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Altered Gene Expression in Murine Placentas in an Infection-Induced Intrauterine Growth Restriction Model: A Microarray Analysis

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Abstract

The biological mechanisms leading to incomplete intrauterine growth are not completely elucidated and few studies have investigated infection-mediated growth restriction. In this investigation we report the alterations induced by maternal infectious challenge in placental gene expression patterns using a murine model. Pregnant dams were challenged at day E7.5 with the oral human pathogen *Campylobacter rectus* to elicit fetal growth restriction. At embryonic day E16.5 placentas were collected to compare placental gene expression patterns from normal fetuses of unchallenged dams and growth restricted fetuses from infected dams. Differential gene expression patterns were determined using Agilent Oligo array (G4121A) with a false discovery rate of $P < 0.05$ and pathway analyses were performed. Seventy four genes were differentially expressed during infection-mediated growth restriction with 9 genes significantly up-regulated, indicating that the effects of maternal infection on gene expression were predominantly suppressive. Pathway analyses indicated that 46 of the 65 genes that were significantly down-regulated were associated with placental/fetal development, and 26 of those were imprinted genes. Among the 9 genes that were up-regulated, 4 are involved in oxygen supply to the fetus and the development of the vascular system. Microarray analysis demonstrated that in the pregnant mouse model, maternal infection that induced growth restriction was associated with down-regulated placental expression of critical growth and developmental related genes, including many imprinted genes. These findings may have significant implications for our understanding of the mechanisms underlying infection-associated human fetal growth restriction and the role of differential placental expression of imprinted genes in fetal growth.

Keywords

Fetal growth; expression array; placenta; infection; development

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1. INTRODUCTION

Low birth weight presents a major public health problem affecting 7–15% of all pregnancies. Low birth weight may occur due to associated preterm birth (PTB) or intrauterine growth restriction (IUGR). In this latter case, the fetuses are small for gestational age (SGA), defined as birth weight less than the 10th percentile for gestational age. Regardless of the etiology, low birth weight increases the risk for perinatal morbidity and mortality as well as the risk for long-term abnormalities and diseases, such as cardiovascular diseases, glucose intolerance, neurodevelopmental and behavioral problems, autism and asthma (CDC et al. 2002; Matthews et al. 2003).

The exact biological mechanism that leads to incomplete intrauterine growth is as yet far from being completely elucidated. However, it seems reasonable to suggest that a reduced nutritional supply from the utero-placental unit impairs fetal growth. This condition, often termed “placental insufficiency”, may be due to abnormal placental development and function. Recent studies, mainly in a number of mouse knock-out and transgenic models, have allowed us to form a list of genes that are critical for placental function and development and, hence, to better understand the possible role of specific genes in IUGR (Hemberger and Cross 2001). Moreover, there is evidence to support that IUGR is associated with the deregulation of the expression of several growth factors involved in placental vascular development, such as vascular endothelial growth factor (*Vegf-A*) and placental growth factor (*Pgf*) (Tsatsaris et al. 2003). Also, IUGR is related to alterations in the activity and/or expression of the placental transport systems of essential amino acids and of the placental nutrient and ion transporters (Mahendran et al. 1993; Jansson et al. 1993). Finally, the role of several imprinted genes, such as *Igf-2*, *Peg-1*, and *Peg-3*, in IUGR has been highlighted (Constância et al. 2002; Li et al. 1999). The majority of imprinted genes that are expressed in the placenta and/or the fetus are considered to regulate fetal demand and maternal supply of nutrients. In humans there is an unbalanced placental expression of these genes in the placenta of IUGR fetuses (McNimm et al. 2006). In addition in mice, targeted deletions and epigenetic alterations of specific alleles of imprinted genes have been shown to affect fetal and placental growth providing important models of imprinting defects leading to IUGR (Constância et al. 2002).

As previously stated, IUGR is not considered a single disorder or disease, but the result of placental insufficiency. Several genetic, metabolic, vascular, autoimmune and infectious factors have been implicated, probably through the induction of alterations in placental/fetal gene expression. Since, the expression patterns of an extensive number of genes may regulate the development of IUGR, expression profiling using the cDNA microarray technology in combination with real-time PCR has proven to be a powerful tool for studying multivariable conditions such as IUGR.

