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Identification of maternally regulated fetal gene networks in the placenta with a novel embryo transfer system in mice

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Senthamaraikannan P, Sartor MA, O’Connor KT, Neumann JC, Klyza JP Jr, Succop FA, Wagner BD, Karyala S, Medvedovic M, Menon AG. Identification of maternally regulated fetal gene networks in the placenta with a novel embryo transfer system in mice. Physiol Genomics 43: 317–324, 2011. First published January 4, 2011; doi:10.1152/physiolgenomics.00078.2010.—The mechanisms for provisioning maternal resources to offspring in placental mammals involve complex interactions between maternally regulated and fetally regulated gene networks in the placenta, a tissue that is derived from the zygote and therefore of fetal origin. Here we describe a novel use of an embryo transfer system in mice to identify gene networks in the placenta that are regulated by the mother. Mouse embryos from the same strain of inbred mice were transferred into a surrogate mother either of the same strain or from a different strain, allowing maternal and fetal effects on the placenta to be separated. After correction for sex and litter size, maternal strain overrode fetal strain as the key determinant of fetal weight (P < 0.0001). Computational filtering of the placental transcriptome revealed a group of 81 genes whose expression was solely dependent on the maternal strain [P < 0.05, false discovery rate (FDR) < 0.10]. Network analysis of this group of genes yielded highest statistical significance for pathways involved in the regulation of cell growth (such as insulin-like growth factors) as well as those involved in regulating lipid metabolism [such as the low-density lipoprotein receptor-related protein 1 (LRP1), LDL, and HDL], both of which are known to play a role in fetal development. This novel technique may be generally applied to identify regulatory networks involved in maternal-fetal interaction and eventually help identify molecular targets in disorders of fetal growth.

fetal growth; maternal-fetal interaction; resource provisioning

ALTHOUGH ELEGANT STUDIES have identified some of the mechanisms by which maternal and maternal interests play different roles in the growth and development of the fetus (16, 31, 39, 40), less mechanistic evidence is available on how the mother and fetus differentially interact to provision resources in the placenta, a tissue of fetal origin that is also the key interface for transport of these resources to the growing fetus. Both the genetic contributions of the fetus as well as in utero factors provided by the mother are suspected to play important roles, although the exact mechanisms are still largely unknown. The principal bottleneck in identifying molecular targets of “maternal regulation” in the placenta is the inability to separate the actions of gene products originating in the fetal genome from those contributed by the maternal genome.

To test the hypothesis that maternally controlled gene networks are present in the placenta (which is derived from the zygote and is therefore a fetal tissue), we developed an embryo transfer system for two inbred strains of mice, a pigmented strain, C57BL/6J (C), and a nonpigmented strain, FVB/NJ (F). These were chosen because 1) they show significant differences in birth weight of offspring, 2) they do not show significant differences in maternal body weight or size, 3) they are differently pigmented, a phenotype that allows the distinct eye color of the embryo at embryonic day (E)18.5 to unequivocally identify its strain of origin before the development of fur coloration, and 4) there are differences in the blood chemistry and metabolic parameters of these two strains (Table 1). C embryos were transferred into C or F surrogate mothers so that maternal genotype was the sole variable (since embryos of identical genotype are implanted in surrogate mothers of each distinct genotype). In corollary studies F embryos were transplanted into C or F surrogate mothers. In each implantation experiment, C embryo “controls” were implanted in C mothers and F embryos implanted in F mothers to provide an internal control for variation due to the embryo transfer technique.

We analyzed the role of maternal and fetal genotype in regulating placental gene expression at E18.5 and identified a subset of genes that are solely regulated by the maternal strain, C, and are differentially expressed between C and F embryos (Fig. 1). The optimal yield of fertilized embryos from the superovulated females was induced by intraperitoneal injection of a donor female mouse with 5 IU of pregnant mare’s serum (National Hormone and Peptide Program, UCLA). Two days later, 5 IU of human chorionic gonadotropin (Sigma) was injected and the female was

