.* 1999), Sal-like 2 (SALL2), which promotes ureteric bud growth (Sampogna & Nigam, 2004), and Wilms tumour 1 (WT1), which promotes ureteric bud outgrowth (Sampogna & Nigam, 2004), are all down-regulated in the NR group (1.4×, 1.3× and 2.0×, respectively). In addition, frizzled homologue 7

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(FZD7), which is thought to play a role in metanephric kidney formation (Farrell & Munsterberg, 2000), homeo box A11 (HOXA11), known to play a role in cell proliferation during early metanephric kidney development (Stuart *et al.* 2001), matrix metalloproteinase 2 (MMP2), which promotes branching and remodelling during kidney development (Sampogna & Nigam, 2004) and glypican-3, which plays a role in late stage branching and tubule maturation, are also all down-regulated in the NR compared to the C kidney (1.5×, 1.5×, 1.4× and 2.5×, respectively). The down-regulation of genes such as these is suggestive of the development of renal hypoplasia in the NR group and is consistent with other models of maternal NR that result in an impaired nephron endowment (Langley-Evans, 1996; Vehaskari *et al.* 2001; Woods *et al.* 2001).

Based on previous studies in characterizing molecular mechanisms of kidney development in rodents, these data present a conflicting picture for the impact of maternal NR on the developing primate kidney. Previous work has shown that complex systems, including gene systems, are often error tolerant for some components of a network (Jeong *et al.* 2001). Studies have also shown that some molecules play contrasting roles at different stages in development. For example, BMP7 can play an activator role at one stage of kidney development and an inhibitory role at another stage (Gupta *et al.* 1999). From this perspective, the apparent inconsistencies in gene expression for the NR baboon kidney at 90 dG compared to C kidney, at least when compared to previous studies on developing kidneys and differentiating kidney cells *in vitro*, may be the result of compensatory responses to NR. It is not yet clear which molecules in the network of molecules for kidney development may fluctuate in expression without phenotypic impact, which have detrimental effects on development, and which demonstrate compensation for environmental deficiencies such as NR.

Expression for apoptosis-related genes

Of the 39 differentially expressed apoptosis-related genes, 11 were up-regulated and 28 were down-regulated. Five genes known to play a role in apoptosis were selected for QRT-PCR validation of the array data: BCL2L13 (B-cell CLL/lymphoma 2 like protein 13) promotes apoptosis via the activation of caspase-3; CREBBP (cAMP responsive element binding protein bind protein) mediates cAMP-gene regulation by binding specifically to phosphorylated CREB protein, thus functioning as a coactivator of cAMP-responsive genes transcription and consequently playing a role in TGF β (transforming growth factor β) signalling, Wnt signalling and apoptosis pathways; RHOB (ras homologue gene family, member B) promotes endothelial cell survival during vascular development; SGK (serum/glucocorticoid regulated kinase) mediates cell survival signals and negatively regulates pro-apoptotic factors; SGK also initiates a cascade that inactivates various channels and transporters such as ENaC (epithelial sodium channel); and VEGF (vascular endothelial growth factor), a growth factor active in angiogenesis, vasculogenesis and endothelial cell growth, induces endothelial cell proliferation, promotes cell migration, and induces permeabilization of blood vessels. All five genes were found to be down-regulated by QRT-PCR, thereby confirming the validity of the array analysis for these particular genes. Given the mix of expression changes found for both pro- and antiapoptotic genes, the impact of maternal NR on programmed cell death in the 90 dG primate kidney remains to be fully elucidated.

Actin cytoskeleton assembly and histological analysis of 90 dG kidney sections

Pathway analysis of C *versus* NR kidney gene expression profiles indicated down-regulation of actin cytoskeleton polymerization and assembly. Consistent with this array data as well as that for genes involved in tubular branching discussed above, histological analysis of 90 dG kidney sections shows a reduction in the percentage of cortex occupied by tubule cross-sections in NR *versus* controls kidneys. The reduction of genes encoding components for actin cytoskeleton assembly and polymerization suggest that nutrient restriction may decrease the number of kidney structural components. These changes in gene expression may also lead to decreased tortuosity and/or decreased length of tubules which manifest as a decrease in cross-sections per unit area.

Perspective

The ability to apply gene array analysis to questions focusing on fetal development exemplifies the utility and importance of our baboon model. Gene array analysis provides a powerful synthesis of system-wide levels of mRNA expression at a given point in development and, as reported here, following perturbation of the intrauterine nutritional milieu. Of the data presented the least clear are those involving cellular activity pathways. KEGG pathways show what is known about *Homo sapiens*; however, some genes are expressed in a tissue specific and/or temporal specific manner. In addition, gene regulation may vary as well. Therefore, pathways such as the MAPK cascade may vary by cell type within a tissue and also between tissues. Thus the apparent contradictions in gene expression from our study may not be contradictory when more is known about gene expression relevant to developing primate kidneys. Our data strongly support the observation that maternal nutrient restriction is associated with decreases in gene expression for components of RNA, DNA, and

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protein biosynthesis and metabolism, as well as decreases in gene expression of cell cycle promoters and increases in gene expression of cell cycle inhibitors. These observations, in conjunction with the decreases in tubular density evident in our histological analysis of the fetal renal cortex, therefore suggest that maternal nutrient restriction inhibits cell division, promotes differentiation and results in a global decrease in structural components within the developing NR kidney. Although the fetal morphometry did not indicate differences between C and NR 90 dG kidneys, gene array, QRT-PCR and histological data all indicate differences in kidney development due to NR, confirming that fetal weight is a poor measure of fetal compromise at mid gestation.

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