Eur. J. Oncol., vol. 14, n. 3, pp. 161-169, 2009

Original studies/Studi originali
General topics/Argomenti generali

Evaluation of genomic imprinting employing the analysis of Loss Of Imprinting (LOI) at the RNA level: preliminary results

Valutazione dell'imprinting genico attraverso l'analisi della Perdita dell'Imprinting (Loss Of Imprinting – LOI) a livello dell'RNA: risultati preliminari

Luca Lambertini*, Andreas I. Diplas*, James Wetmur**, ***, Men-Jean Lee****, Jia Chen*, *****, ******
* Department of Preventive Medicine, Mount Sinai School of Medicine, New York, USA
** Department of Microbiology, Mount Sinai School of Medicine, New York, USA
*** Department of Genetics and Genomic Sciences, Mount Sinai School of Medicine, New York, USA
**** Department of Obstetrics, Gynecology, and Reproductive Science, Mount Sinai School of Medicine, New York, USA
***** Department of Pediatrics, Mount Sinai School of Medicine, New York, USA
****** Department of Oncological Sciences, Mount Sinai School of Medicine, New York, USA

Summary

Aim. **This manuscript summarizes the first results obtained on the investigation of the relationship between genomic imprinting dysregulation and intra-uterine growth restriction (IUGR). Genomic imprinting refers to the silencing of one parental allele in the zygotes depending upon the parent of origin; this silencing occurs *via* epigenetic processes such as DNA methylation and histone modification resulting in monoallelic expression of the affected genes in the offspring. Genomic imprinting plays a critical role in placental and fetal development. Emerging evidence implicates Loss Of Imprinting (LOI) in reproductive and developmental diseases, neurological disorders and cancer. IUGR accounts for**

Riassunto

Finalità. **Questo manoscritto riassume i risultati preliminari dell'analisi sperimentale sulla relazione fra imprinting genico e restrizione intrauterina della crescita (IUGR). L'imprinting genico si riferisce al silenziamento di uno degli alleli parentali nello zigote in relazione al genitore di origine; questo fenomeno è regolato da processi epigenetici quali la metilazione del DNA e la modificazione degli istoni e risulta nella espressione monoallelica di questi geni nella prole. L'imprinting genico gioca un ruolo cruciale nello sviluppo del feto e della placenta. Dati recenti hanno dimostrato la perdita dell'imprinting genico (LOI) in patologie della riproduzione e dello sviluppo fetale, disordini neurologici e**

Received/Pervenuto 17.6.2009 - Accepted/Accettato 3.8.2009
Address/Indirizzo: Dr. Luca Lambertini, Mount Sinai School of Medicine, Department of Preventive Medicine, 17 East 102nd Street, Box 1057, New York, NY 10029, USA - E-mail: luca.lambertini@mssm.edu

Questo studio è stato supportato dai finanziamenti NO1 AI50028, U19 AI06231 e P50 ES09584 dei National Institutes of Health, R827039 del U.S. Environmental Protection Administration e dal Venture Capital Research Funding Program del Mount Sinai Children's Environmental Health Center e Mount Sinai Child Health and Development Institute

~10% of all pregnancies in the US, it is associated with major postnatal morbidity and mortality in the newborn period and it has been associated with abnormalities in fetal growth that have been linked with developmental origins of many adult disorders, such as obesity and breast cancer. Some developmental syndromes have been moreover associated with known imprinted genes often seen in IUGR pregnancies that may therefore themself be associated with genomic imprinting dysregulation. *Materials and Methods*. We developed a functional and highly sensitive assay at the RNA level for measuring LOI. We also analyzed imprinted gene expression in normal and IUGR placentas by real-time PCR. *Results*. We showed that LOI is a common phenomenon in placenta that preferentially affects specific imprinted genes in IUGR compared to control placentas; however, this does not correlate with changes in expression of genes with a perturbed imprinting profile. *Conclusions*. Genomic imprinting dysregulation plays a role in the etiology of IUGR. Eur. J. Oncol., 14 (3), 161-169, 2009