Embryo transfer. All experimental procedures involving the use of live mice were approved by the Institutional Animal Care and Use Committee review board of the University Of Cincinnati College Of Medicine. The scheme for the embryo transfer procedure is presented in Fig. 1. The optimal yield of fertilized embryos from the superovulated females was induced by intraperitoneal injection of a donor female mouse with 5 IU of pregnant mare’s serum (National Hormone and Peptide Program, UCLA). Two days later, 5 IU of human chorionic gonadotropin (Sigma) was injected and the female was
placed with a male of the same genotype (for example, superovulated C57BL/6J females with C57BL/6J males). After a vaginal plug was confirmed on the following day the females were killed (CO2 followed by cervical dislocation) to harvest the embryos (53). The embryos at this point were typically in the pronucleus stage and considered 12 h old [or 0.5 day postcoitum (dpc)] (41, 59). The embryos were harvested from the ampullary region of the oviduct tube and collected in M2 medium (HEPES buffered at pH 7.4). The embryos were then transferred into a surrogate mother with a laparotomy incision through the opening of the oviduct of an anesthetized pseudopregnant female. We have refined the microsurgical techniques to implant the harvested embryos into the ampullary segment of the fallopian tube of the surrogate mother and are routinely able to attain an almost 50–80% successful implantation rate in both C57BL/6J and FVB/NJ mice by using flame-polished micropipettes and handling techniques that minimize damage to the transplanted embryos (43). Surrogate mothers were “prepared” by having them naturally mate with vasectomized males the night before embryo transfer (13). Embryos from both strains (C57BL/6J and FVB/NJ) were mixed in culture medium and transferred into C57BL/6J surrogates. Similarly, a mixture of embryos (C57BL/6J and FVB/NJ) was transferred via laparotomy incision to a FVB/NJ surrogate. The pregnancy was allowed to proceed for 18.5 days, and then pregnant mothers were killed by cervical dislocation to harvest placentas and fetuses. The embryo transfer model that we have developed has the following advantages: 1) identical fetal genotypes are transferred into two different maternal environments, which allows us to study the maternal genotype effect, and 2) the eye color and coat color of the strains we selected for our studies have advantages in identification of the offspring at prenatal and postnatal stages, without any genotyping. C57BL/6J and FVB/NJ embryos have black pigmented eyes, whereas FVB/NJ embryos have nonpigmented eyes even at E18.5. Four types of embryos (and placentas) were obtained: C/embryo/C/mother (C/C), C/embryo/F/mother (C/F), F/embryo/C/mother (F/C), and F/embryo/F/mother (F/F).

**Analysis of placental and fetal weight.** Embryo-transferred pregnant mice were killed at 18.5 dpc. Fetuses with attached placentas were removed. The placentas were then detached from the fetal membranes; the fetuses and placentas were blotted with Kim wipes and dried on tissues before being weighed on a Mettler AJ100 balance allowed to proceed for 18.5 days, and then pregnant mothers were killed by cervical dislocation to harvest placentas and fetuses. The embryo transfer model that we have developed has the following advantages: 1) identical fetal genotypes are transferred into two different maternal environments, which allows us to study the maternal genotype effect, and 2) the eye color and coat color of the strains we selected for our studies have advantages in identification of the offspring at prenatal and postnatal stages, without any genotyping. C57BL/6J and FVB/NJ embryos have black pigmented eyes, whereas FVB/NJ embryos have nonpigmented eyes even at E18.5. Four types of embryos (and placentas) were obtained: C/embryo/C/mother (C/C), C/embryo/F/mother (C/F), F/embryo/C/mother (F/C), and F/embryo/F/mother (F/F).

**RNA extraction.** Total RNA was extracted from E18.5 placentas of all four groups of embryos with Tri reagent (Molecular Research Center, Cincinnati, OH) according to the manufacturer’s instructions. Subsequently, the RNA samples were purified with RNeasy columns (Qiagen, Valencia, CA) after DNase I digestion. All RNA samples were tested for quality with an Agilent 2100 bioanalyzer, and the intensities of both 28S and 18S ribosomal bands were assessed.

**Microarray hybridization and analysis.** The microarray experiments were carried out as described previously (28, 37, 50) with the mouse 70-mer oligonucleotide library version 3.0 (31,775 optimized oligos) (Qiagen, Alameda, CA). More details of slide preparation can be found at [http://microarray.uc.edu](http://microarray.uc.edu).