One of the infectious factors associated with IUGR is periodontal disease (Boggess et al. 2003), which is a chronic infectious disease of the gingiva that, through bacteremia, may have systemic effects and affect the feto-placental unit (Jared et al. 2009). A recent meta-analysis evaluating the possible association between periodontal disease and adverse pregnancy outcomes, such as low birth weight and PTB concludes that there is a positive association (Polyzos et al. 2009). Similarly, experiments on various animal models have revealed that infection with periodontal bacteria at a site distant to the placenta induces IUGR (Yeo et al. 2005; Lin et al. 2003; Offenbacher et al. 2005). Moreover, these bacteria are found to be present in the placental and fetal tissues (Yeo et al. 2005; Lin et al. 2003; Offenbacher et al. 2005). Furthermore, histological evaluation of the placentas of IUGR fetuses have shown structural alterations with most important the decrease of the labyrinth layer in the placentas of IUGR fetuses (Offenbacher et al. 2005). Since, the labyrinth is the layer of nutrient and waste exchange between the mother and the fetus these changes may signify placental insufficiency.

The goal of the present study was to assess differences in the expression profiles of placentas from infected and IUGR fetuses and from unchallenged normal fetuses. Moreover, we tried to evaluate the mechanism by which infection induces IUGR by analyzing the activated expression signaling pathways.

2. MATERIALS AND METHODS

2.1 Animal Husbandry

All procedures were in accordance with the animal welfare guidelines and approved by the University of North Carolina-Chapel Hill Institutional Animal Care and Use Committee. BALB/c mice were housed under controlled and standardized conditions, with 12-hour light-dark cycles (0700-1900 light). Regular mouse diet and water were provided *ad libitum*. The mouse infection model used in this study was similar to that described elsewhere (Yeo et al. 2005). Briefly, female Balb/c mice were enrolled in the experiments at approximately 6 weeks of age. At that time a stainless steel, open-ended cylindrical coil spring (chamber) approximately 1.0 cm × 0.4 cm was implanted subcutaneously into the dorsolumbar region of each mouse. After a 2-week period of healing, they were mated overnight with males of the same strain. The next morning, females were removed from the male cages and examined for vaginal plugs. If a plug was found, that day was recorded as embryonic day E0.5.

2.2 Challenge with *C. rectus* and Bacterial Culture

Pregnant mice were randomly divided into two groups at embryonic day E7.5. An E7.5 mouse embryo features an incipient decidual sac (initial implantation) without significant embryo development, making this an ideal stage to disturb fetal development without affecting embryo implantation.

The control group received an intra-chamber injection of 0.1 ml of phosphate buffered saline (PBS), while the test group received an intra-chamber injection of 0.1 ml of 10⁹ CFU/ml live *C. rectus* strain 314. Pilot ascending dosage studies with *C. rectus* 314 have demonstrated that *C. rectus* 314 is well tolerated by the animals and pre-treatment with heat-killed bacteria is not needed. Mice were infected at E7.5, as we have previously shown that at this time point *C. rectus* affects normal fetoplacental development as evidenced by fetal intrauterine growth restriction, abnormal placental architecture and placental edema (Yeo et al. 2005, Offenbacher et al. 2005, Bobetsis et al. 2007).

Bacteria were previously cultured under anaerobic conditions at 37° C on enriched tryptic soy agar plates (Anaerobe Systems, Morgan Hill, CA), and were used at their log phase of growth.

2.3 Sample Collection

On day E16.5 pregnant mice were sacrificed and placentas and fetuses were collected. Fetuses were weighed to determine whether they were growth restricted. IUGR was defined as fetal weight <0.37 g, which was 2 SD below the pooled average weight of fetuses from unchallenged dams (144 fetuses from 27 unchallenged dams). Placentas were weighed on a microbalance and processed for RNA extraction.