Key words: **Loss Of Imprinting (LOI), Intra-Uterine Growth Restriction (IUGR), human placenta, gene expression, quantitative Allele-Specific PCR (qASPCR)**

cancro. Negli Stati Uniti circa il 10% delle gravidanze sono IUGR; questa sindrome è responsabile della maggior parte della morbidità e mortalità postanatale ed è stata associata ad anomalie della crescita fetale a loro volta connesse a disordini in età adulta, come obesità e carcinoma mammario. Alcune sindromi legate allo sviluppo sono state inoltre associate a geni con imprinting genico in gravidanze IUGR, suggerendo una correlazione tra IUGR e deregolazioni dell'imprinting genico. *Materiali e Metodi.* Un saggio funzionale altamente sensibile per la misurazione del LOI è stato appositamente sviluppato a livello del RNA. Il profilo di espressione dei geni con imprinting genico è stato inoltre analizzato in placente normali e con IUGR attraverso real-time PCR. *Risultati.* Questo studio ha dimostrato che il LOI è un fenomeno comune nella placenta che, in gravidanze IUGR, colpisce specificamente alcuni geni; tuttavia questo fenomeno non è direttamente correlato a specifiche variazioni dell'espressione genica negli stessi campioni. *Conclusioni.* Modificazioni dell'imprinting genico contribuiscono all'eziologia dell'IUGR. Eur. J. Oncol., 14 (3), 161-169, 2009

Parole chiave: **perdita dell'impriting genico, restrizione intrauterine della crescita, placenta umana, espressione genica, PCR quantitativa allele-specifica**

Introduction

Genomic imprinting refers to the silencing of one parental allele in the zygotes *via* DNA methylation, histone modification and RNA silencing, leading to monoallelic expression of these genes in the offspring. This process results in a reversible parent-of-origin specific marking of the genome that ultimately produces a functional difference between the genetic information contributed by each parent (1, 2).

It has been unequivocally demonstrated from a mouse model that maternal and paternal contributions to the zygote are not equivalent, and importantly, there is an absolute requirement for a genetic contribution from both sexes in order for development to proceed normally (3). The prevailing hypothesis to explain such conservation is the "parental conflict hypothesis" (4, 5) which proposes that the purpose of the imprinting is to assure appropriate allocation of maternal resources to each conceptus. The model postulates that paternally expressed imprinted genes promote growth of the offspring, either *in utero* or in the perinatal period, while imprinted genes with the opposite direction of imprinting have the opposite effect (6).

Compared to other mammalian genomes, like that of the mouse, the human genome is imprinted to a much lesser extent, possibly due to lack of competition for maternal resources because human pregnancies are typically singletons (7). In humans, the estimated number of imprinted genes is around 1% of the genome, or ~200 genes (8, 9). While genomic imprinting is tissue specific, most imprinted genes are expressed in extraembryonic tissues, such as the placenta (10).

Imprinted genes cluster together to share common regulatory elements that rely on the correct and timely placement of epigenetic signals across specific genomic areas. Such signals are represented by: DNA methylation, histone modification and RNA silencing (11, 12).

The genomic areas that overlook the imprinting status of a given cluster are known as imprinting control regions (ICRs). ICRs contain areas showing an allele-specific methylation profile embedded in consensus sequences that often display signaling repeats for the binding of regulatory proteins. Consistently, the reactivation of the silent allele, known as Loss Of Imprinting (LOI), has mostly been assessed by measuring loss of DNA methylation (13-15), however there is evidence that maintenance of imprinting not only depends on methylation but also on histone modification (12, 16). For example, there is imprinting maintenance in the absence of DNA methylation for several genes in the mouse *Kcnq1* domain (17) suggesting that certain imprinted genes may be exclusively controlled by histone modification. However the two phenomena are more often thought to work together as demonstrated by a recent study on mice sperm cells that showed allele-specific patterns of histone modification overlapping the allele-specific DNA methylation profile in some ICRs of imprinted genes (18). Much less is instead known about the RNA silencing mode of action. Non-coding RNAs (ncRNAs) like *Air* and *Kcnq1ot1* have been described as essential for the maintenance of the imprinting status of specific ICRs, while other studies reported an unusually high ncRNA transcription rate at imprinted *loci* reportedly associated with allele-specific expression which is being studied for its role in the imprinting signals' setting (19).

