A complex association between DNA methylation and gene expression in human placenta at first and third trimesters

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Abstract

The human placenta is a maternal-fetal organ essential for normal fetal development and maternal health. During pregnancy, the placenta undergoes many structural and functional changes in response to fetal needs and environmental exposures. Previous studies have demonstrated widespread epigenetic and gene expression changes from early to late pregnancy. However, on the global level, how DNA methylation changes impact on gene expression in human placenta is not yet well understood. We performed DNA methylome analysis by reduced representation bisulfite sequencing (RRBS) and gene expression analysis by RNA-Seq for both first and third trimester human placenta tissues. From first to third trimester, 199 promoters (corresponding to 189 genes) and 2,297 gene bodies were differentially methylated, with a clear dominance of hypermethylation (96.8% and 93.0% for promoters and gene bodies, respectively). A total of 2,447 genes were differentially expressed, of which 77.2% were down-regulated. Gene ontology analysis using differentially expressed genes were enriched for cell cycle and immune response functions. The correlation between DNA methylation and gene expression was non-linear and complex, depending on the genomic context (promoter or gene body) and gene expression levels. A wide range of DNA methylation and gene expression changes were observed at different gestational ages. The non-linear association between DNA methylation and gene expression indicates that epigenetic regulation of placenta development is more complex than previously envisioned.

Introduction

The human placenta is a temporary maternal-fetal organ essential for normal fetal development. It serves several functions such as exchange of oxygen, nutrients and waste products between the mother and fetus. During pregnancy, the human placenta undergoes tremendous changes in size, morphology and structure to cope with the development of the fetus [1–3]. Not surprisingly, extensive molecular changes occur during placenta development. A number of studies have investigated gene expression profiles at different structural locations of the
placenta [4], and at different gestational ages of the placenta, with gene expression changes often correlating with functional changes at different gestational ages [5–8]. However, the molecular mechanisms underlying such drastic gene expression changes remain to be elucidated.

Epigenetics is considered as a fundamental mechanism regulating gene expression during development. The placenta has long been a favourite organ for the study of epigenetics, particularly in genomic imprinting [9–15]. Epigenetics is also widely considered as a mechanism for environmental factors to impact on development. For this reason, studying the epigenetics of the human placenta such as DNA methylation is particularly interesting as the placenta serves as the portal for the fetus to experience the external environment. Aberrant DNA methylation in placenta was found to be associated with pregnancy complications such as preclampsia [16,17], IUGR [18] and fetal abnormalities [19]. Recent work by others have investigated the DNA methylation changes of the placenta at different gestational ages [20], with main focus made on promoter regions.

In this study, we systematically analysed the transcriptomes and the DNA methylomes of human placenta samples derived from different gestational ages. Furthermore, we studied the dynamic correlations between gene expression and DNA methylation at different gestational ages and genomic locations.

Materials and methods

Ethics statement

Informed written consent was obtained under the ethics approval from the SingHealth CRIB Committee.

Clinical samples

Women with euploidy pregnancies who attended KK Women’s and Children’s Hospital, Singapore, were recruited.

Chorionic villus samples from subjects at the first or early second trimesters of pregnancy were collected by chronic villus sampling (CVS). Placenta villi samples (fetal side) were collected from third trimester of pregnancy after delivery. All tissue samples were washed with diethylpyrocarbonate (Sigma-Aldrich, USA) treated water. For DNA analysis, tissues were stored at -80˚C. For RNA analysis, tissues were incubated with RNA later (Life Technologies, USA) at 4˚C overnight, and then stored at -80˚C. Genomic DNA extraction from tissues was performed with QIAamp DNA Mini Kit (QIAGEN GmbH, Germany), according to manufacturer’s instructions. Total RNA was extracted from frozen tissues using TRIZOL protocol (Life Technologies).

Reduced representation bisulfite sequencing (RRBS)