Imaging and data generation were carried out with GenePix 4000A and GenePix 4000B (Axon Instruments; Union City, CA) and associated software from Axon Instruments (Foster City, CA). The data representing background-subtracted spot intensities generated by GenePix Pro version 5.0 software were analyzed to identify differentially expressed genes. The data were preprocessed and normalized as previously reported (28, 37, 50). The statistical analysis was performed for C/C vs. C/F and F/C vs. F/F, and for each gene separately by fitting the following linear model (23): $Y_{ijk} = \mu + A_j + S_k + C_{ij} + \varepsilon_{ijk}$, where $Y_{ijk}$ corresponds to the normalized log-intensity on the $i$th array, with the $j$th maternal genotype (F or C), and labeled with the $k$th dye ($k = 1$ for Cy5 and 2 for Cy3); $\mu$ is the overall mean log-intensity;

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**Table 1. Blood chemistry and metabolic parameters of C57BL/6J and FVB/NJ mouse strains**

<table>
<thead>
<tr>
<th></th>
<th>Male</th>
<th>Female</th>
<th>Male</th>
<th>Female</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Blood chemistry</strong></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Total cholesterol, mg/dl</td>
<td>100 ± 11.9</td>
<td>79.2 ± 8.83</td>
<td>163 ± 10.8</td>
<td>120 ± 9.64</td>
</tr>
<tr>
<td>Plasma triglycerides, mg/dl</td>
<td>79.5 ± 18.1</td>
<td>92.9 ± 22.2</td>
<td>186 ± 43.2</td>
<td>238 ± 45.0</td>
</tr>
<tr>
<td>High-density lipoprotein cholesterol, mg/dl</td>
<td>89.9 ± 10.9</td>
<td>67.3 ± 7.95</td>
<td>142 ± 9.36</td>
<td>105 ± 7.65</td>
</tr>
<tr>
<td>Plasma glucose (4-h fast), mg/dl</td>
<td>156 ± 29.5</td>
<td>176 ± 31.4</td>
<td>177 ± 36.2</td>
<td>138 ± 17.3</td>
</tr>
<tr>
<td>Plasma total protein, g/dl</td>
<td>6.04 ± 0.552</td>
<td>6.09 ± 0.439</td>
<td>5.88 ± 0.286</td>
<td>5.42 ± 0.286</td>
</tr>
<tr>
<td>Plasma phosphorus, mg/dl</td>
<td>10.3 ± 2.29</td>
<td>10.3 ± 1.91</td>
<td>11.4 ± 2.05</td>
<td>9.30 ± 0.921</td>
</tr>
<tr>
<td>Plasma calcium, mg/dl</td>
<td>10.4 + 0.826</td>
<td>10.6 ± 0.797</td>
<td>10.5 ± 0.577</td>
<td>9.77 ± 0.340</td>
</tr>
<tr>
<td>Plasma blood urea nitrogen, mg/dl</td>
<td>24.2 ± 3.11</td>
<td>27.1 ± 4.00</td>
<td>24.9 ± 3.45</td>
<td>18.3 ± 1.42</td>
</tr>
<tr>
<td><strong>Metabolic parameters</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Daily caloric intake adjusted for body wt, kcal·g⁻¹·day⁻¹</td>
<td>9.82 ± 1.02</td>
<td>15.5 ± 1.09</td>
<td>17.4 ± 3.87</td>
<td>14.9 ± 1.57</td>
</tr>
<tr>
<td>Daily food intake, g</td>
<td>2.62 ± 0.219</td>
<td>2.92 ± 0.198</td>
<td>4.24 ± 0.984</td>
<td>3.00 ± 3.063</td>
</tr>
<tr>
<td>Daily volume of oxygen consumption, ml·kg⁻¹·h⁻¹</td>
<td>2.907 ± 211</td>
<td>3.792 ± 309</td>
<td>3.819 ± 355</td>
<td>3.871 ± 432</td>
</tr>
<tr>
<td>Water intake, ml</td>
<td>2.86 ± 0.438</td>
<td>2.96 ± 0.672</td>
<td>2.91 ± 0.704</td>
<td>2.81 ± 1.00</td>
</tr>
</tbody>
</table>

Values are means ± SD (open source data courtesy of Mouse Phenome Database, Jackson Laboratory, [http://www.jax.org/phenome](http://www.jax.org/phenome)).