DNA methylation and histone modification signals are established during fetal development with a carefully planned schedule of events. The DNA methylation profile undergoes a reprogramming phase that takes place during the early phases of embryonic development. Epigenetic reprogramming acts bimodally during preimplantation affecting differentially the embryo and the primordial phase of gametogenesis (20). During preimplantation, the embryo undergoes a genome-wide demethylation. Both parental genomes are remethylated around the time of implantation. Concomitantly methylated imprinted genes are protected from the de-/re-methylation wave. Later in fetal development a subset of cells are selected to become primordial germ cells (PGCs). These cells undergo to a second de-/re-methylation wave, this time targeted to imprinted domains, erasing the parental contribution in PGCs. Imprinting marks are then replaced with new signals consistent with the sex of the developing embryo.

Histone programming relies instead on an asymmetric chromatin remodeling that is required for the completion of fertilization. At fertilization the oocyte chromatin is organized in complexes with histones, while sperm chromatin carries protamines resulting in an asymmetric distribution of chromatin in the fertilized oocyte. Exchange of nucleoprotamine for nucleohistone occurs during the first hour after fertilization concurrently with the beginning of the first DNA de-/re-methylation wave involving specific histone methyltransferases that later also contribute to the placement of imprinting signals (21).

This sequence of events highlights the epigenetic differences between maternal and paternal chromosomes arising during the zygote programming that may well remain throughout the preimplantation period of development and influence the proper genomic imprinting programming. This unique setting clearly defines a window of vulnerability particularly during the early phases of the fetal development that extends into the later phases, exerting a trans-generational effect by perturbing the PCGs.

Drawing from experiments in mice, a first classification has been attempted that groups imprinted genes into three categories based on their activity in placental/embryonic development and fetal programming (22): 1) imprinted genes exclusively

expressed in placenta that act in the allocation of maternal resources to the fetus; 2) imprinted genes that act on metabolism in the early postnatal period; and 3) an imprinted gene network expressed both in placenta and embryo that act prenatally setting the metabolism of developing metabolic organs such as the pancreas, muscle, fat cells and the hypothalamus.

IUGR is a clinical term used to describe a fetus that has not reached its growth potential. Infants, who are the smallest ones are at substantially increased risk of neonatal and infant mortality (23-25). The implanted embryo, called the blastocyst, is comprised of an inner cell mass that develops into the fetus, and an outer cell layer, the trophoblast, that develops into the placenta. Placentation, occurring between gestation weeks 6 and 18, refers to invasion of the maternal circulatory system by the outer trophoblast cell layer of the implanted blastocyst. Abnormal or insufficient placentation has been implicated in the pathogenesis of IUGR (26, 27).

IUGR accounts for ~10% of all pregnancies in the US. Birthweights in the range of 1,500 grams have neonatal mortality risks 50-100 times greater than infants of optimal birthweight. The greatest risk of perinatal morbidity and mortality occurs among growth restricted fetuses/infants with weights below the 3rd percentile for gestational age (28, 29). Additionally IUGR has been found to predispose the infant to the development of hypertension, hyperlipidemia, coronary heart disease, and diabetes mellitus in adulthood (Barker Hypothesis) (30, 31).

Well known risk factors for IUGR include external factors affecting the intrauterine environment such as maternal nutrition (28, 32), abnormal placental implantation into the uterus (27), poor oxygenation of the uterus and placenta, or exposure to exogenous chemicals (33). This may result in classic vascular lesions of the placenta leading to uteroplacental insufficiency and IUGR. These changes in the placenta are likely mediated by epigenetic factors (*e.g.* LOI), rather than genetic.

Several exogenous chemicals with estrogenic power, highly prevalent in the US population (34), can act *in utero* or interfere with hormone synthesis as shown in animals (35). Detected both in the amniotic fluid and in umbilical cord blood (36-38), they have been shown to cause reproductive effects up to the 4th generation in experimental animals (35). This extraordinary phenotype penetrance strongly suggests that the likely mechanism of action involves epigenetic rather than genetic phenomena. The ability of an external agent to induce transgenerational effects requires a stable chromosomal alteration or an epigenetic phenomenon in the germline including imprinting (39).

The rôle of genomic imprinting in IUGR however, has been implicated but not systematically studied. In a recent study by Guo et al. (40), perturbations in *H19* expression and methylation in placenta were associated with IUGR; there was a significant correlation between the relative expression level of *IGF2* in placenta and birthweight percentile (but not gestational age) with a significant down-regulation of *IGF2* in IUGR placentas in comparison to controls. These data suggest that down-regulation of the imprinted *IGF2* gene in placenta likely plays a role in the development of IUGR.