Six DNA samples from first trimester of pregnancies reported previously [19] and five samples from third trimester of pregnancies carrying normal fetuses were chosen for DNA methylation analysis by RRBS (S1 Table), following previously described method [19,21]. Briefly, 1–5 μg of genomic DNA was used for each library preparation. Each DNA sample was sequentially digested by MspI and Taq’I (New England Biolabs). The digested product was purified with the QIAquick PCR Purification Kit (QIAGEN GmbH), and was end-repaired, 3’-end-adenylated, and adapter-ligated using reagents from ChIP-Seq Sample Preparation Kit (Illumina, USA), except that the methylation adapter oligonucleotides were used in the adapter-ligation step. Two different sizes of fragments (150–197 bp and 207–230 bp) were selected by gel...
electrophoresis, and were then bisulfite treated using the EZ DNA Methylation-Gold Kit (Zymo Research, USA). The converted DNA was amplified using HotStarTaq DNA Polymerase Kit (QIAGEN GmbH), with 1x reaction buffer, 1.5 mM of additional MgCl₂, 300 μM of dNTP mix, 500 nM each of PCR primer PE 1.0 and 2.0, and 2.5 U of HotStarTaq DNA polymerase. The thermocycling condition was 15 min at 94°C for heat activation, and 8–12 cycles of 20 sec at 94°C, 30 sec at 65°C and 30 sec at 72°C, followed by a 5 min final extension at 72°C. The amplified fragments were purified by gel electrophoresis and further quantified by the Agilent 2100 Bioanalyzer (Agilent Technologies, USA). Each DNA library was analyzed by two lanes of paired-end sequencing (2 × 36 bp) read on an Illumina Genome Analyzer IIx. The paired-end 36 bp reads were analyzed using in-house developed pipeline, as previously described [19, 21].

Differential DNA methylation analysis
A total of 1,707,910 autosomal CpGs with sequencing depth ≥10 and covered in at least 3 first and 3 third trimester samples were used in all subsequent analyses. Differential methylation analysis was mainly performed at regional levels. Core promoters are defined as 1 kb upstream and +500 bp downstream from a transcription start site while a genomic region is generated by merging nearby CpGs of less than 500 bp together.

A 2-sided Mann Whitney U test was first performed at single CpG level and p values were adjusted within regions using Benjamini Hochberg. A promoter was considered significantly differentially methylated if 1) methylation difference between average first trimester and third trimester samples was at least 10% and 2) contained at least 2 CpGs with FDR corrected p < 0.05. For gene bodies, in addition to the above two criteria, we require that all significantly differential methylated fragments mapped to the gene body be regulated in the same direction (either all hypermethylated or hypomethylated).

All statistical analyses were performed using R package.

mRNA sequencing (mRNA-SEQ)
Five RNA samples from first and second trimesters of pregnancies reported previously [19] and 4 samples from third trimester of pregnancies carrying normal fetuses were chosen for mRNA-seq analysis (S1 Table). Briefly, 2–5 μg of total RNA was used for each library preparation. Each RNA sample was treated with DNase I (Life Technologies). Messenger RNA purification and fragmentation, complementary DNA synthesis, end-repair, 3’-end-adenylation, and adapter-ligation were performed using Illumina’s mRNA-Seq Sample Preparation Kit. Manufacturer’s instructions were followed, except that the SuperScript III First-Strand Synthesis SuperMix (Life Technologies) was used for first strand cDNA synthesis. Adapter-ligated cDNA fragments were size-selected using a 3% agarose gel (200 ± 25 bp). The DNA samples were then amplified by PCR for 15–16 cycles. The PCR products were purified using 3% agarose gels and further quantified by the Agilent 2100 Bioanalyzer (Agilent Technologies). Each library was analyzed by one lane of either 36 bp single read or 2 × 36 bp paired-end sequencing on an Illumina Genome Analyzer IIx.

Differential gene expression analysis
High quality reads from RNA-seq were processed using Illumina RNA-seq pipeline, CASAVA software version 1.7, following the steps described previously [19]. The normalized gene expression level for a gene was represented by reads per kilobase per million mapped reads.
(RPKM) value, using the formula below:

\[
\text{RPKM} = \frac{\text{Number of aligned read for a gene of interest}}{\text{Number of total aligned reads} \times \text{Transcript length for the gene (kb)}} \times 10^6
\]

Average RPKM values for each gene in each sample group (first and third trimester) were calculated. When the average RPKM for a gene is less than 0.5, the value was adjusted to 0.5. A gene was considered to be differentially expressed between first and third samples when: 1) two-sided Mann Whitney U test p-value less than 0.05; and 2) the ratio of (Average third trimester/Average first trimester) ≥ 2 or ≤ 0.5. R package was used for all statistical analyses.