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![Fig. 1. Schematic illustration of the mouse embryo transfer model used in this study to determine maternal genotype and fetal genotype effect on fetal weight. F/F, FVB/NJ (F) embryo transferred into F foster mother; C/F, C57BL/6J (C) embryo transferred into F foster mother; C/C, C embryo transferred into C foster mother; F/C, F embryo transferred into C foster mother. Transferred embryos were gestated in foster mothers for 18.5 days (E18.5) and then collected to record fetal and placental weights.](http://microarray.uc.edu)
A_1 is the effect of the \( i \)th array, \( S_j \) is the effect of the \( j \)th treatment; and \( C_k \) is the gene-specific effect of the \( k \)th dye. Resulting \( t \)-statistics from each contrast were modified with an empirical Bayesian moderated \( t \)-test (55). Estimates of fold change were calculated, and genes with false discovery rate (FDR) \( < 0.15 \) for C/C vs. F/F (107 genes) and FDR \( < 0.10 \) for F/C vs. F/F (190 genes) emerged from the filtering process. We then limited the lists to genes that had an absolute change > 1.5-fold, resulting in no change in the number for C/C vs. C/F (107) and filtering out 12 genes from the original 190 genes for F/C vs. F/F to yield a final number of 178 genes. The sets of 107 and 178 genes thus identified are considered to be significantly differentially expressed and are represented in the Venn diagram shown in Fig. 3. The intersection of these sets resulted in 81 unique genes.

Analysis was performed with R statistical software and the limma Bioconductor package (55). All data sets have been deposited in the EMBL-EBI database (ArrayExpress accession no. E-MEXP-2479).

Pathway analysis. Concepts significantly enriched with the list of 81 overlapping genes were tested with the software platform ConceptGen, which tests predefined gene sets from a broad range of biological knowledge types. Significantly enriched Gene Ontology (GO) terms were determined for differences caused by mothers (C/C vs. C/F and F/C vs. F/F) with EASE (35). Transcripts that had a \( P \) value \( < 0.01 \) and fold change \( > 1.5 \) were considered differentially expressed for the purposes of this test. Fisher’s exact test was used (22) with the FDR \( P \) value adjustment (10). Categories with FDR \( < 0.10 \) and containing at least three differentially expressed genes are displayed in Supplemental Fig. S2.1 Gene lists were also uploaded into MetaCore pathway analysis software (GeneGo). Enrichment analysis on the pathway maps in GeneGo was performed by using two different cutoffs, \( P < 0.005 \) and \( P < 0.05 \), to identify pathways that are changed under stringent and less stringent conditions.

Sex determination. To determine the sex of the E18.5 embryos, polymerase chain reaction (PCR) assay was carried out with the male-specific Sry gene (38). Male-specific Sry forward primer 5’-TGGGACTGGTGACAATTGTC-3’ and reverse primer 5’-GAGTACAGGTTGTCAGCTCT-3’ were used to amplify the DNA extracted from tail and liver tissues. Autosomal gene apolipoprotein AIV was used as an internal control in all PCR reactions.

Statistical analysis. The initial analysis of the data set provided was a \( t \)-test of the mean fetal weight in a fetal genotype of C57BL/6J (variable = 1) versus FVB/NJ (variable = 0). There was a significant difference (\( P < 0.0001 \)) between the means in both the pooled and the Satterthwaite analysis methods. We also performed a \( t \)-test of the mean fetal weight for maternal genotype of C57BL/6J (variable = 1) versus FVB/NJ (variable = 0). There was a significant difference (\( P < 0.0001 \)) between the means in both the pooled and the Satterthwaite analysis methods. Both methods were used since the variances were unequal in fetal weight. Further analysis of the data set was necessary to account for the two confounder effects (sex and litter size) and also to quantify the effect of the variables of interest (maternal and fetal genotype). For this reason, we analyzed the data by means of multivariate analysis of covariance (MANCOVA) of the primary independent variables (maternal and fetal genotype) with two confounders (sex and litter size) for the outcome variable (fetal weight). This model was used because there were continuous outcome variables, three dichotomous (only 2 values) independent variables (fetal/maternal genotype and sex), and one continuous independent variable (litter size). We tested for all possible interactions of the variables. Backward elimination was used to remove the least significant of the highest-level interactions and rerun the model. This process was repeated, leaving no significant interactions in the final model. For example, the interaction between fetal and maternal genotype was not significant with either the placental weight or the fetal weight outcomes (\( P = 0.1505 \) and \( P = 0.3107 \), respectively). The final model used was highly significant (\( P < 0.0001 \)). We tested the model for homogeneity of variance, normality, and collinearity. The two primary variables and two confounders were highly significant for the overall model (Wilks \( \lambda < 0.0001 \) in all variables).