Genomic imprinting has been more directly linked to many human diseases with parent-of-origin effects, which includes Angelman, Prader-Willi and Rett syndromes, and expression phenotypes of such diseases as Huntington's disease and cystic fibrosis (41-43). Recently, there has been compelling evidence also linking genomic imprinting to autism spectrum disorder (44).

This background of scientific knowledge prompted us to start a research line intended to study the LOI profiles in IUGR compared to appropriate-for-gestational age (AGA) and otherwise clinically normal placentas. Here we summarize the work accomplished in elaborating a highly sensitive methodology for measuring LOI that takes into account all epigenetic mechanisms by working at the RNA level, the final product of the fine tuning operated by genomic imprinting. We also report the first results obtained while exploring the significance of LOI as method for controlling the gene expression of the imprinted genes expressed in placenta.

Materials and Methods

Details on materials and methods have been previously published (45, 46). Here following be briefly summarize the approach.

Placenta collection

Normal AGA and IUGR placentas were collected from the Labor and Delivery Unit of the Department of Obstetrics, Gynecology and Reproductive Sciences at the Mount Sinai Medical Center. AGA placentas were retrieved from pregnancies delivered at ≥37 weeks with fetal weight estimated by ultrasound >10th percentile with no IUGR evidences. Severe IUGR placentas were collected with fetal weight that <5th percentile for gestational age, with either absent end-diastolic flow or reversed end-diastolic flow of the umbilical artery.

Placentas were sterilely collected immediately after being cleared by the post-partum morphological analysis. The organ was biopsied and biopsies free of maternal decidua were washed with PBS dried with sterile gauze, placed in tubes, frozen in liquid nitrogen and stored at -80°C. A control experiment was run to determine possible differences in LOI and/or gene expression between different areas of the sampled placentas. To carry out this experiment, smaller biopsies were retrieved, 1 for each placenta quadrant, and consistently treated. The study was approved by the Institutional Review Board of the Mount Sinai School of Medicine (New York, NY).

Nucleic acid extraction and cDNA synthesis

DNA and RNA were extracted from placental tissue for the planned analyses. In order to carry the gene expression and LOI tests out, total RNA extracted was converted into cDNA.

LOI measurements

LOI profiling was run on a set of 14 imprinted genes of which 7 were paternally expressed/maternally imprinted (*IGF2*, *MEST*, *PEG3*, *PEG10*, *PLAGL1*, *DLK1*, *SNRPN*), 5 maternally expressed/paternally imprinted (*MEG3*, *H19*, *TP73*, *PHLDA2*, *SLC22A18*) and 2 of yet unknown parent of origin (*EPS15*, *CD44*).

Quantitative RT-PCR

Gene expression was conducted by designing gene specific primers that were used for amplifying cDNA samples. The assays were run in a quantitative PCR (qPCR) setting using a LightCycler480™ (Roche). All assays were run in triplicate under previously optimized PCR conditions.

Statistical analysis

Statistics were conducted using the software SPSS 16.0 (SPSS Inc., Chicago, IL). We use the t-test to analyze gene expression differences between the AGA and IUGR placentas. We also applied the non-parametric Wilcoxon Signed-Rank test to assess differences in gene expression levels between different placenta quadrants.

Results

Developing a sensitive and functional assay for measuring LOI

We have previously shown that quantitative Allele-Specific PCR (qASPCR) was able to accurately determine allele frequencies in pooled DNA samples (47). Based on similar technology, we developed a sensitive and functional assay for measuring allelic imbalance of imprinted genes, i.e. LOI, at the RNA level (45). This RNA-based assay can achieve the sensitivity of 1% LOI. Briefly, a common readout polymorphism residing in the transcript of the studied gene is selected. After RT-PCR to amplify the sequence containing the readout polymorphism, qASPCR is applied to measure the relative abundance of the two heterozygous alleles that allows quantification of LOI. In individuals heterozygous for this readout polymorphism, LOI is a measurement of the presence of the supposedly silenced allele. We have set up LOI assays for 14 suitable imprinted genes (i.e. presence of a readout SNP with minimun allele frequency >20%) expressed in placentas; 7 are paternally expressed, 5 maternally expressed, and 2 of yet unknown parent-of-origin (See Materials and Methods – LOI measurement).