**Effect of promoter methylation on gene expression determined by dual luciferase assays**

Selected gene promoters were PCR amplified with target-specific primers (S2 Table) and cloned into pGL3-Basic Luciferase Reporter Vector (Promega) with appropriate restriction enzyme digestions. Five microgram of each promoter construct was treated with 32 units of M.SssI methyltransferase (New England Biolabs) for 1.5 hours at 37°C and purified by the QIAquick PCR Purification Kit (QIAGEN GmbH). HEK293FT cells (Life Technologies) were cultured in Dulbecco’s Modified Eagle Medium (Life Technologies) supplemented with 10% FBS. Twenty-four hours before transfection, 1 X 10^5 HEK293FT cells were plated into 24-well plates with 500 μl medium in each well. DNA mixture used for transfection contained 450 ng of individual promoter constructs with or without M.SssI treatment or pGL3-Basic empty vector control, and 50 ng Renilla plasmid DNA. The DNA mixture was transfected into each well of a 24-well plate containing pre-plated HEK293FT cells in duplicates, using Jetprime transfection reagent (Polyplus-transfection SA), and media were changed 4 hours after addition of transfection reagents. Cells were harvested 48 hours post-transfection, and lysed in 100 μl passive lysis buffer (Promega) for 15 minutes at room temperature. Twenty microliter of the lysate was loaded and subjected to luciferase and renilla activity measurements on a luminometer (Promega, Glomax multi-detection system). Firefly luciferase activity was divided by Renilla luciferase activity to normalize for transfection efficiency. Ratio of luciferase:Renilla activity of experimental wells was normalized to empty vector control by subtracting value of luciferase:Renilla ratio of empty vector from luciferase:Renilla ratio of gene promoter construct. Each assay was repeated 3 times.

**Results**

Using an improved method of reduced representation bisulfite sequencing (RRBS) [19,21], we quantified DNA methylation of six first trimester and five third trimester placenta villi samples. Using a minimum sequencing depth of 10 as the cutoff, we obtained on average 1.8 million CpGs per sample (S1 Table and S1 Fig). To facilitate cross gestation comparison, we further removed CpG sites that were on the sex chromosomes or present in less than three samples in either the first or the third trimester group, resulting in 1.7 million CpG sites for further analysis. These CpGs represented about 3% of Hg19 autosomal CpGs, 78% of CGIs, 70.8% of core promoters (defined as -1kb upstream and +500bp downstream from a transcription start site) and 64.2% of gene bodies (defined as +1kb downstream from a transcription start site to the transcription termination site).

The distribution of individual CpG methylation levels were drastically different for CpG island (CGI) and non-CpG island (non-CGI) regions, as were shown in many earlier studies in placenta and other cell types [19,22–26] (Fig 1A and 1B). Similar distribution was observed for DNA fragments from merging neighbouring CpGs (Fig 1C and 1D).
In non-CGI regions, there was an apparent enrichment of highly methylated CpGs (95–100% methylation) in the third trimester samples (Fig 1B and 1D), indicative of global differences in DNA methylation at different gestation ages. Principal component analysis showed distinct separation of samples based on gestation age (Fig 2A). Additionally, there was a significant increase in mean CpG methylation in third trimester ($p = 0.028$, 2-sided Mann-Whitney U test) (Fig 2B). Furthermore, at both individual CpG and genomic fragment level, hypermethylation was consistently more frequent than hypomethylation (Fig 2C and 2D).

DNA methylation changes in promoters and gene bodies were further analysed as methylation of these regions have been demonstrated to be associated with gene expression. A total of 199 promoters (corresponding to 189 genes) were found to be significantly differentially methylated between the first and third trimester samples, with 193 (183 genes) (96.8%) showing higher methylation in the third trimester group. We also identified 2,297 gene bodies to be significantly differentially methylated, with 2,136 (93.0%) being hypermethylated in the third trimester samples.
We next carried out RNA-Seq analysis on five first/second trimester and four third trimester placenta villi samples (S1 Table). Genes with low expression levels (RPKM < 0.5) in both sample groups were filtered out, leaving 13,756 genes for differential gene expression analysis. A total of 2,447 genes were significantly differentially expressed between first/second and third trimester samples, of which, 1,889 (77.2%) were down-regulated and 588 (22.8%) were up-regulated in the third trimester samples (Fig 3A). Gene ontology analysis with multiple testing correction (p < 0.05) using a commercial database (MetaCore from GeneGo Inc.) was performed separately on the down-regulated (Fig 3B) and up-regulated gene lists (Fig 3C). The down-regulated genes were enriched mainly in the cell cycle pathways, with the top three pathways being “Cell cycle_The metaphase checkpoint”, “Cell cycle_Role of APC in cell cycle regulation” and “Apoptosis and survival_DNA-damage-induced apoptosis”. The up-regulated genes, on the other hand, were mainly related to immune response, with the top three...
Fig 3. Expression changes during placental development across gestational age. (A) Distribution of gene expression changes from first to third trimester for the genes that showed at least 2 fold changes. There
is a general gene repression from first to third trimester, indicated by higher green bars. (B) Metacore analysis on the repressed genes at third trimester. Genes were mostly involved in cell cycle. (C) Metacore analysis on the activated genes at third trimester. Genes were mostly involved in immune response and signalling pathways.