RESULTS

Maternal genotype overrides fetal genotype in controlling fetal growth in utero. To determine fetal and maternal genotype control over fetal weights, embryo-transferred surrogate mothers (F and C) were dissected at E18.5 (18.5 dpc) (Fig. 1) and fetal weights were recorded. A MANCOVA was performed to analyze the maternal and fetal genotypic effects on fetal weight while controlling for sex and litter size (Table 2). Fetal weight was significantly determined by the maternal genotype (\( P < 0.0001 \)). Fetal genotype was not a determining factor on fetal weight itself (\( P = 0.6242 \)). Both C (C/C) and F (F/C) embryos were smaller in C mothers compared with C (C/F) and F (F/F) embryos from F mothers (Fig. 2). Fetal and maternal genotype interactions were not statistically significant in determining fetal weights (\( P = 0.3107 \)). These results show that the maternal genotype significantly overrides the fetal genotype to control fetal weight. On average, fetuses from F mothers weighed 115.64 mg more than fetuses from C mothers. The sex variable of being female versus male decreased the fetal weight by 107.24 mg (\( P < 0.0001 \)) and a decrease in fetal weight by 21.28 mg per pup was attributed to the litter size variable (\( P < 0.0001 \)). The average maternal weight of the FVB/NJ mothers (23.50 g) was not significantly different from that of the C57BL/6J mothers (22.15 g), showing that the difference in the fetal weight of F/F and C/F embryos was not due to heavier F mothers.

Fetal weight of transferred embryos is indistinguishable from naturally conceived embryos in both mouse strains. The fetal weights of the naturally conceived and embryo-transferred E18.5 embryos of the C57BL/6J and FVB/NJ strains were analyzed by using MANCOVA to see whether the embryo transfer technique introduced any artifacts into the fetal weight analysis. Results of the analysis show that the fetal weights of the naturally conceiving and embryo-transferred mothers of both strains did not show a significant difference (source \( \times \) strain interaction \( P = 0.72 \)). The fetal weights at E18.5 (18.5 dpc) of the naturally conceived and embryo-transferred embryos of the FVB/NJ strain were 1.25 \( \pm 0.04 \) g and 1.23 \( \pm 0.04 \) g, respectively; for the C57BL/6J strain, the fetal weights of the naturally conceived and embryo-transferred embryos were 1.11 \( \pm 0.04 \) g and 1.07 \( \pm 0.04 \) g, respectively. None of the other interactions with source was significant for fetal weight (Supplementary Fig. S1 and Supplementary Table S1).

Table 2. MANCOVA analysis of maternal and fetal genotype effects on E18.5 embryos

<table>
<thead>
<tr>
<th>Dependent Variable</th>
<th>Effect per Unit Change, g</th>
<th>Standard Error</th>
<th>( P ) Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Maternal genotype</td>
<td>(-0.12)</td>
<td>(\pm 0.02)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Fetal genotype</td>
<td>(-0.01)</td>
<td>(\pm 0.02)</td>
<td>0.6242</td>
</tr>
<tr>
<td>Sex</td>
<td>(-0.11)</td>
<td>(\pm 0.02)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Litter size</td>
<td>(-0.02)</td>
<td>(\pm 0.003)</td>
<td>&lt;0.0001</td>
</tr>
</tbody>
</table>

\(P = 0.005\) and \(P < 0.01\) in all variables.

1 Supplemental Material for this article is available online at the Journal website.

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with ConceptGen, which uses a modified Fisher’s exact test.

3.4

vs. data set 1

F/F vs. F/C) and comparison of these two data sets (Fig. 3). Stringent statistical filtering of the 27,000 probe total were maternally regulated according to the same criteria (Fig. 4). Only 6 genes out of the set of 81 maternally regulated genes fit this pattern (differentially expressed but in different directions in the 2 comparisons). These are 1) IL-14 (NM_001005), 2) the ABI gene family member 3 (NESH) binding protein (XM_359276), 2) the transformation/transcription domain-associated protein (NM_133901), 4) the chromobox homolog 2 Drosophila Pc class protein (NM_007623), 5) serine/threonine kinase 40 (NM_028800), and 6) the microphthalmia-associated transcription factor BY747759. None of these genes was picked up by the subsequent pathway and concept analysis, suggesting that their influence is likely to be lower in ordinal ranking than that for the growth factors and lipid metabolism pathways. To better examine the interaction network represented by the maternally regulated genes involved in regulation of growth, we used the software tool MetaCore (GeneGo) and Dijkstra’s “shortest paths” algorithm (with up to 2 steps; Fig. 5). We analyzed top scoring pathways containing differentially regulated genes of the F/F vs. F/C and C/C vs. C/F placentas separately, using GO analysis and MetaCore software from GeneGo to visualize the interaction network for the genes in these categories. On the basis of GO term enrichment analyses, the strongest statistical significance where maternal genotype controlled placental processes in both comparisons involved 1) growth factor pathways and 2) pathways that affect lipid metabolism (Table 3 and Supplemental Fig. S2). Pathway analysis results showed that marked enrichment for genes associated with lipid metabolism pathway was affected in both data set comparisons (F/F vs. F/C and C/C vs. C/F) (P < 0.005). The MetaCore pathway program allows for selection of different levels of stringency in selecting differentially ex-

