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human reproduction **ORIGINAL ARTICLE** ***Reproductive genetics***

Altered gene expression in human placentas after IVF/ICSI

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STUDY QUESTION: Is gene expression in placental tissue of IVF/ICSI patients altered when compared with a spontaneously conceived group, and are these alterations due to loss of imprinting (LOI) in the case of imprinted genes?

SUMMARY ANSWER: An altered imprinted gene expression of *H19* and Pleckstrin homology-like domain family A member 2 (*PHLDA2*), which was not due to LOI, was observed in human placentas after IVF/ICSI and several biological pathways were significantly overrepresented and mostly up-regulated.

WHAT IS KNOWN ALREADY: Genomic imprinting plays an important role in placental biology and in placental adaptive responses triggered by external stimuli. Changes in placental development and function can have dramatic effects on the fetus and its ability to cope with the intrauterine environment. An increased frequency of placenta-related problems as well as an adverse perinatal outcome is seen in IVF/ICSI derived pregnancies, but the role of placental epigenetic deregulation is not clear yet.

STUDY DESIGN AND PARTICIPANTS: In this prospective cohort study, a total of 115 IVF/ICSI and 138 control couples were included during pregnancy. After applying several exclusion criteria (i.e. preterm birth or stillbirth, no placental samples, pregnancy complications or birth defects), respectively, 81 and 105 placentas from IVF/ICSI and control pregnancies remained for analysis. Saliva samples were collected from both parents.

METHODS: We quantitatively analysed the mRNA expression of several growth-related imprinted genes [*H19*, insulin-like growth factor 2 (IGF2), PHLDA2, cyclin-dependent kinase inhibitor 1C (CDKN1C), mesoderm-specific transcript homolog (MEST) isoform α and β by quantitative PCR] after standardization against three housekeeping genes [Succinate dehydrogenase A (SDHA), YWHAZ and TATA-binding protein (TBP)]. A quantitative allele-specific expression analysis of the differentially expressed imprinted genes was performed to investigate LOI, independent of the mechanism of imprinting. Furthermore, a microarray analysis was carried out ($n = 10$ in each group) to investigate the expression of non-imprinted genes as well.

MAIN RESULTS AND THE ROLE OF CHANCE: Both *H19* and *PHLDA2* showed a significant change, respectively, a 1.3-fold ($P = 0.033$) and 1.5-fold ($P = 0.002$) increase in mRNA expression in the IVF/ICSI versus control group. However, we found no indication that there is an increased frequency of LOI in IVF/ICSI placental samples. Genome-wide mRNA expression revealed 13 significantly overrepresented biological pathways involved in metabolism, immune response, transmembrane signalling and cell cycle control, which were mostly up-regulated in the IVF/ICSI placental samples.

LIMITATIONS, REASONS FOR CAUTION: Only a subset of samples was found to be fully informative, which unavoidably led to lower sample numbers for our LOI analysis. Our study cannot distinguish whether the reported differences in the IVF/ICSI group are exclusively attributable to the IVF/ICSI technique itself or to the underlying subfertility of the patients.

WIDER IMPLICATIONS OF THE FINDINGS: Whether these placental adaptations observed in pregnancies conceived by IVF/ICSI might be connected to an adverse perinatal outcome after IVF remains unknown. However, it is possible that these differences affect fetal development and long-term patterns of gene expression, as well as maternal gestational physiology.

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Introduction

The placenta is a highly specialized and adaptive organ, which supports fetal growth and development. Changes in placental development and function can have dramatic effects on the fetus and its ability to cope with the intrauterine environment. Placental adaptive responses that are triggered by in- or extrinsic factors may have long-lasting consequences, as intrauterine growth and size at birth are critical determinants for the successful adaptation to post-natal life. The origin of chronic diseases in later life, such as hypertension, type 2 diabetes, coronary heart disease and stroke, can be traced back to intrauterine growth, according to the developmental origins of health and disease concept (Barker *et al.*, 2009).