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pathways being "Immune response_Alternative complement pathway", "Immune response_Classical complement pathway" and "Immune response_Lectin induced complement pathway".

Eleven imprinted genes were also differentially expressed, of which ten (SLC22A18, PEG10, MEST, NAPIL5, MIMT1, PSIMCT-1, PEG3, LIN28B, DGCR6, PLAGL1) showed higher expression level for the first trimester samples and one (ANO1) showed higher expression in the third trimester (Table 1).

To explore the correlation between DNA methylation and gene expression, we first separated the genes into 50 equal size bins with increasing expression levels. The average DNA methylation level of the promoters in each bin was then calculated. Interestingly, for both CGI promoters (promoters overlapping with CGIs) and non-CGI promoters, a non-linear correlation between DNA methylation and gene expression was observed (Fig 4A and 4B). There was a clear anti-correlation for genes with lower expression levels (expression bins 1 to 20). However, for genes at higher expression levels (bins 21 and above), DNA methylation levels were largely similar regardless of gene expression level.

The correlation between DNA methylation at gene bodies and gene expression was also non-linear, with CGI gene bodies and non-CGI gene bodies behaving somewhat differently (Fig 4C and 4D). For CGI gene bodies, there was a positive correlation between DNA methylation and gene expression, for genes in bin 1 to bin 40, followed by a seemingly negative correlation for genes in bin 41 to 50. For non-CGI gene bodies, the positive correlation was only observed for genes in bin 1 to bin 25. For genes in bin 26 to about 40, DNA methylation levels were largely similar. Similarly, there was a seemingly negative correlation for genes in bin 41 to 50 (Fig 4A-4D). We did not observe a difference in exonic and intronic regions (S2 Fig). However, the DNA methylation levels of CGI exons were consistently higher than CGI introns, regardless of gene expression levels (S3A and S3B Fig).

Lastly, we asked how changes in DNA methylation from early gestation to late gestation in human placenta affects gene expression. A total of 25 genes (Table 2) showed both differential gene expression and differential DNA methylation in promoters when comparing the two trimesters.

Table 1. List of the imprinted genes showing expression changes between first and third trimester. The list was obtained from http://www.geneimprint.com. Expression was given relative to the third trimester samples.

| Gene         | Average first trimester (RPKM) | Average third trimester (RPKM) | log₂(3rd trimester/1st trimester) | Maternal/Paternal imprint |
|--------------|-------------------------------|-------------------------------|----------------------------------|---------------------------|
| SLC22A18     | 23.25                         | 3.90                          | -2.58                            | Maternal                  |
| PEG10        | 612.10                        | 188.34                        | -1.70                            | Paternal                  |
| MEST         | 291.60                        | 93.04                         | -1.65                            | Paternal                  |
| NAPIL5       | 4.26                          | 1.48                          | -1.53                            | Paternal                  |
| MIMT1        | 1.34                          | 0.50                          | -1.42                            | Paternal                  |
| PSIMCT-1     | 3.246                         | 1.35                          | -1.26                            | Paternal                  |
| PEG3         | 194.33                        | 81.51                         | -1.25                            | Paternal                  |
| LIN28B       | 23.84                         | 10.08                         | -1.24                            | Paternal                  |
| DGCR6        | 1.38                          | 0.64                          | -1.11                            | Unknown                   |
| PLAGL1       | 119.87                        | 56.75                         | -1.08                            | Paternal                  |
| ANO1         | 3.90                          | 8.07                          | 1.05                             | Maternal                  |

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gestation groups (Fig 5A). Of those, 19 genes (78%) showed anti-correlation between changes in gene expression and changes in promoter DNA methylation. There was a statistically significant difference between the positive and negative correlations (2-sided binomial test, p = 0.015). We validated the results in five genes (GJB5, LOC401109, BRDT, BIN2 and ANGPTL2) using the dual luciferase assay by cloning the respective promoters into the reporter vectors. Gene expression repression was observed in all five genes when the vectors were treated with the methyltransferase M.SssI (Fig 5B).

A total of 370 genes showed both differential gene expression and differential DNA methylation in gene bodies when comparing the two gestation groups (Fig 5C). Of those, 233 (63%) showed negative correlation between changes in gene expression and changes in gene body DNA methylation. Similar to the promoters, there was a statistically significant difference between the positive and negative correlations (2-sided binomial test, p = 6.85 x 10^{-7}). Given
that the correlation between gene body methylation and gene expression was positive for genes with relatively lower expression, but negative for genes with higher expression (Fig 4C and 4D), we hypothesized that the negatively correlated genes (233 genes) were of higher expression levels than the positively correlated genes. RNA-seq data confirmed our hypothesis (Fig 5D).