LOI in human placentas

We have applied our newly developed LOI method mentioned above to 36 placenta samples that

we have collected from various sources. Among them, 14 were from uncomplicated AGA pregnancies and 22 from IUGR (<5th percentile). Within the set of 14 imprinted genes, there were 149 heterozygosities in the 36 placentas; 40 of them (~27%) showed LOI > 3%, indicating that LOI is a common phenomenon in human placenta. The cutoff level of 3% LOI (compared the sensitivity of 1% of the assay) was chosen because it is comparable to the latest studies indicating that common environmental changes (such as dietary changes) resulted in variations in the epigenome at the level of 4–5% (48).

When we examine the LOI profile by the clinical phenotype of the placentas, a distinct pattern of LOI emerges. The 14 tested genes can be classified into 3 groups. The group 1 contains genes *CD44*, *EPS15*, *MEG3*, *PHLDA2* and *PEG10*, which are rarely perturbed in placentas regardless of the phenotype; group 2 genes contain *IGF2*, *TP73*, *MEST*, *PEG3* and *SLC22A18*, which showed widespread LOI across phenotypes; and lastly the group 3 genes, i.e. *PLAGL1*, *DLK1*, *H19* and *SNRPN*, uniquely demonstrated LOI only in IUGR placentas. A similar LOI profile on group 3 genes was found in a limited number of preeclampsia samples analyzed (data not shown) in agreement with the notion of shared etiology for IUGR and preeclampsia. We acknowledge that these data are preliminary and the small samples size prevents meaningful statistical analyses. Nevertheless, a specific LOI pattern in IUGR placentas is clearly distinguishable from the AGA placentas.

One additional important consideration for a quantitative LOI assay is the extent of maternal contamination of placental tissue which is of fetal origin. This consideration has been largely neglected in previous studies of LOI or RNA-based studies. The major source of maternal contamination comes from maternal lymphocytes since the placenta is perfused with maternal circulation. We have conducted pilot studies to examine the contribution of maternal DNA/RNA in fetal DNA/RNA that was isolated from placentas. For a given tested gene, we chose a case that was homozygous in the placental DNA but heterozygous in the corresponding maternal blood. Maternal contamination can then be evaluated by the presence of the heterozygous allele. We tested three genes, *GNAS1*, *TXK* and *OSBPL5,* each on two different placentas. We found that the level of maternal contamination was <0.1%.

Differential expression of imprinted genes in normal and IUGR placentas

We recently published our results of a pilot study directed to examine the expression profile of imprinted genes in AGA and IUGR placentas (46). We selected 74 genes from those experimentally shown to be imprinted and verified their placenta-specific gene expression in the Unigene/NCBI database. We tested the expression levels of these genes using quantitative RT-PCR in 10 AGA and 7 clinically confirmed IUGR (<5th percentile) placentas chosen among those providing with enough tissue to run the test. The amount of starting template was normalized against *18S rRNA*; the housekeeping gene *ACTB* was used as reference to compare expression levels of imprinted genes. For each gene, we concomitantly tested AGA and IUGR placenta samples in the same RT-PCR plate to minimize intra-plate variation.

Of the 74 genes tested, 22 were not expressed in placental tissues. Comparative expression levels between AGA and IUGR placentas for the remaining 52 genes allowed for several key observations from this experiment: 1) all imprinted genes are expressed at modest or low level compared to the housekeeping gene *ACTB*; 2) for the 52 imprinted genes that are expressed in placentas cycle threshold (Ct) values across these genes ranged between 21 and 34 cycles, corresponding to ~8,000 fold difference in expression levels; 3) inter-person variations of imprinted genes are small, with mean standard deviation <20%, suggesting these genes are tightly regulated; and 4) there are 9 genes that have significantly different expression levels between AGA and IUGR placentas; 5 of them are up-regulated (*PHLDA2*, *ILK2*, *NNAT*, *CCDC86* and *PEG10*) and 4 are down-regulated (*PLAGL1*, *DHCR24*, *ZNF331* and *CDKAL1*). In summary, our results indicate that ~70% of imprinted genes are expressed in placentas; imprinted genes are tightly regulated; and 17% of placentally imprinted genes are differentially expressed in normal *vs* IUGR placentas.

It is important to notice that in running this analysis we concomitantly investigated the potential

bias brought about by changes in gene expression levels with the sampling area of the placenta (49). We found that there were no significant expression differences with respect to the placenta sampling site.