2.4 RNA Extraction from whole Placenta Tissues

Total RNA from placental tissues was isolated using the Agilent Total RNA Isolation Mini Kit (Agilent Technologies) and the RNeasy Mini Kit (Qiagen, Valencia, CA), following the manufacturer's protocol. The quality and quantity of RNA were evaluated spectrophotometrically at 260 nm and 280 nm using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE). Also, samples were examined with the Agilent

2100 Bioanalyzer at the Lineberger Cancer Center (UNC Medical School, Department of Genetics).

2.5 Microarray Analysis

Pooled RNA from 15 placentas from 8 different unchallenged dams was compared to a reference RNA (Ambion) in triplicate. RNA from 4 challenged dams from IUGR placentas were individually compared with the same reference RNA giving a total of 7 arrays (3 technical replicates for controls plus 4 biological replicates for infected placentas). Specifically, starting with the total RNA from the placental tissues and the reference RNA, the mRNA was amplified and fluorescently labeled using the Agilent Low RNA Input Fluorescent Linear Amplification kit (Agilent Technologies). Then, the fluorescently labeled mRNA was hybridized on an Agilent Oligo array (G4121A). All procedures were performed based on the manufacturer's protocol. The glass slides were scanned using an Agilent G2565BA microarray scanner. Raw data were collected using the Feature Extraction software (Agilent Technologies) and normalized by applying the LOWESS normalization. Finally, data were analyzed using the Permutation test at a false positive discovery rate of 0.0486, using the Significance Analysis of Microarrays software (SAM) v.2.23 (Stanford University, CA) (Tusher et al. 2001). Moreover, in order for each gene to be included in the statistical analysis, only the data from one array per group could be "flagged". Hence, the analysis used data from a minimum of 5 out of the 7 arrays (2 control and 3 challenged).

Activated signaling pathways were evaluated using GeneSpring software. Each gene was classified according to its gene ontology (GO), in which genes are organized into hierarchical categories based on biological process, molecular function, and cellular component.

Moreover, genes were clustered in five groups using the K-Means algorithm (Stekel 2003). Finally, to determine whether the expression of the 12 known placental/fetal growth and development genes was significantly altered as a cluster of 12 genes we used the relative gene expression for unchallenged or challenged IUGR placentas using the O'Brien non-parametric rank-sum test for uniformity of the multivariate end-point (O'Brien 1984).

2.6 Real Time PCR data validation

Genes showing significant differential expression were confirmed by means of qPCR reactions. Briefly, 1 µg of total RNA was reversely transcribed using the Omniscript system (Qiagen, CA). Real-time PCR was performed with 1 µL of the cDNA reaction in a 7500 Sequence Detection System (ABI Prism, Applied Biosystems, CA). TaqMan pre-inventoried assays were used for relative gene expression quantification. Reactions were performed in duplicate and in two independent assays for each gene. Gapdh was used as an endogenous control for each sample. Results were evaluated using the delta-delta Ct method. Differences of the Ct value between the tested gene and *Gapdh* (ΔCt) for each sample were calculated and then the calibrated ΔCt value ($\Delta\Delta Ct$) and positive control $\Delta\Delta Ct$ was calculated ($\Delta\Delta Ct = \Delta Ct$ test sample - ΔCt control).

3. RESULTS

We applied the mouse cDNA microarray analysis to evaluate, simultaneously, differences in the expression of 22,000 genes among placentas of IUGR fetuses from dams challenged with the periodontal pathogen *C. rectus*, and placentas from normal fetuses from unchallenged dams (control). We analyzed a total of 7 microarrays, 4 from IUGR and challenged fetuses and 3 from control fetuses (pooled). Based on the array data analysis, we found that the expression of 74 genes was statistically different among the two groups. From these differentially expressed genes 9 were up-regulated while the rest (65) were down-regulated, indicating that

the effects of maternal infection on gene expression were predominantly suppressive rather than stimulatory. The fold change of these genes ranged from 1.2 to 5.5. Most of these 74 genes were related to hypothetical proteins, RIKEN cDNA sequences, different clone images and other proteins with no specific or known function. However, there were also some genes of known function, but only a few had an obvious role in placental/fetal growth and development. A list of genes, accession numbers, main function, and fold changes of all annotated genes that are possibly related to placental/fetal growth and development are presented in Table 1. The list of genes, accession numbers, and fold changes of all annotated, differentially expressed genes are available in Appendix 1.