### Discussion

In this study, we applied next generation sequencing techniques to study the gene expression (by RNA-Seq) and DNA methylation profiles (by RRBS) of human placenta tissues derived from early and late gestations.

A total of 2,477 genes, including 11 imprinted genes, were found to be differentially expressed between the early and late gestational age placenta samples (Table 1).

Imprinted genes are essential to the normal growth and development of the mammalian fetus. Paternally and maternally expressed genes have been known to promote and repress fetal growth, respectively.[27]. Alterations in imprinted genes have been implicated in pregnancy complications such as intrauterine growth restriction (IUGR) [28,29], preeclampsia (PE) [30,31] and lethality [32,33]. Prospectively, even if the fetus survives to birth, these effects may be exhibited chronically and are linked to increased risks for hypertension [34], cardiovascular disease [35–37], abnormalities in neuro [38] and renal development [39,40].

Table 2. List of genes having both significant promoter DNA methylation difference and significant gene expression between first and third trimester placenta samples.

| Gene   | Promoter DNA methylation change (3T-1T) | Gene Expression (log$_{2}$(3T/1T)) |
|--------|----------------------------------------|-----------------------------------|
| ANGPTL2| 22.32                                  | -2.20                             |
| BIN2   | 18.44                                  | -3.19                             |
| BRDT   | 12.26                                  | -3.01                             |
| CCRL2  | 23.06                                  | -1.69                             |
| CYP2W1 | 15.00                                  | -3.72                             |
| FAM111A| 14.58                                  | -1.10                             |
| FBXO17 | 16.93                                  | -1.49                             |
| FGL2   | 26.25                                  | -1.49                             |
| GJB5   | -24.29                                 | 2.51                              |
| GREB1  | 17.90                                  | -1.06                             |
| HSPB2  | 10.37                                  | -1.28                             |
| ISLR   | 18.62                                  | -1.23                             |
| LOC100289019 | -14.03 | 1.06 |
| LOC401109 | -13.71 | 1.16 |
| MAL    | 10.43                                  | 2.36                              |
| PLEKHA6| 20.11                                  | 1.58                              |
| PTPRE  | 10.36                                  | -1.03                             |
| RAB42  | 18.59                                  | -1.51                             |
| RHOBTB2| 14.66                                  | -1.38                             |
| SEMA6D | 24.79                                  | -1.56                             |
| SNORD110| 13.68                                | -1.85                             |
| SNRPF  | 14.89                                  | -1.43                             |
| ST5    | 19.15                                  | 1.36                              |
| STRA6  | 16.80                                  | 1.71                              |
| SYNPO  | 23.10                                  | 1.27                              |

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Our RNA-seq results show that eleven imprinted genes were significantly differentially expressed, of which eight were paternally expressed, two were maternally expressed and the remaining one being random. All of the eight paternally expressed genes were down-regulated in the third/term trimester, which is coherent with the fully developed status of the fetus.

Gene ontology analysis revealed that down- and up-regulated genes during placenta development were associated with cell cycles and immune responses, respectively.

An overall DNA hypermethylation was observed in placental tissues at later gestational ages, consistent with earlier work using a much lower throughput method (Illumina Infinium HumanMethylation 27 Beadchip) [20]. The overall DNA hypermethylation changes were observed in both promoters and gene bodies, which also coincided with largely gene expression repression.
The association between DNA methylation and gene expression was found to be complex and dependent on at least two factors: genomic context (promoters or gene bodies) and gene expression level. Consistent with previous findings from placenta [20] and other biological systems [41–43], we found a negative correlation between gene expression level and promoter methylation level in both early and late pregnancies, as well as a positive correlation between gene expression level and gene body methylation level [24,44–46]. However, these correlations were no longer present in genes with higher expression levels (Fig 5). In contrast, for genes with the highest expression levels, there was a negative correlation between gene expression and DNA methylation (Fig 5).

There are a few limitations in our study. First, the placenta is a complex organ with different cell types at different structural locations with different expression profiles [4]. Secondly, placenta samples from additional time points during pregnancy may provide more dynamic and detailed changes in gene expression and DNA methylation. Thirdly, although majority of the genes with both significant differential methylation and gene expression were anti-correlated, the minority set which showed positive correlation might be regulated by other epigenetic mechanisms such as histone modification, transcriptional factor binding and nucleosome positioning.