Discussion

Herein we summarized our work in exploring the role that LOI plays in the development of IUGR. We started out by developing a new comprehensive, highly sensitive and functional assay for measuring LOI at the RNA level using RNA expression of each allele of an imprinted gene set in placental tissue. This method is able to account for all epigenetic mechanisms that act on regulating the gene expression of the imprinted genes tested. The assay represents an innovative approach if compared to the current methods for measuring LOI that only rely on the determination of DNA methylation patterns of paternal and maternal alleles (35). DNA methylation in fact represents only one of the processes involved in imprinting regulation and it is not necessarily correlating directly with the overall imprinting status of a specific gene (7, 50).

Limitations of the use of readout polymorphisms in heterozygotes are twofold: 1) not all genes contain suitable readout polymorphisms; 2) we can determine LOI for a given imprinted gene at best 50% of the time for a given sample. However the advantage of using functional polymorphisms outweighs the disadvantages because: 1) the inclusion of genes measurable by our functional assay and not by methylation analysis can balance out the genes lost to analysis by the absence of readout polymorphisms; 2) we can use the LOI data to develop a functional genomic markers.

Secondly we measured genomic imprinting in AGA and IUGR samples and, for the first time, we were able to describe a possible baseline LOI profile and its modification in growth restricted placentas. The absence of detectable LOI in genes of group 1 (*CD44*, *EPS15*, *MEG3*, *PHLDA2*, *PEG10*) could be interpreted as a highly conserved signal: the maintenance of the allele-specific expression status for these genes could be somehow critical. Group 2 genes (*IGF2*, *TP73*, *MEST*, *PEG3*, *SLC22A18*) could instead represent genes that, as the human genome gets less imprinted, are under a lower selective pressure for maintaining their genomic imprinting status unaltered. Group 3 genes (*PLAGL1*, *DLK1*, *H19*, *SNRPN*) are possibly group 1 genes that, if perturbed on their genomic imprinting status, can contribute to the IUGR etiology.

This interpretation of the LOI profile obtained is however very preliminary because of the limited number of samples available that does not allow for the data reaching a significant threshold. Also, in constructing the LOI profile presented, we treated measurements as independent while some samples were more informative than others because they had more measurable heterozygous polymorphisms than others. This condition could introduce a bias related to the specific characteristics of each sample analyzed.

Finally we tested the possible correlation between LOI and gene expression. Based on the "parental conflict hypothesis", paternally expressed (maternally imprinted) genes should show up as down-regulated in IUGR. Of the four down-regulated genes detected, *PLAGL1*, *DHCR24*, *ZNF331* and *CDKAL1,* two (*PLAGL1* and *ZNF331*) had known imprinting origin and both are paternal expressed genes. However, among the up-regulated genes, where only maternally expressed (paternally imprinted) genes were expected, two (*NNAT* and *PEG10*) are paternally expressed. These results indicate that not all imprinted genes fit into the "parental conflict hypothesis".

Nevertheless, our results are largely consistent with published results. We compared our results with that of McMinn et al. (51) in which 27 imprinted genes were analyzed in IUGR and non-IUGR placentas. Both studies observed a significant up-regulation of the maternally expressed *PHLDA2* in IUGR placentas and down-regulation of paternally expressed *PLAGL1*. Both studies also showed indication of down-regulation of the paternally expressed *IGF2*. In addition, 17% (9 out of 52) imprinted genes in placenta appear to be differentially expressed in our pilot study, which is similar to 22% (6 out 27) reported by McMinn et al. (51).

It is noticeable that LOI and imprinted gene expression perturbation are largely uncorrelated. For

example, while the frequent LOI of *SNRPN* in IUGR placentas corresponds to an increased expression of the gene, *PHLDA2* showed significant up-regulation in IUGR placentas without significant LOI of the gene. In the meantime, the expression of *IGF2* was up-regulated in IUGR placentas but the LOI was observed in normal placentas. These results suggest that the role of LOI in gene expression regulation could be mediated by other regulatory mechanisms not yet discovered.

Our analysis was mainly limited by the small sample size even though the mean standard deviation of 1.8 of the mean expression levels across 52 genes was really consistent allowing for the detection of differential expression level greater than 2.9% assuming 80% power and type I error of 5%.

These preliminary results strongly support the critical role of genomic imprinting in the etiology of IUGR.