From the nine genes that were up-regulated, four are involved in the supply of oxygen to the fetus and the development of the vascular system. These included genes encoding the embryonic hemoglobins Z and Y, the prolactin-like protein B (*Prlpb*) and glypican 1 (*Gpc1*). Genes that were down-regulated and had known functions could generally be separated into two groups based on their biological actions: a) genes that mainly affect the development of the fetus, and b) genes that mainly affect the development of the placenta. Specifically, the first group included genes that encode the DNA binding protein *Desrt*, the type VIIa1 collagen, the olfactory receptors MOR 31-3 and MOR 256-3, the synaptotagmin X (*Syt10*), and the neuropeptide galanin (*Gal*) and its receptor (*Galr3*). Genes that were included in the second group included the reticulon 4 receptor (*Rtn4r*) and the angiogenic factor placental growth factor (*Pgf*).

To further investigate the biological processes involved in the development of IUGR, we performed a gene ontology analysis using the GeneSpring software. However, after assigning the 79 genes to the various functional subsets, there was no indication of any obvious association of these genes with the activation of specific molecular pathways (data not shown).

A different approach to cluster genes that are possibly involved in common signaling pathways is by using the K-Means algorithm. This algorithm clusters genes not based on their biological functions, but based on similar gene expression profiles. The assumption behind this analysis is that genes with similar expression patterns may participate in common or related signaling pathways. From the 5 clusters generated, all genes that were up-regulated and involved in nutrient supply and blood vessel development were found in the same cluster. From the genes that were down-regulated and thought to be involved in fetal development, all except for galanin and its receptor were in the same cluster, a cluster different from that of the up-regulated genes. Finally, in a third cluster of genes we could identify the genes that are considered important for placental development. In this cluster galanin and its receptor also were included. All other genes, mainly of unknown function, were found throughout the 5 clusters (Figure 1).

Besides the useful information that can be extracted using the various data analysis softwares, valuable insight about the effects of maternal infection in placental development can also be generated by studying the microarray raw data. This approach revealed a differential expression of several inflammatory mediators. Some genes responsible for pro-inflammatory molecules such as *Il-1* and *S100a* and *S100b* were up-regulated, while others such as *Tnf-α* were slightly down-regulated. From the genes encoding Th1 and Th2 types of inflammatory cytokines, *Ifn-γ* and *Il-4* were up-regulated, while *Il-2* and *Il-10* were down-regulated.

When genes important for placental/fetal growth and development were evaluated, using the raw data again, there was a clear pattern of down-regulation of the expression of these genes in the IUGR placentas (data not shown). Moreover, when genes essential for placental development, as determined with knockout mouse models, were evaluated as a cluster, there was a significant depression in the expression of the group that exceeded 50%, $p=0.012$ (Table 2) (Hemberger and Cross 2001).

Microarray analysis also revealed the effect of maternal infection on imprinted genes. As shown in Table 3, the microarray data demonstrated that the majority of these genes known to be involved in growth and development are down-regulated in placentas from IUGR fetuses. We also sought to validate these observations on specific imprinted genes by means of real-time PCR reactions using TaqMan chemistry in pre-inventoried assays. All of the analyzed imprinted genes were down-regulated and also showed a similar expression pattern when normalized to GAPDH expression and when compared to non-infected controls (Indexed footnotes on Figure 3).

Finally, we weighed the placentas in order to evaluate whether the observed differences in gene expression among placentas from IUGR and normal fetuses are due to variations in their size. As indicated in Table 4 there were no significant differences in the size of the placentas between both groups.

4. DISCUSSION

We found that the expression of only 74 genes was statistically different in the placentas of IUGR fetuses compared to those of normal and unchallenged fetuses. The relatively low number of genes that were differentially expressed can be attributed to the very stringent parameters used in our analysis in order to gain more reliable results. Specifically, we analyzed the data using the permutation test at a false positive discovery rate (FDR) of less than 0.05. Moreover, in order for each gene to be included in the statistical analysis, only the data from one array per group could be “flagged”. Hence, the analysis used data from a minimum of 5 out of the 7 arrays (2 control and 3 challenged).

From the 74 differentially expressed genes 9 were up-regulated while the rest were down-regulated, indicating that the effects of maternal infection on gene expression were predominantly suppressive rather than stimulatory. Some of these genes had a known function, but only a few had an obvious role in placental/fetal growth and development. The fact that the size of the placentas between the two groups studied did not differ, demonstrates that the decrease in the expression of the genes is not due to a smaller total number of cells in the placentas. However, in previous reports we have demonstrated, by histological analysis, that the zone of the labyrinth is significantly smaller in the IUGR placentas (Offenbacher et al. 2005).