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Fig. 2. Representative figure showing morphology of E18.5 embryos obtained from embryo-transfered mothers. F/F and C/F are littermates gestated in a F mother, and C/C and F/C are littermates gestated in a C mother. F/F and C/F are bigger compared with C/C and F/C. Litter size was 9 in the C mother and 8 in the F mother. Note eye color of the fetuses: C has black-pigmented eye, while F has nonpigmented eye.

The placental transcriptome contains 81 genes that are principally regulated by the mother. To identify the components of the placental transcriptome that are regulated by the mother we interrogated microarrays with cDNA prepared from 18.5 dpc placent from C/C vs. C/F (and separately F/F vs. F/C), thus keeping the placental genotype constant and isolating the changes in which maternal genotype is the sole variable. Although the placenta is a tissue that originates in the zygote, and therefore harbors the fetal genotype, our results revealed a small subset of genes that are regulated solely by the mother. In the comparison of placental transcriptomes of C/C vs. C/F 107 genes or 0.39% of the total were maternally regulated (with a threshold of ≥1.5 fold change with an FDR <0.10), and in the F/F vs. F/C comparison 178 or 0.6% of the total were maternally regulated according to the same criteria (Fig. 3). Stringent statistical filtering of the 27,000 probe microarray data sets (data set 1 = C/C vs. F/F and data set 2 = F/F vs. F/C) and comparison of these two data sets (data set 1 vs. data set 2) allowed the identification of 81 genes (Supplemental Table S2) that are turned on or off by changing the genotype of the surrogate mother. We specifically chose FDR cutoffs for both experiments and used the intersection between the two independently performed experiments to arrive at the 81-gene list to maximize the stringency of filtering and minimize the risk of false positives (Fisher’s test P value = 3.4 × 10^-169).

These 81 genes were also used to build a heat map (Fig. 4) with ConceptGen, which uses a modified Fisher’s exact test (the same used by the DAVID program developed by NIH), to identify predefined gene sets from several different databases enriched with the input list. In the heat map shown in Fig. 4 each row represents a gene cluster (gene set), while each column represents a gene.

To gain further insight into how these 81 “core maternally regulated genes” may be organized into specific signaling pathways, we identified enriched GO terms (3) and other biological concepts. Analysis using ConceptGen (51) also showed that the set of 81 maternally regulated genes is significantly enriched in concepts such as regulation of cell growth, insulin-like growth factor binding, and extracellular matrix (Fig. 4). GeneGo to visualize the interaction network represented by the maternally regulated genes involved in regulation of growth, we used the software tool MetaCore (GeneGo) and Dijkstra’s “shortest paths” algorithm (with up to 2 steps; Fig. 5). We analyzed top scoring pathways containing differentially regulated genes of the F/F vs. F/C and C/C vs. C/F placentas separately, using GO analysis and MetaCore software from GeneGo to visualize the interaction network for the genes in these categories. On the basis of GO term enrichment analyses, the strongest statistical significance where maternal genotype controlled placental processes in both comparisons involved 1) growth factor pathways and 2) pathways that affect lipid metabolism (Table 3 and Supplemental Fig. S2). Pathway analysis results showed that marked enrichment for genes associated with lipid metabolism pathway was affected in both data set comparisons (F/F vs. F/C and C/C vs. C/F) (P < 0.005). The MetaCore pathway program allows for selection of different levels of stringency in selecting differentially ex-

Fig. 3. Venn diagram illustrations of maternal genotype control of 81 placental genes between C/C vs. C/F and F/F vs. F/C data sets (Fisher’s test P value = 3.4 × 10^-169). The common genes from the microarray experiments were either up- or downregulated at least 1.5-fold [false discovery rate (FDR) 0.10] in the placenta. The complete gene list of the 81 genes in the overlap is provided in Supplemental Table S2.
pressed genes by changing the \( P \) value threshold. To reveal wider biological processes that might be governed by maternal signals in the placenta, we repeated enrichment analysis by lowering stringency of the \( P \) value cutoff (from \( P < 0.005 \) to \( P < 0.05 \)). Enrichment analysis for pathway maps with decreasing stringency (a \( P \) value cutoff \( P < 0.05 \)) revealed pathways that are involved in glucose handling, cytoskeletal remodeling, and immune response in both data sets, suggesting that these pathways may be subject to a broader, yet weaker influence by maternal genotype compared with the pathways for cell growth and lipid metabolism (Supplemental Table S3).