Supporting information
S1 Fig. The histogram gives the average number of CpGs sites for 11 samples, with varying minimum sequencing depths. Error bars represents standard deviation for 11 samples.
(TIF)

S2 Fig. Correlation between DNA methylation and gene expression. Genes were grouped into 50 bins, in order of increasing gene expression. DNA methylation of the exons/introns fragments within each gene expression group was then averaged to obtain the relationship. Scatterplot of the DNA methylation of gene body exons and introns against the gene expression showed positive correlation. The DNA methylation in exons and introns did not exhibit clear differences.
(TIF)

S3 Fig. Correlation between DNA methylation and gene expression. Genes were grouped into 50 bins, in order of increasing gene expression. DNA methylation of the exons/introns fragments within each gene expression group was then averaged to obtain the relationship. The genes were divided into 4 groups where non-CGI introns and exons showed similar pattern while differences were observed between exons and introns in CGI gene bodies. First and third trimester samples showed similar patterns and trends.
(TIF)

S1 Table. Sample information.
(XLSX)

S2 Table. Information on dual luciferase assays on selected genes.
(XLSX)

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**References**

1. Jansson T, Powell TL (2007) Role of the placenta in fetal programming: underlying mechanisms and potential interventional approaches. Clin Sci (Lond) 113: 1–13.

2. Maccani MA, Marsit CJ (2009) Epigenetics in the placenta. Am J Reprod Immunol 62: 78–89. https://doi.org/10.1111/j.1600-0897.2009.00716.x PMID: 19614624

3. John R, Hemberger M (2012) A placenta for life. Reprod Biomed Online 25: 5–11. https://doi.org/10.1016/j.rbmo.2012.03.016 PMID: 22578825

4. Sood R, Zehnder JL, Druzin ML, Brown PO (2006) Gene expression patterns in human placenta. Proc Natl Acad Sci U S A 103: 5478–5483. https://doi.org/10.1073/pnas.0508035103 PMID: 16567644

5. Sitras V, Fenton C, Paulssen R, Vartun A, Acharya G (2012) Differences in gene expression between first and third trimester human placenta: a microarray study. PLoS One 7: e33294. https://doi.org/10.1371/journal.pone.0033294 PMID: 22442682

6. Mikheev AM, Nabekura T, Kaddoumi A, Bammlier TK, Govindarajan R, et al. (2008) Profiling gene expression in human placentae of different gestational ages: an OPRU Network and UW SCOR Study. Reprod Sci 15: 866–877. https://doi.org/10.1177/1933719108322425 PMID: 19050320

7. Winn VD, Haimov-Kochman R, Paquet AC, Yang YJ, Madhusudhan MS, et al. (2007) Gene expression profiling of the human maternal-fetal interface reveals dramatic changes between midgestation and term. Endocrinology 148: 1059–1079. https://doi.org/10.1210/en.2006-0683 PMID: 17170095

8. Uuskula L, Mannik J, Rull K, Minajeva A, Koks S, et al. (2012) Mid-gestational gene expression profile in placenta and link to pregnancy complications. PLoS One 7: e49248. https://doi.org/10.1371/journal.pone.0049248 PMID: 23145134

9. Das R, Lee YK, Strogantsev R, Jin S, Lim YC, et al. (2013) DNMT1 and AIM1 Imprinting in human placenta revealed through a genome-wide screen for allele-specific DNA methylation. BMC Genomics 14: 685. https://doi.org/10.1186/1471-2164-14-685 PMID: 24094292

10. Magalhaes HR, Leite SB, Paz CC, Duarte G, Ramos ES (2013) Placental hydroxymethylation vs methylation at the imprinting control region 2 on chromosome 11p15.5. Braz J Med Biol Res 46: 916–919. https://doi.org/10.1590/1414-431X20133035 PMID: 24270911

11. Buckberry S, Bianco-Miotto T, Hiendleder S, Roberts CT (2012) Quantitative allele-specific expression and DNA methylation analysis of H19, IGF2 and IGF2R in the human placenta across gestation reveals H19 imprinting plasticity. PLoS One 7: e51210. https://doi.org/10.1371/journal.pone.0051210 PMID: 23227253

12. Barbaux S, Gascoin-Lachambre G, Buffat C, Monnier P, Mondon F, et al. (2012) A genome-wide approach reveals novel imprinted genes expressed in the human placenta. Epigenetics 7: 1079–1090. https://doi.org/10.4161/epi.21495 PMID: 22894909

13. Lamberti L, Marsit CJ, Sharma P, Maccani M, Ma Y, et al. (2012) Imprinted gene expression in fetal growth and development. Placenta 33: 480–486. https://doi.org/10.1016/j.placenta.2012.03.001 PMID: 22465419