Of the 9 genes that were up-regulated, most were involved in the supply of oxygen to the fetus and the development of the vascular system. Specifically, the embryonic hemoglobins Z and Y are known to be produced by the yolk sac and the embryo and are critical for the transport of oxygen towards the fetus via the blood circulation. The finding that these hemoglobins are up-regulated in our arrays suggests that these proteins may also be produced in the mouse placenta. The impaired transfer of nutrients such as oxygen, due to the small size of the labyrinth, may have stimulated the placenta to increase its efficiency by producing more molecules that enhance this process. Two other genes that were also up-regulated included the genes that encode *Prlpb* and *Gpc1*. *Prlpb* is related to prolactins that are expressed in the placenta and uterus. In general, prolactins have important modulatory actions during the establishment of pregnancy and the initiation of parturition and they consist of three groups of proteins: the placental lactogens, the prolactins involved in parturition, and the proliferins. No specific function has been attributed to *Prlpb*, but since it belongs to the proliferins, it is thought to modulate blood vessel development (Muller et al. 1998). *Gpc1* is a proteoglycan expressed in the placenta. It regulates the function of FGF by binding to this growth factor and acts as a chaperon for various VEGF molecules. Hence, *Gpc1* is thought to be involved in growth and differentiation and especially in angiogenesis (Gengrinovitch et al. 1999). The increase in expression of *Prlpb* and *Gpc1* may indicate that the placentas of IUGR fetuses are trying to

restore the defective labyrinth and enhance vascularization. The extension of vascular branching will increase the surface area of nutrient exchange and therefore the amount of nutrients transferred to the fetus. For the already growth restricted fetuses this placental response may be very critical, especially considering that a significant portion of fetal growth is yet to come.

Surprisingly, none of the 9 genes that were up-regulated included genes that encode inflammatory mediators. This result was not anticipated considering the increase of the inflammatory infiltrate observed in the histological sections of these placentas (Tusher et al. 2001). Moreover, in previous reports where pregnant dams were challenged with the periodontal pathogen *P. gingivalis*, there was an increase in the expression of pro-inflammatory Th1 type cytokines and of *Tnf- α* in placentas of infected and IUGR fetuses (Lin et al. 2003). It is possible that the majority of the innate inflammatory response against *C. rectus* took place at an earlier stage before E16.5 and had subsided so that it was not detected with the microarray data analysis. It is also possible that the technique used is not able to detect a statistical difference in the expression in these genes even though a difference is actually present.

Genes that were down-regulated and had known functions could generally be separated into two groups based on their biological actions: a) genes that mainly affect the development of the fetus, and b) genes that mainly affect the development of the placenta. The first group included the majority of down-regulated genes; however, their possible role in placental development has not been studied and therefore should not be excluded.

Specifically, genes in this group included genes that encode the DNA binding protein Desrt, the type VIIa1 collagen, the olfactory receptors MOR 31-3 and MOR 256-3, the synaptotagmin X (Syt10), and the neuropeptide galanin (Gal) and its receptor (Galr3). Desrt is important for growth and normal development of the reproductive organs. Knockout mice that do not express the *Desrt* gene have reduced viability, pronounced growth retardation, and a high incidence of abnormalities of the female and male reproductive organs (Lahoud et al. 2001). Collagen VIIa1 is the main collagen type present in the skin, and mutation of this gene leads to skin disorders (dystrophic epidermolysis bullosa) in humans (Kern et al. 2006). The olfactory receptors are important for the olfactory function and this is impaired or even lost with the reduction of these receptors (Rawson 2006). Interestingly, in mice, the olfactory system has been shown to be critical for mate recognition and sexual behavior in females (Keller et al. 2006). Syt10 regulates the secretion of neurotransmitters stored in the synaptic vesicles and thus affects the signaling between neurons (Chapman et al. 1996). Impairment of the nervous system is also found in mutants that do not express galanin. Galanin is a neuropeptide, and mice lacking this peptide show lower numbers of sensory neurons and reduced capability for nerve regeneration (Holmes et al. 2000). Moreover, galanin and its receptor are considered to have functions related to energy and nutrient balance, since administration of galanin to experimental animals stimulates food intake (Kyrkouli et al. 1990).