**DISCUSSION**

Survival and fitness of offspring in animals depend on complex systems of provisioning resources between parents and offspring, resulting in intricate coadaptations to variations in supply and demand. In eutherian mammals, fetal growth and...
epigenetic preadaptive responses for birth depend on the proper function of the placenta, which acts as an interface between the mother and fetus. Many excellent studies describing various facets of this interaction reveal that a fine balance exists between fetal demand and maternal supply of nutrients and gases (1, 15, 18, 30, 47, 48). It is also known that maternal nutrition plays a key role in determining fetal growth and can result in either abnormally increased fetal size (25, 56) or growth-restricted fetuses (17, 60). In humans, significant deviation from “normal” birth weight has its consequences: individuals in the highest and lowest quintiles of birth weight are at increased risk of developing a spectrum of diseases many decades later, in adulthood (7, 8, 11). Indeed, either maternal undernutrition or overnutrition can be correlated with significantly increased risk for cardiovascular and metabolic disorders (6, 12). Experimental studies in animal models assessing the role of maternal metabolic environment on fetal growth corroborate the major findings from the human studies (27, 54). One explanation for these phenomena is the “fetal origins of adult disease hypothesis,” also called the “Barker hypothesis” in recognition of one of its early proponents (David J. P. Barker, University of Southampton, UK) (5).

Evidence from animal and human studies shows that many adult diseases can be traced back to events that occurred in utero (2, 9). In humans, an adverse maternal uterine environment due to gestational diabetes, type 1 diabetes mellitus, preeclampsia, and small-for-gestational age pregnancies affects placental gene expression and fetal growth (24, 45, 46, 49, 58). Animal models mimicking these maternal uterine environments also show changes in placental gene expression and fetal growth (27, 61). Since the placenta is an interface receiving signals from both mother and fetus and a platform for maternal-fetal interaction, we isolated maternal from fetal genotype effects on placental gene expression with an embryo transfer system using two inbred strains of mice (C57BL/6J and FVB/NJ), based on coat color variation that allowed easy identification of the genotype of the offspring as well as the fact that the metabolic parameters (lipid profile and metabolic parameters) of the parental strains had been extensively characterized by the Mouse Phenome Project (Table 1) (42, 52).

As an initial assessment of the maternal effect on the fetus we analyzed fetal weight. Our results show that at E18.5 the fetal weights of the embryos were significantly regulated by the maternal genotype but not the fetal genotype. This finding correlates with previous embryo transfer studies in mice and cattle that show that maternal genotype is the key in determining fetal weight (26, 44). Although major changes in the placental weight and morphology in embryo-transferred animals were not observed [as reported in cloned mice (57) and in genetic crosses between different mouse strains (62)], it is likely that higher-resolution studies of the cellular components of the placenta may reveal important changes that reflect molecular changes that affect placental function, for example, in nutrient transport and fuel-sensing circuits. Historically, animal breeding studies have focused on two important factors that are known to play a significant role in influencing fetal weight, litter size and sex. The effects of litter size and sex have been extensively studied in the mouse and described previously (19, 36, 44). Each of these studies convincingly shows that increased litter size is correlated with lower birth weight (presumably due to sharing fixed maternal resources among a larger group of fetal recipients). These studies also show that male sex is correlated with increased birth weight, a trend that appears to be observed in mammals, including humans (20). Multivariate analysis of our own data from embryo transfer corroborates these earlier reports: male sex increased fetal weight by ~10% (107.24 mg) (P < 0.0001), and increased litter size decreased fetal weight by ~2% per littermate (21.28 mg) (P < 0.0001). The embryo transfer approach has allowed a third, very important, variable to be added to what is already known about litter and sex effects on fetal weight by making it possible to make a quantitative estimate of the role played by the mother in influencing fetal weight: on average, fetuses from F mothers weighed 115.64 mg (~10%) more than fetuses from C mothers (P < 0.0001).

