Placenta-Imprinted Gene Expression Association of Infant Neurobehavior

Carmen J. Marsit, PhD[1,2], Luca Lambertini, PhD[3,4], Matthew A. Maccani, PhD[7], Devin C. Koestler, PhD[2], E. Andres Houseman, ScD[8], James F. Padbury, MD[9], Barry M. Lester, PhD[9,10], and Jia Chen, ScD[3,5,6]

Objective To identify links between altered gene imprinting in the placenta and infant neurobehavioral profiles.
Study design Quantitative reverse-transcription polymerase chain reaction was used to examine the expression of 22 imprinted candidate genes in a series of 106 term human primary placenta tissues. The expression pattern uncovered was associated with Neonatal Intensive Care Unit Network Neurobehavioral Scales summary scores in the corresponding infants. Clustering of the expression data was used to define distinct classes of expression.
Results Significant associations were identified between classes of expression and the Neonatal Intensive Care Unit Network Neurobehavioral Scales quality of movement (P = .02) and handling (P = .006) scores. Multivariate regression demonstrated an independent effect of imprinted gene expression profile on these neurobehavioral scores after controlling for confounders.
Conclusion These results suggest that alterations in imprinted gene expression in the placenta are associated with infant neurodevelopmental outcomes, and suggest a role for the placenta and genomic imprinting in the placenta beyond intrauterine growth regulation. *(J Pediatr 2012;160:854-60)*.

The paradigm of the developmental origins of health and disease posits that the period of intrauterine growth is critical to determining health outcomes throughout life. The focus is on outcomes associated with metabolic diseases and related disorders, including diabetes, obesity, and coronary heart disease.[1,2] The concept of fetal programming and the importance of the intrauterine environment beyond metabolic outcomes have now been linked to behavioral and psychosocial outcomes,[3,4] including schizophrenia[5] and depression.[6,7] Most previous studies have linked birth size with these health outcomes, but it is generally accepted that birth size is a proxy for a complex interplay of underlying etiologic mechanisms and that certain common factors influence intrauterine growth as well as adult physiological systems.[8] Defining the molecular basis of fetal programming is critical to understanding the mechanistic basis of these observational findings. Such understanding can provide insight into the early identification of individuals who might benefit from intervention and help guide the design of novel approaches to preventing and treating health problems later in life.

Numerous adverse effects related to the intrauterine environment may result in epigenetic alterations.[9-11] A key mode of epigenetic regulation is genomic imprinting, which refers to the monoallelic expression of a subset of genes in a conserved parent-of-origin fashion. This mode of expression control is orchestrated by the timely placement of epigenetic signals, including DNA methylation and histone modification. The key functions of imprinted genes include controlling the allocation of maternal resources to the fetus, regulating metabolism in the early postnatal period, and determining the metabolic states of developing metabolic organs such as the pancreas, muscle, adipocytes, and hypothalamus.[12] It is likely that imprinted genes play a crucial role in neurodevelopment. A high proportion of imprinted genes are expressed in the central nervous system. Maternally expressed imprinted genes are thought to favor the development of larger brains.[13] Children with Beckwith-Wiedemann syndrome, which results in part from inappropriate imprinting of specific imprinted gene clusters, demonstrate greater-than-expected proportions of abnormal scores on emotional and behavioral scales.[14] Female mice engineered to be null for the paternal *Peg3* gene, exhibit a reduced number of oxytocin-producing neurons in the hypothalamus.[15]

The placenta serves as a central regulator of the intrauterine environment, and may be important in appropriate infant neurodevelopment through its immune-endocrine role.[16,17] Using a population-based birth cohort, we examined the expression of 22 imprinted genes in 106 human term placenta samples and associated the profiles of expression of these genes with measures of newborn neurodevelopment. Our operative hypothesis was that altered gene expression profiles of imprinted genes have a quantifiable impact on neurobehavioral development in an unbiased, population-based cohort.

NNNS	Neonatal Intensive Care Unit Network Neurobehavioral Scales
qRT-PCR	Quantitative, real-time reverse-transcription polymerase chain reaction

From the Departments of [1]Pharmacology and Toxicology and [2]Community and Family Medicine, Dartmouth Medical School, Hanover, NH; Departments of [3]Preventive Medicine and [4]Obstetrics, Gynecology and Reproductive Science, [5]Pediatrics, and [6]Oncological Sciences, Mount Sinai School of Medicine, New York, NY; [7]Center for Alcohol and Addiction Studies and [8]Community Health Center for Environmental Health and Technology, Brown University; and [9]Department of Pediatrics and the [10]Brown Center for the Study of Children at Risk, Women and Infants Hospital, Providence, RI

Supported by the National Institute of Mental Health (grant R01 MH094609), the National Center for Research Resources (grant P20 RR018728), and the Venture Capital Research Funding Program of the Mount Sinai Children's Environmental Health Center and Mount Sinai Child Health and Development Institute. The authors declare no conflicts of interest.