From all the above, it is clear that these genes are involved in the normal development and function of a variety of adult tissues. However, it is not known whether the observed down-regulation of these genes in the placenta can have a direct effect on the developing fetus. It is likely that during gestation the immature fetal tissues may not be able to produce sufficient amounts of all proteins related to development and thus rely on the placenta for their supplementation. In this case, down-regulation of such genes in the placenta may be critical for the developing fetus and may contribute to anomalies found in the offspring later in life.

Of the genes that were down-regulated only two could be related to the development of the placenta and included *Rtn4r* and *Pgf*. *Rtn4r* is the receptor of reticulon, also known as neuroendocrine-specific protein. Its exact function is unknown, but it is thought to be associated

with transport processes in the endoplasmic reticulum (ER). Although, the importance of Rtn4r in these processes remains to be elucidated, it is well established that at a cellular level the ER is important for the secretion of proteins. Hence, even though the reduction of Rtn4r may not directly affect a distinct placental function, it may compromise the normal function of cells within the placenta.

A more specific effect on placental development may be associated with the down-regulation of *Pgf*. The growth factor encoded by this gene belongs to the VEGF family of proteins and is expressed only in placental tissues. It is an important angiogenic factor that along with the other VEGFs regulates vascularization and vascular permeability, while it is thought to participate also in the regulation of the vascular tone and blood flow (Torry et al. 2003). Mice that do not express *Pgf* show impaired angiogenesis, and in humans, down-regulation of *PGF* is associated with pregnancy complications such as preeclampsia. Also, cross-sectional studies in normal pregnancies indicate that *PGF* levels rise during the second trimester and peak at the early third trimester (Torry et al. 1998). If similar events are true in mice, the observed down-regulation of *Pgf* may explain in part the deficiency in the growing labyrinth. In fact, this is the first report demonstrating that growth factors important for the growth and development of the placenta are down-regulated in association with maternal infection with bacteria.

Several other proteins, such as different kinases were also down-regulated. Since these kinases are considered to participate in a variety of signaling pathways we were not able to associate them with a specific function and hence could not assign them to either of the two groups described.

Although, as mentioned earlier, these two groups were determined based on the biological functions of the genes, further microarray data analysis could not identify specific pathways that were either activated or suppressed within these groups. It is very likely that although some signaling pathways are differentially activated, the small number of genes involved in this analysis made these pathways non-detectable.

A different approach to cluster genes that are possibly involved in common signalling pathways is by using the K-Means algorithm. This algorithm clusters genes not based on their biological functions, but based on similar gene expression profiles in terms of magnitude and direction of response. The assumption behind this analysis is that genes with similar expression patterns may participate in common or related signalling pathways. Of the 5 clusters generated, all genes that were up-regulated and involved in nutrient supply and blood vessel development were found in the same cluster. Of the genes that were down-regulated and thought to be involved in fetal development all except for galanin and its receptor were in the same cluster, different though from that of the up-regulated genes. Finally, in a third cluster of genes we could identify the genes that are considered important for placental development. In this cluster galanin and its receptor were also included. All other genes, mainly of unknown function, were found throughout the 5 clusters. The fact that this clustering method grouped the genes in a similar fashion as we had grouped them based on their biological functions may imply a common regulation of these genes. Although the genes involved in fetal development may not be obviously related, since they affect different types of tissues, it is possible that they may have a common up-stream control.

Microarray analysis of the raw data also revealed a differential expression of several inflammatory mediators. Some genes responsible for pro-inflammatory molecules such as *Il-1* and *S100a* and *S100b* were up-regulated, while others such as *Tnf- α* were slightly down-regulated. From the genes encoding Th1 and 4 Th2 type of inflammatory cytokines *Ifn- γ* and *Il-4* were up-regulated, while *Il-2* and *Il-10* were down-regulated. Hence, although a previous

study, where pregnant dams were challenged with *P. gingivalis*, reported a shift in placental Th1/Th2 cytokine balance towards a Th1 response, our data were not able to clearly support a similar shift (Lin et al. 2003).