14. Lamberti L, Lee TL, Chan WY, Lee MJ, Diplas A, et al. (2011) Differential methylation of imprinted genes in growth-restricted placentas. Reprod Sci 18: 1111–1117. https://doi.org/10.1177/1933719111404611 PMID: 21693779
15. Hamada H, Okae H, Toh H, Chiba H, Hiura H, et al. (2016) Allele-Specific Methylome and Transcriptome Analysis Reveals Widespread Imprinting in the Human Placenta. Am J Hum Genet 99: 1045–1058. https://doi.org/10.1016/j.ajhg.2016.08.021 PMID: 27843122

16. Yeung KR, Chiu CL, Pidsley R, Makris A, Hennessy A, et al. (2016) DNA methylation profiles in pre-eclampsia and healthy control placentas. Am J Physiol Heart Circ Physiol 310: H1295–1303. https://doi.org/10.1152/ajpheart.00958.2015 PMID: 26968548

17. Chu T, Bunce K, Shaw P, Shridhar V, Althouse A, et al. (2014) Comprehensive analysis of preeclampsia-associated DNA methylation in the placenta. PLoS One 9: e107318. https://doi.org/10.1371/journal.pone.0107318 PMID: 25247495

18. Rolfman M, Choufani S, Turinsky AL, Drewlo S, Keating S, et al. (2016) Genome-wide placental DNA methylation analysis of severely growth-discordant monochorionic twins reveals novel epigenetic targets for intrauterine growth restriction. Clin Epigenetics 8: 70. https://doi.org/10.1186/s13148-016-0238-x PMID: 27330572

19. Jin S, Lee YK, Lim YC, Zheng Z, Lin XM, et al. (2013) Global DNA hypermethylation in down syndrome placenta. PLoS Genet 9: e1003515. https://doi.org/10.1371/journal.pgen.1003515 PMID: 23754950

20. Novakovic B, Yuen RK, Gordon L, Penaherrera MS, Sharkey A, et al. (2011) Evidence for widespread changes in promoter methylation profile in human placenta in response to increasing gestational age and environmental/stochastic factors. BMC Genomics 12: 529. https://doi.org/10.1186/1471-2164-12-529 PMID: 22032438

21. Lee YK, Jin S, Duan S, Lim YC, Ng DP, et al. (2014) Improved reduced representation bisulfite sequencing for epigenomic profiling of clinical samples. Biol Proced Online 16: 1. https://doi.org/10.1186/1480-9222-16-1 PMID: 24406024

22. Zhang Y, Rohde C, Tierling S, Jurkowski TP, Bock C, et al. (2009) DNA methylation analysis of chromosome 21 gene promoters at single base pair and single allele resolution. PLoS Genet 5: e1000438. https://doi.org/10.1371/journal.pgen.1000438 PMID: 19325872

23. Eckhardt F, Lewin J, Cortese R, Rakyan VK, Attwood J, et al. (2006) DNA methylation profiling of human chromosomes 6, 20 and 22. Nat Genet 38: 1378–1385. https://doi.org/10.1038/ng1909 PMID: 17072317

24. Laurent L, Wong E, Li G, Huynh T, Tsirigos A, et al. (2010) Dynamic changes in the human methylome during differentiation. Genome Res 20: 320–331. https://doi.org/10.1101/gr.101907.109 PMID: 20133333

25. Stadler MB, Murr R, Burger L, Ivanek R, Lienert F, et al. (2011) DNA-binding factors shape the mouse methylome at distal regulatory regions. Nature 480: 490–495. https://doi.org/10.1038/nature10716 PMID: 22170606

26. Meissner A, Mikkelsen TS, Gu H, Wernig M, Hanna J, et al. (2008) Genome-scale DNA methylation maps of pluripotent and differentiated cells. Nature 454: 766–770. https://doi.org/10.1038/nature07107 PMID: 18600261

27. Moore GE, Ishida M, Demetriou C, Al-Olabi L, Leon LJ, et al. (2015) The role and interaction of imprinted genes in human fetal growth. Philos Trans R Soc Lond B Biol Sci 370: 20140074. https://doi.org/10.1098/rstb.2014.0074 PMID: 25602077

28. Diplas AI, Lambertini L, Lee MJ, Sperling R, Lee YL, et al. (2009) Differential expression of imprinted genes in normal and IUGR human placentas. Epigenetics 4: 235–240. PMID: 19483473

29. McMinn J, Wei M, Schufl N, Cusma-J, Johnson EB, et al. (2006) Unbalanced placental expression of imprinted genes in human intrauterine growth restriction. Placenta 27: 540–549. https://doi.org/10.1016/j.placenta.2005.07.004 PMID: 16125225

30. Yu L, Chen M, Zhao D, Yi P, Lu L, et al. (2009) The H19 gene imprinting in normal pregnancy and pre-eclampsia. Placenta 30: 443–447. https://doi.org/10.1016/j.placenta.2009.02.011 PMID: 19342096