In human IVF/ICSI offspring, a lower birthweight (Pandey *et al.*, 2012) as well as characteristics of metabolic syndrome, such as vascular dysfunction, increased systolic blood pressure, fasting glucose level and skin fold thickness (Ceelen *et al.*, 2007; Ceelen *et al.*, 2008; Sakka *et al.*, 2010; Scherrer *et al.*, 2012), have been reported. An important role for the placenta herein is plausible as an increased frequency of placenta-related problems is observed in IVF/ICSI derived pregnancies, such as an increased risk of hypertensive disorders of pregnancy, placenta praevia, abruption and third trimester vaginal bleeding (Pandey *et al.*, 2012). Recently, a higher placental weight/birthweight ratio among pregnancies conceived by assisted reproduction technologies (ART) compared with spontaneous pregnancies was found, which persisted after adjustment for length of gestation, offspring birthweight, parity, fetal sex, maternal age, pre-eclampsia and diabetes (Haavaldsen *et al.*, 2012).

Genomic imprinting plays an important role in placental biology and in the placental adaptive responses (Sandovici *et al.*, 2012). Imprinted genes, which are expressed in a parent-of-origin-dependent way, are abundantly expressed in the placenta and are indispensable for proper placental morphology and function (Kawahara *et al.*, 2009; Nelissen *et al.*, 2011). Transcription of either the paternal or the maternal gene is regulated by epigenetic marks, such as DNA methylation, histone modifications or non-coding RNAs (Nelissen *et al.*, 2011). Around conception, genome-wide epigenetic reprogramming takes place, but imprinted genes are protected to preserve the parental imprints in the developing embryo (Reik and Walter, 2001; van Montfoort *et al.*, 2012). This period of epigenetic reprogramming is known to be sensitive to epigenetic disturbances (Fraga *et al.*, 2005; Jirtle and Skinner, 2007), which might potentially make ART treatment a risk.

An important question is whether IVF/ICSI induces epigenetic deregulation of imprinted genes in placental tissue. Indeed, animal models have shown that for instance preimplantation embryo culture can affect methylation and expression of imprinted genes (Mann *et al.*, 2004; Fauque *et al.*, 2010; Market-Velker *et al.*, 2010). Moreover, animal studies suggest that placental tissues are more sensitive to preimplantation epigenetic disturbance of imprinted genes than embryonic tissues (Mann *et al.*, 2004; Rivera *et al.*, 2008). This can lead to abnormal placental development and function with possible consequences for the developing fetus.

In humans, several studies performed in the oocyte, preimplantation embryo, peripheral blood, umbilical cord blood, amniotic membrane, cord and buccal smears have shown conflicting data about epigenetic deregulation (Geuns *et al.*, 2003; Sato *et al.*, 2007; Tierling *et al.*, 2010; Feng *et al.*, 2011; Hiura *et al.*, 2012; Oliver *et al.*, 2012; Puumala *et al.*, 2012; Huntriss *et al.*, 2013). However, these studies, although of great value, cannot easily be compared with each other and transferred to placenta biology because genomic imprinting can be tissue-specific, especially in extra-embryonic tissues (Tabano *et al.*, 2010; Prickett and Oakey, 2012).

Only a few studies have focused on the epigenetic deregulation after IVF/ICSI in human placenta. DNA methylation differences of imprinted genes and the *H19*/insulin-like growth factor 2 (IGF2) imprinting control region (*H19 ICR1*) have been reported (Katari *et al.*, 2009; Turan *et al.*, 2010; Rancourt *et al.*, 2012). In a previous study, we identified aberrant DNA methylation at some differentially methylated regions (DMRs), which in some cases did (*H19*) and some cases did not (mesoderm-specific transcript homolog: *MEST*) lead to aberrant gene expression (Nelissen *et al.*, 2013). Therefore, it is important to not only investigate DNA methylation, but also to focus on placental gene expression.

Furthermore, it is currently unknown whether the reported changes in imprinted gene expression are the result of loss of imprinting (LOI), i.e. expression from the normally silenced allele. There is evidence that histone methylation and non-coding RNAs rather than DNA methylation might be important for imprinting maintenance in the mouse placenta (Lewis *et al.*, 2004; Wagschal and Feil, 2006; Pandey *et al.*, 2008; Wagschal *et al.*, 2008; Redrup *et al.*, 2009). Therefore, we investigated LOI, independently of the mechanism of imprinting, using an allele-specific expression analysis.