On the contrary, when genes important for placental/fetal growth and development were evaluated, there was a very clear pattern of expression, with the majority of the genes down-regulated to various degrees. Moreover, when genes essential for placental development, as determined with knockout mouse models, were evaluated as a cluster, there was a significant depression in the expression of the group. The decrease in the expression of multiple growth-related genes may act synergistically to impair placental and fetal development, as noted with the smaller labyrinth of IUGR fetuses. In addition, if growth factors produced in the placenta are also shared by the fetus, a shortage of these factors, due to maternal infection, may contribute to possible abnormalities in the offspring. This would further support the notion that the *in utero* environment is critical for the future well being of the offspring.

Additional important information revealed from the microarrays is the effect of maternal infection on imprinted genes. The majority of these genes are involved in growth and development. Genomic imprinting represents a unique epigenetic regulatory mechanism operating in placental mammals and in general, these genes are expressed only from one of the parental alleles, while the other allele is silenced due to a methylation pattern that is imprinted on the DNA. The regulation of growth factor expression by imprinting is considered to allow for the control of resource allocation. Most of the imprinted genes are expressed in the placenta and some of them have been identified, directly or indirectly, to be critical for placental/fetal growth and transplacental nutrient transport. Differential DNA methylation at imprinted loci serves both to mark the parental origin of the alleles and to regulate their expression. Although alteration in expression could be expected in placental tissues, our results indicated through microarray data, confirmed by qPCR, that the bulk of these imprinted genes are down-regulated in placentas from IUGR fetuses. Consistent with our bacterial infection-induced murine model of IUGR, array data from human placental tissues of term IUGR fetuses also demonstrate down-regulation of several imprinted genes including *IGF-2*, *PEG-3* and *GNAS* (McMinn et al. 2006). The down-regulation of imprinted genes may imply that maternal insult with bacteria may alter the expression of these genes possibly by affecting their imprinting mechanism. This idea is also strengthened by the observation that the expression of the gene for cytosine DNA methyltransferase is increased, suggestive of an enhanced DNA methylation activity. In fact, our group has shown with real-time PCR that *C. rectus*-infected IUGR placentas have a significantly lower expression of *Igf-2*, very similar to that revealed by the raw microarray data. Moreover, the placental promoter (P0) of this gene is shown to be hyper-methylated in a site-specific manner in four CG dinucleotides (Bobetsis et al. 2007). Although it has not been proven that the observed hyper-methylation contributes to the reduction in the expression of this imprinted gene, it presents a plausible biological mechanism by which infection may lead to IUGR.

In conclusion, we have demonstrated, by microarray data analysis and real-time PCR validation that placentas of infection-induced IUGR fetuses show an attenuation of the expression of growth related genes. Expression profiling using a cDNA microarray approach is a convenient way of identifying a large number of candidate genes that may be implicated in IUGR. However, microarray data should be interpreted with caution, since verification of the results with better quantitative techniques (e.g., real-time PCR) is necessary.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.
Distribution of differentially expressed genes in 5 clusters using the K-Means algorithm.

Table 1

Fold change in the expression of genes related to placental/fetal growth and development in IUGR placentas. The main function of each gene is also listed.

Gene	Symbol	Function (abnormality)	Accession number	Fold change
Prolactin-like protein B	<i>Prlpb</i>	Blood vessel development	NM_011166	+ 3.3
Hemoglobin Y	<i>Hbb-y</i>	Transport of oxygen	NM_008221	+ 1.8
Hemoglobin Z	<i>Hbb-z</i>	Transport of oxygen	NM_008219	+ 1.7
Glypican	<i>1 Gpc-1</i>	Angiogenesis	NM_016696	+ 1.6
Placental growth factor	<i>Pgf</i>	Angiogenesis, vascular tone and flow	NM_008827	– 1.6
Reticulon 4 receptor	<i>Rtn4r</i>	Transport process in E.R.	NM_022982	– 1.6
Type VIIa1 collagen	<i>Col7a1</i>	Collagen in skin (dystrophic epidermolysis bullosa)	NM_007738	– 1.7
Olfactory receptors	<i>Mor</i>	Olfactory function (mating)	NM_147101.1	– 1.9
Synaptogamin 10	<i>Syt10</i>	Signaling between neurons	NM_018803	– 2.2
Developmentally and sexually retarded with transient abnormalities	<i>Desrt</i>	Development of reproductive organs (IUGR, abnormal r.o.)	NM_023598	– 2.3
Galanine	<i>Gal</i>	Neuropeptide (reduced nerve regeneration and less sensory neurons) nutrient balance	NM_010253	– 2.4
Galanine receptor 3	<i>Galr3</i>	Galanine Receptor	NM_015738	– 2.4