 10.1016/j.jpeds.2011.10.028

Methods

The study subjects are part of the ongoing Rhode Island Child Health Study, which is enrolling mother–infant pairs after delivery at Women and Infants Hospital of Rhode Island. Term infants born small for gestational age (ie, lowest 10th percentile) or large for gestational age (ie, highest 10th percentile), based on birth weight and gestational age and calculated from the Fenton growth chart,[18] were selected. Infants born appropriate for gestational age matched on sex, gestational age (±3 days), and maternal age (±2 years) were enrolled as well. Only singleton, viable infants were included in the study. Other exclusion criteria were maternal age <18 years, a life-threatening medical complication of the mother, and a congenital or chromosomal abnormality of the infant. A structured chart review was used to collect information from the maternal inpatient medical record from delivery. Mothers completed an interviewer-administered structured questionnaire for information on lifestyle, demographics, and exposure histories.

Neurodevelopmental status at birth was assessed with the Neonatal Intensive Care Unit Network Neurobehavioral Scales (NNNS), administered by certified psychometrists blinded to the study hypothesis. The test was administered during the newborn inpatient stay after the day of birth but before discharge. Initially designed for the observation of high-risk term and preterm infants, the NNNS provide a comprehensive evaluation of neurobehavioral performance, including neurologic and behavioral measures and signs of stress.[19,20] This test has demonstrated predictive validity for medical outcomes, such as cerebral palsy diagnosis, neurologic abnormalities, and diseases with risks to the brain, as well as developmental outcomes, such as mental and motor functioning, behavior problems, school readiness, and IQ.[21] Norms have been developed for this examination in healthy, full-term infants.[22]

Items on the NNNS were scored using previously established summary scores. We focused on the 9 summary scores (attention, arousal, excitability, hypertonicity, stress/abstinence, self-regulation, nonoptimal reflexes, handling, and quality of movement) found to be the most sensitive in previous studies.[21] A total of 106 subjects, enrolled between September 2009 and May 2010, were examined. All subjects provided written informed consent as approved by the Institutional Review Boards for Women and Infants Hospital and Brown University.

Placenta Sample Collection and RNA Extraction

For each subject, within 2 hours of delivery, 12 biopsy specimens of placenta tissue, 3 from each of 4 quadrants (totaling approximately 1 g of tissue) were excised from the maternal side of the placenta 2 cm from the umbilical cord insertion site. All samples were from the placenta parenchyma and were homogenous, without areas of calcification, hemorrhage, or clots and were free of maternal decidua. The samples were immediately placed in RNA later and stored at 4°C. At least 72 hours later, the samples were removed from RNAlater, blotted dry, snap-frozen in liquid nitrogen, homogenized using a mortar and pestle, and stored in sample tubes at −80°C until examination. RNA was isolated from approximately 100 mg of placental sample using the RNeasy system (Qiagen, Valencia, California) including on-column DNAse digestion to ensure removal of residual DNA contamination. Extracted RNA was quantified using a NanoDrop spectrophotometer (Thermo Scientific, Wilmington, Delaware) and stored at −80°C.

Quantitative Reverse-Transcription Polymerase Chain Reaction Analysis for Imprinted Genes

Twenty-two imprinted genes were chosen for this investigation, including 20 that were found to be dysregulated in our previous gene expression experiments comparing severe growth-restricted placentas with appropriate-for-gestational-age placentas.[23,24] Two homeobox genes, *HOXA11* and *HOXD10*, which are putatively imprinted genes fundamental for proper fetal development, were included as well. A complete list of genes, along with the primer sets used for the gene expression analysis, is provided in **Table I** (available at www.jpeds.com).

Quantitative, real-time reverse-transcription polymerase chain reaction (qRT-PCR) was performed at the Mount Sinai School of Medicine Real-Time Polymerase Chain Reaction facility, which uses automated fluid handling systems for running 384-well plates. Expression values were cascade-normalized against the 3 housekeeping genes *RPS11*, *ACTB*, and *TUBB*. The copy number for each imprinted gene for each sample was then calculated as

$$\mathbf{CN = k\ x\ eff}^{(Ct_{cn} - Ct_{gt})},$$

where CN is the copy number, k is the equation constant, *eff* is the average replication efficiency, and Ct_{cn} and Ct_{gt} are the Ct values for each specific sample for the 3 housekeeping genes cascade-normalized and each gene-tested, respectively.

Statistical Analyses

The correlation of expression between imprinted genes was evaluated using the Spearman rank correlation. Because of the high degree of correlation in expression among imprinted genes, and to allow for subsequent inference, subjects were clustered based on gene expression data using a recursively partitioned mixture modeling[25] fit with a mixture of Gaussian distributions on log-transformed gene expression data. This modeling strategy allows clustering of samples into groups based on similarity in the gene expression profiles, and for association of membership in those groups with variables of interest. The robustness of the recursively partitioned mixture modeling was assessed by comparison with a principle components analysis, using singular value decomposition, which revealed a similar class structure based on gene expression pattern. Class membership was obtained from the mixture model, and subsequent bivariate associations were tested via permutation test with 10 000 permutations each. The