Besides imprinted genes, also non-imprinted genes are important for placental development (Nelissen *et al.*, 2011). Katari *et al.* (2009) found that *in vitro* conception-associated DNA methylation differences are associated with gene expression differences at both imprinted and non-imprinted genes. Furthermore, it is known that gene expression changes in placental transporter systems can be induced by intrinsic (i.e. genetic and developmental) and extrinsic (i.e. environmental) factors (Sandovici *et al.*, 2012).

Our hypothesis is that epigenetic aberrations occur in placental tissue of IVF/ICSI patients compared with placental tissue of spontaneous pregnancies. Therefore, we carried out a microarray analysis to investigate genome-wide which pathways are deregulated in IVF/ICSI placentas. Regarding the importance of imprinted genes in placental function, special attention was given to several growth-related imprinted genes [*H19*, *IGF2*, Pleckstrin homology-like domain family A member 2 (*PHLDA2*), cyclin-dependent kinase inhibitor 1C (*CDKN1C*), *MEST*

isoform α and β] by analysing the expression in a larger number of samples using quantitative real-time PCR (qPCR) after standardization against three housekeeping genes [Succinate dehydrogenase A (*SDHA*), *YWHAZ* and TATA-binding protein (*TBP*)]. Finally, a quantitative allele-specific expression analysis of the differentially expressed imprinted genes was performed to investigate LOI, independent of the mechanism of imprinting.

Materials and Methods

Study population and sample collection

In this prospective cohort study, IVF/ICSI patients were recruited at the IVF centre of the Maastricht University Medical Centre (MUMC), The Netherlands, after an ultrasound examination of a viable singleton pregnancy at 7 weeks' gestation in the period between May 2008 and October 2009. Only pregnancies resulting from fresh embryo transfers after standard IVF or ICSI procedures were included. Excluded were patients who had performed PGD or required donor gametes. IVF and ICSI treatments were performed as described previously (Dumoulin *et al.*, 2010), with the exception that in this study only Vitrolife G1.5 medium (Göteborg, Sweden) was used.

Non-IVF control pregnant couples were recruited during their pregnancy in the period between April 2008 and July 2009 at the obstetric outpatients' clinic in the MUMC (52%) and at cooperating midwife practices (48%), and were defined as having conceived spontaneously without the use of any kind of hormones, other medication or ART. Also in this group only singleton pregnancies were included.

In both groups, saliva samples were collected from both parents for genomic DNA extraction using the Oragene® DNA Self-Collection kit (DNA Genotek Inc., Ottawa, Canada). Participants were not allowed to eat, drink, smoke or chew gum for half an hour before producing the saliva sample (2 ml). The couples were asked to fill in a short questionnaire about their height and weight before pregnancy and the smoking and drinking habits of the woman before and during the pregnancy.

Placental tissue was collected within 30 min after delivery of the placenta by the gynaecologist, nurse or midwife according to a standardized protocol. The biopsies (~5 mm^3) were taken from the fetal side near the umbilical cord insertion point, rinsed extensively in cold phosphate-buffered saline to remove blood and stored in RNAlater® (Applied Biosystems/Ambion, USA). The samples were sent to the department of Obstetrics and Gynaecology at the Maastricht University Medical Centre together with a copy of the delivery report. After receiving the samples, the amnion and chorionic membranes were removed (which also served as an extra verification that the biopsies were taken from the fetal side) and the villi were stored until further use at −20°C according to the guidelines provided by the RNAlater manufacturer. Samples from deliveries before 37 weeks' gestation were excluded from analysis. Also samples from patients with pregnancy complications, such as gestational diabetes, hypertension and pre-eclampsia, or from children with birth defects, were excluded.

This study was approved by the Ethical Review Board of the Maastricht University Medical Centre and all participants gave written informed consent.

RNA isolation from placental tissues

Total RNA was extracted using Trizol reagent (Invitrogen, Carlsbad, USA) according to the manufacturer's instructions with some minor adaptations. After adding 800 µl Trizol with 10% β-mercaptho-ethanol, the samples were ground using a pestle and a mini bead beater (Biospec, USA). RNA was precipitated with isopropyl alcohol at room temperature for 2 h and the RNA pellet was washed three times with 70% ethanol. Total RNA was resuspended in 20 µl RNAse free water and stored at −80°C. RNA quantity and quality were determined using a Nanodrop ND-1000 spectrophotometer (Nanodrop Technologies, Wilmington, USA) and RNA integrity was measured using a Bioanalyzer 2100 (Agilent technologies, Palo Alto, USA).

cDNA synthesis

After a DNAse I treatment (Invitrogen), total RNA was converted to cDNA using the High Capacity cDNA Reverse transcription kit (Applied Biosystems, Foster City, USA) according to the manufacturer's instructions.