Table 2

Fold change in the expression of genes that are essential for placental development in placentas of IUGR fetuses.*

Placental function	Gene	Gene product	Fold Change**
Songiotrophoblast maintenance	<i>Ascl2</i> (<i>Mash2</i>)	bHLH transcription factor	-1.96
Regulation of fetal-placental growth	<i>Igf2</i>	Insulin-like growth factor 2	-1.92
Chorioallantoic branching and labyrinthine development	<i>Gcm1</i>	Transcription factor	-1.85
Vascularization of labyrinth	<i>Esx1</i>	Homeobox transcription factor	-1.69
Chorioallantoic branching and labyrinthine development	<i>Pparg</i>	Nuclear hormone receptor	-1.67
Chorioallantoic branching and labyrinthine development	<i>Dlx3</i>	Homeobox transcription factor	-1.59
Trophoblast giant cell differentiation	<i>Hand1</i>	bHLH transcription factor	-1.51
Chorioallantoic branching and labyrinthine development	<i>Pdgfra</i>	Platelet-derived growth factor receptor α	-1.48
Nutrient transport across labyrinth	<i>Gjb2</i> (<i>Cx26</i>)	Connexin, gap junction protein	-1.41
Trophoblast lineage development	<i>Fgf4</i>	Fibroblast growth factor 4	-1.31
Ectoplacental cone function	<i>Ets2</i>	Ets domain transcription factor	-1.29
Chorioallantoic branching and labyrinthine development	<i>Fzd5</i>	Frizzled 5, Wnt receptor	-1.25

* Genes determined with knockout mouse models

** Raw microarray data

Table 3

Fold change in the expression of imprinted genes known to be essential for placental development in placentas of IUGR fetuses.

Accession number	Gene	Fold change
NM_172119.1	<i>Dio3</i>	-3.29
NM_027052	<i>Slc38a4</i>	-2.18
NM_008554	<i>Mash2 (Ascl2)</i>	-1.96 ¹
NM_010514	<i>Igf2</i>	-1.92 ²
AK033735	<i>Meg3</i>	-1.91
NM_009876	<i>Cdkn1c</i>	-1.65 ³
NM_011395	<i>Slc22a3</i>	-1.56
NM_008817	<i>Peg3</i>	-1.44
NM_023123	<i>H19 fetal liver</i>	-1.43 ⁴
	<i>Gatm</i>	-1.37
NM_010345	<i>Grb10</i>	-1.35
NM_021323	<i>Usp29</i>	-1.31
	<i>Nap1/4</i>	-1.23
	<i>Slc221/</i>	-1.23
NM_024289	<i>Obph1</i>	-1.19
NM_011769	<i>Zim1</i>	-1.17
NM_010052	<i>Dlk1</i>	-1.10
NM_020285	<i>Tssc4</i>	-1.09
NM_008387	<i>Ins2</i>	-1.02
AK052949	<i>U2af1-rs1</i>	-1.01
NM_008378	<i>Impact</i>	1.02
NM_017478	<i>Copg2</i>	1.27
NM_013667	<i>Slc22a2</i>	1.35
D85430	<i>Murr1</i>	1.35
NM_008579	<i>Meg1</i>	1.51
X61453	<i>H19 clone</i>	1.96

¹ qPCR Fold change= (-3.31±0.75)

² qPCR Fold change= (-2.3±0.38)

³ qPCR Fold change= (-2.03±0.65)

⁴ qPCR Fold change= (-1.97±0.14)

All qPCR validations were normalized to GAPDH expression. Related fold differences were calculated to non-infected murine placentas.

Table 4

Placental weight from normal and *C. rectus*-infected dams.

	Unchallenged	Challenged	Challenged and IUGR
Placental weight Mean $\pm$ s.e.m (g)	0.132 $\pm$ 0.002	0.14 $\pm$ 0.005	0.141 $\pm$ 0.0
Number of placentas	106	105	20
Number of dams	19	19	11 out of 19