31. Tycko B (2006) Imprinted genes in placental growth and obstetric disorders. Cytogenet Genome Res 113: 271–278. https://doi.org/10.1159/000090842 PMID: 1657190

32. Blake WJ, MKA, Cantor CR, Collins JJ (2003) Noise in eukaryotic gene expression. Nature 422: 633–637. https://doi.org/10.1038/nature01546 PMID: 12687005

33. Fraser HB, Hirsh AE, Giaever G, Kumm J, Eisen MB (2004) Noise minimization in eukaryotic gene expression. PLoS Biol 2: e137. https://doi.org/10.1371/journal.pbio.0020137 PMID: 15124029

34. Spence D, Stewart MC, Alderdice FA, Patterson CC, Halliday HL (2012) Intra-uterine growth restriction and increased risk of hypertension in adult life: a follow-up study of 50-year-olds. Public Health 126: 561–565. https://doi.org/10.1016/j.puhe.2012.03.010 PMID: 22607982

35. Hecher K, Campbell S, Doyle P, Harrington K, Nicolaides K (1995) Assessment of fetal compromise by Doppler ultrasound investigation of the fetal circulation. Arterial, intracardiac, and venous blood flow velocity studies. Circulation 91: 129–138. PMID: 7805194
36. Crispi F, Hernandez-Andrade E, Pelsers MM, Plasencia W, Benavides-Serralde JA, et al. (2008) Cardiac dysfunction and cell damage across clinical stages of severity in growth-restricted fetuses. Am J Obstet Gynecol 199: 254–258.

37. Comas M, Crispi F, Cruz-Martinez R, Martinez JM, Figueras F, et al. (2010) Usefulness of myocardial tissue Doppler vs conventional echocardiography in the evaluation of cardiac dysfunction in early-onset intrauterine growth restriction. Am J Obstet Gynecol 203: 45–47.

38. Fattal-Valevski A, Toledano-Alhadef H, Leitner Y, Geva R, Eshel R, et al. (2009) Growth patterns in children with intrauterine growth retardation and their correlation to neurocognitive development. J Child Neurol 24: 846–851. https://doi.org/10.1177/0883073808331082 PMID: 19617460

39. Rodriguez MM, Gomez AH, Abitbol CL, Chandar JJ, Duara S, et al. (2004) Histomorphometric analysis of postnatal glomerulogenesis in extremely preterm infants. Pediatr Dev Pathol 7: 17–25. https://doi.org/10.1016/s10024-003-3029-2 PMID: 15255031

40. Teeninga N, Schreuder MF, Bokenkamp A, Delemarre-van de Waal HA, van Wijk JA (2008) Influence of low birth weight on minimal change nephrotic syndrome in children, including a meta-analysis. Nephrol Dial Transplant 23: 1615–1620. https://doi.org/10.1093/ndt/gfm829 PMID: 18065792

41. Lim YC, Chia SY, Jin S, Han W, Ding C, et al. (2016) Dynamic DNA methylation landscape defines brown and white cell specificity during adipogenesis. Mol Metab 5: 1033–1041. https://doi.org/10.1016/j.molmet.2016.08.006 PMID: 27689016

42. Bell JT, Pai AA, Pickrell JK, Gaffney DJ, Pique-Regi R, et al. (2011) DNA methylation patterns associate with genetic and gene expression variation in HapMap cell lines. Genome Biol 12: R10. https://doi.org/10.1186/gb-2011-12-1-r10 PMID: 21251332

43. Pai AA, Bell JT, Marioni JC, Pritchard JK, Gilad Y (2011) A genome-wide study of DNA methylation patterns and gene expression levels in multiple human and chimpanzee tissues. PLoS Genet 7: e1001316. https://doi.org/10.1371/journal.pgen.1001316 PMID: 21383968

44. Jjingo D, Conley AB, Yi SV, Lunyak VV, Jordan IK (2012) On the presence and role of human gene-body DNA methylation. Oncotarget 3: 462–474. https://doi.org/10.18632/oncotarget.497 PMID: 22577155

45. Ball MP, Li JB, Gao Y, Lee JH, LeProust EM, et al. (2009) Targeted and genome-scale strategies reveal gene-body methylation signatures in human cells. Nat Biotechnol 27: 361–368. https://doi.org/10.1038/nbt.1533 PMID: 19329990

46. Rauch TA, Wu X, Zhong X, Riggs AD, Pfeifer GP (2009) A human B cell methylome at 100-base pair resolution. Proc Natl Acad Sci U S A 106: 671–678. https://doi.org/10.1073/pnas.0812399106 PMID: 19139413