References

1. Sasaky H, Ishino I, eds. Cytogenetic Genome Res - Complete Issue, 2006.
2. Tilghman SM. The sins of the fathers and mothers: genomic imprinting in mammalian development. Cell 1999; 96: 185-93.
3. McGrath J, Solter D. Completion of mouse embryogenesis requires both the maternal and paternal genomes. Cell 1984; 37: 179-83.
4. Haig D, Graham C. Genomic imprinting and the strange case of the insulin-like growth factor II receptor. Cell 1991; 64: 1045-6.
5. Moore T, Haig D. Genomic imprinting in mammalian development: a parental tug-of-war. Trends Genet 1991; 7: 45-9.
6. Tycko B, Morison IM. Physiological functions of imprinted genes. J Cell Physiol 2002; 192: 245-58.
7. Monk D, Arnaud P, Apostolidou S*, et al.* Limited evolutionary conservation of imprinting in the human placenta. Proc Natl Acad Sci USA 2006; 103: 6623-8.
8. Luedi PP, Dietrich FS, Weidman JR*, et al.* Computational and experimental identification of novel human imprinted genes. Genome Res 2007; 17: 1723-30.
9. Maynard ND, Chen J, Stuart RK*, et al.* Genome-wide mapping of allele-specific protein-DNA interactions in human cells. Nat Methods 2008; 5: 307-9.
10. Coan PM, Burton GJ, Ferguson-Smith AC. Imprinted genes in the placenta—a review. Placenta 2005; 26 Suppl A: S10-20.
11. Chotalia M, Smallwood SA, Ruf N*, et al.* Transcription is required for establishment of germline methylation marks at imprinted genes. Genes Dev 2009; 23: 105-17.
12. Lewis A, Reik W. How imprinting centres work. Cytogenet Genome Res 2006; 113: 81-9.
13. Eads CA, Danenberg KD, Kawakami K*, et al.* MethyLight: a high-throughput assay to measure DNA methylation. Nucleic Acids Res 2000; 28: E32.
14. Gonzalgo ML, Jones PA. Rapid quantitation of methylation differences at specific sites using methylation-sensitive single nucleotide primer extension (Ms-SNuPE). Nucleic Acids Res 1997; 25: 2529-31.
15. Gonzalgo ML, Jones PA. Quantitative methylation analysis using methylation-sensitive single-nucleotide primer extension (Ms-SNuPE). Methods 2002; 27: 128-33.
16. Wagschal A, Feil R. Genomic imprinting in the placenta. Cytogenet Genome Res 2006; 113: 90-8.
17. Lewis A, Mitsuya K, Umlauf D*, et al.* Imprinting on distal chromosome 7 in the placenta involves repressive histone methylation independent of DNA methylation. Nat Genet 2004; 36: 1291-5.
18. Dindot SV, Person R, Strivens M*, et al.* Epigenetic profiling at mouse imprinted gene clusters reveals novel epigenetic and genetic features at differentially methylated regions. Genome Res 2009; 19(8): 1374-83.
19. Royo H, Cavaille J. Non-coding RNAs in imprinted gene clusters. Biol Cell 2008; 100: 149-66.
20. Reik W, Dean W, Walter J. Epigenetic reprogramming in mammalian development. Science 2001; 293: 1089-93.
21. Santos F, Dean W. Epigenetic reprogramming during early development in mammals. Reproduction 2004; 127: 643-51.
22. Charalambous M, da Rocha ST, Ferguson-Smith AC. Genomic imprinting, growth control and the allocation of nutritional resources: consequences for postnatal life. Curr Opin Endocrinol Diabetes Obes 2007; 14: 3-12.
23. Alexander GR, Kogan M, Bader D*, et al.* US birth weight/gestational age-specific neonatal mortality: 1995-1997 rates for whites, hispanics, and blacks. Pediatrics 2003; 111: e61-6.
24. Basso O, Wilcox AJ, Weinberg CR. Birth weight and mortality: causality or confounding? Am J Epidemiol 2006; 164: 303-11.
25. Wilcox AJ, Skjaerven R. Birth weight and perinatal mortality: the effect of gestational age. Am J Public Health 1992; 82: 378-82.
26. Chaddha V, Viero S, Huppertz B*, et al.* Developmental biology of the placenta and the origins of placental insufficiency. Semin Fetal Neonatal Med 2004; 9: 357-69.
27. Smith GC. First trimester origins of fetal growth impairment. Semin Perinatol 2004; 28: 41-50.
28. Godfrey KM, Barker DJ. Fetal nutrition and adult disease. Am J Clin Nutr 2000; 71: S1344-52.