The results from ConceptGen (Fig. 4) show that the signaling pathways that are most significantly changed in the placenta by the mother involve two important pathways. First, pathways involved in regulation of cell growth, extracellular matrix, and insulin-like growth factor binding were affected. The fact that they were identified in an unbiased screen of the placental transcriptome suggests that the gene networks for maternal-fetal interaction overlap with those for fetal growth (such as IGF-1 and IGF-2) (18, 21). The expressions of these genes were significantly downregulated in the placentas of the embryos gestated in C57BL/6J mothers, irrespective of the genotype of the embryo, and correlate to the smaller fetal size and weight seen in C57BL/6J mothers. We also tested for canonical pathways that are significantly enriched in the placental microarrays, using the MetaCore pathway analysis platform (GeneGo).

Analysis of the smaller set of 81 genes with GeneGo and ConceptGen resulted mainly in enrichment for signal pathways that were associated with lipid metabolism in both C/C vs. C/F and F/C vs. F/F comparisons (P < 0.005). These pathways were enriched in genes that interact with low-density lipoprotein receptor-related protein 1 (Lrp1) (Table 3). GeneGo identified the specific pathways of “chylomicron, VLDL, and LDL metabolism,” “HDL metabolism,” and “XR-dependent regulation of lipid metabolism via PPAR, RAR, and VDR.” Lipid

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Table 3. List of placental genes regulated by maternal genotype interactions with LRP1 as identified by ConceptGen

<table>
<thead>
<tr>
<th>No.</th>
<th>Gene Name</th>
<th>Accession No.</th>
<th>C/C vs. C/F</th>
<th>F/C vs. F/F</th>
<th>P Value C/C vs. C/F</th>
<th>P Value F/C vs. F/F</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Thrombospondin 1 (THBS1)</td>
<td>NM_01580</td>
<td>−2.43</td>
<td>−1.59</td>
<td>0.000271</td>
<td>7.00E-04</td>
</tr>
<tr>
<td>2</td>
<td>Connective tissue growth factor (CTGF)</td>
<td>NM_010217</td>
<td>−2.92</td>
<td>−2.46</td>
<td>0.007469</td>
<td>0</td>
</tr>
<tr>
<td>3</td>
<td>Apolipoprotein E (APOE)</td>
<td>NM_009606</td>
<td>−1.92</td>
<td>−1.87</td>
<td>0.000796</td>
<td>7.00E-04</td>
</tr>
<tr>
<td>4</td>
<td>Serine (or cysteine) proteasome inhibitor, clade A, member 3N (SERPINA3)</td>
<td>NM_009252</td>
<td>−2.15</td>
<td>−1.96</td>
<td>0.000279</td>
<td>0</td>
</tr>
</tbody>
</table>

C, C57BL/6J; F, FVB/NJ; C/[embryo]/C/[mother], C/F/[embryo]/F/[mother], F/F/[embryo]/F/[mother], F/C/[embryo]/C/[mother]; LRP1, low-density lipoprotein receptor-related protein 1.
metabolism-related sets were also identified by GeneGo and Gene Ontology with the individual lists from the two experiments. The lipid genes clustered into two groups: 1) LRP-related genes such as thrombospordin and Apo E (Table 3) and 2) lipoprotein-related genes such as VLDL and HDL. Both these groups are well-described components of endocytic receptor-triggered cellular processes, including intracellular signaling and synthesis and clearance of lipids (14).

We also performed computational enrichment analysis using the software tool “GeneGo pathway maps” with a less stringent P value cutoff (P < 0.05) and identified other processes such as cytoskeletal remodeling and immune response as significant in both C/C vs. C/F and F/C vs. F/F comparisons. These results serve as internal controls in our studies as they corroborate an extensive literature that shows the role of cytoskeletal reorganization (32) and immunologic responses due to maternal sensitization in response to fetal antigens during mouse placentogenesis (4, 29, 33).

In a broader evolutionary context, our molecular network data in placental mammals are strikingly concordant with a recently published study of parent-offspring provisioning in birds (nonplacental animals) that elegantly shows that parental control of provisioning increases prenatal adaptation to increase the fitness of offspring (34).

In conclusion, we have developed a novel technique that can be broadly applied to identification of key signaling pathways in any tissue from the offspring that are principally regulated by the mother rather than the fetus. This is especially relevant in identifying signaling pathways by which fetal growth is regulated, as there is mounting evidence that early events in centogenesis (4, 29, 33).

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

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