29. McIntire DD, Bloom SL, Casey BM*, et al.* Birth weight in relation to morbidity and mortality among newborn infants. N Engl J Med 1999; 340: 1234-8.
30. Resnik R. Intrauterine growth restriction. Obstet Gynecol 2002; 99: 490-6.
31. Svensson AC, Pawitan Y, Cnattingius S*, et al.* Familial aggregation of small-for-gestational-age births: the importance of fetal genetic effects. Am J Obstet Gynecol 2006; 194: 475-9.
32. Savitz DA, Kaufman JS, Dole N*, et al.* Poverty, education, race, and pregnancy outcome. Ethn Dis 2004; 14: 322-9.
33. Wilcox AJ. Birth weight and perinatal mortality: the effect of maternal smoking. Am J Epidemiol 1993; 137: 1098-104.
34. Third National Report on Human Exposure to Environmental Chemicals. Department of Health and Human Services - Centers for Disease Control and Prevention - National Center for Environmental Health - Division of Laboratory Sciences - Atlanta, GA, USA - NCEH Pub. No. 05-0570, 2005.
35. Anway MD, Cupp AS, Uzumcu M*, et al.* Epigenetic transgenerational actions of endocrine disruptors and male fertility. Science 2005; 308: 1466-9.
36. Latini G, De Felice C, Presta G*, et al.* In utero exposure to di-(2-ethylhexyl)phthalate and duration of human pregnancy. Environ Health Perspect 2003; 111: 1783-5.
37. Schonfelder G, Wittfoht W, Hopp H*, et al.* Parent bisphenol A accumulation in the human maternal-fetal-placental unit. Environ Health Perspect 2002; 110: A703-7.
38. Silva MJ, Reidy JA, Herbert AR*, et al.* Detection of phthalate metabolites in human amniotic fluid. Bull Environ Contam Toxicol 2004; 72: 1226-31.
39. Rakyan V, Whitelaw E. Transgenerational epigenetic inheritance. Curr Biol 2003; 13: R6.
40. Guo L, Choufani S, Ferreira J*, et al.* Altered gene expression and methylation of the human chromosome 11 imprinted region in small for gestational age (SGA) placentae. Dev Biol 2008; 320: 79-91.
41. Falls JG, Pulford DJ, Wylie AA*, et al.* Genomic imprinting: implications for human disease. Am J Pathol 1999; 154: 635-47.
42. Walter J, Paulsen M. Imprinting and disease. Semin Cell Dev Biol 2003; 14: 101-10.
43. Horike S, Cai S, Miyano M*, et al.* Loss of silent-chromatin looping and impaired imprinting of DLX5 in Rett syndrome. Nat Genet 2005; 37: 31-40.
44. Schanen NC. Epigenetics of autism spectrum disorders. Hum Mol Genet 2006; 15 Spec No 2: R138-50.
45. Lambertini L, Diplas AI, Lee MJ*, et al.* A sensitive functional assay reveals frequent loss of genomic imprinting in human placenta. Epigenetics 2008; 3: 1-9.
46. Diplas AI, Lambertini L, Lee MJ*, et al.* Differential expression of imprinted genes in normal and IUGR human placentas. Epigenetics 2009; 4: 235-40.
47. Chen J, Germer S, Higuchi R*, et al.* Kinetic polymerase chain reaction on pooled DNA: a high-throughput, high-efficiency alternative in genetic epidemiological studies. Cancer Epidemiol Biomarkers Prev 2002; 11: 131-6.
48. Heijmans BT, Tobi EW, Stein AD*, et al.* Persistent epigenetic differences associated with prenatal exposure to famine in humans. Proc Natl Acad Sci USA 2008; 105: 17046-9.
49. Wyatt SM, Kraus FT, Roh CR, *et al*. The correlation between sampling site and gene expression in the term human placenta. Placenta 2005; 26: 372-9.
50. Byun HM, Wong HL, Birnstein EA, *et al*. Examination of IGF2 and H19 loss of imprinting in bladder cancer. Cancer Res 2007; 67: 10753-8.
51. McMinn J, Wei M, Schupf N, *et al*. Unbalanced placental expression of imprinted genes in human intrauterine growth restriction. Placenta 2006; 27: 540-9.