Quantitative PCR

Each cDNA sample was diluted 500-fold. All assays were run in triplicate in a reaction mixture containing 1 µl of the diluted sample, 5 µl reaction buffer of mesa green qPCR MasterMix plus for SYBR assay (Eurogentec, San Diego, USA), 1 µl of each primer ([1 µM]), 1.9 µl nuclease-free water and 0.1 µl Uracil-N-Glycosylase (UNG, Eurogentec). The investigated genes of interest were *H19*, *IGF2*, *PHLDA2*, *CDKN1C*, *MEST* isoform α and β, which are all growth-related genes. The primers are listed in Supplementary data, Table SI and were chosen to span an exon-exon barrier wherever possible. qRT–PCR amplification was performed using an Applied Biosystems Prism 7900 sequence detection system with the following thermal cycler conditions: 2 min at 50°C for UNG treatment, 10 min at 95°C to activate Taq polymerase and 40 cycles of denaturation at 95°C for 15 s and annealing and elongation at 60°C for 1 min. A dissociation curve analysis was performed to verify the absence of genomic DNA contamination and to control whether the reaction was specific for one PCR product.

The expression of each gene was related to the expression of a combination of reference genes as described previously (Nelissen *et al.*, 2013). The mean of the Ct values of the reference genes *SDHA*, *YWHAZ* and *TBP* was used as a normalization factor and subtracted from the Ct values of the genes of interest to obtain normalized Ct values (ΔCt). The fold change in expression of each gene of interest was analysed using the ΔΔCt method (Livak and Schmittgen, 2001).

Microarray analysis

Two hundred nanogram of total RNA was amplified using the Ambion WT Expression Kit (Life Technologies, Carlsbad, CA, USA). The Affymetrix WT Terminal Labelling Kit was used for fragmentation and labelling (Affymetrix, Santa Clara, USA). Hybridization was performed using 5 µg of biotinylated target, which was incubated with the GeneChip® Human Gene 1.0 ST array (Affymetrix) at 45°C for 16–18 h, covering 36 079 transcripts. After hybridization, non-specifically bound material was removed by washing and specifically bound target was detected using the GeneChip Hybridization, Wash and Stain kit and the GeneChip Fluidics Station 450 (Affymetrix). The arrays were scanned using the GeneChip Scanner 3000 7G (Affymetrix) and raw data were extracted from the scanned images. The samples were processed in two separate batches of both IVF/ICSI and control samples (4 IVF/ICSI + 2 controls and 6 IVF/ICSI + 8 controls).

Genomic DNA extraction from placenta and saliva

DNA from placental tissue was extracted using the Gentra PureGene tissue kit (Qiagen, Venlo, The Netherlands) according to the manufacturer's guidelines. The AutoGenFlex STAR (Autogen, Holliston, USA) was used for fully automated extraction of DNA from saliva samples according to the manufacturer's instructions.

Allele-specific expression analysis

Single nucleotide polymorphisms (SNPs) were used to distinguish between the parental alleles of *H19* and *PHLDA2*. Genotyping of SNPs in genomic DNA of placental tissue and saliva samples from both parents was done by PCR and sequencing according to the Sanger (chain-termination) method.

The PCR primers, which contained an M13 tail for sequencing, and the analysed SNPs are listed in Supplementary data, Table SII. PCR amplification of *H19* was performed using an initial denaturation step at 94°C for 3 min followed by 35 cycles of denaturation at 94°C for 30 s, annealing for 40 s at 61°C and elongation at 72°C for 1 min. The programme ended with a final extension step at 72°C for 10 min. For *PHLDA2*, the thermal cycling conditions were the same except for the annealing temperature of 55°C, an elongation time of 3 min and 45 cycles of amplification. DNA sequencing of the PCR products after amplification was performed by using the Big Dye terminator cycle sequencing ready reaction mix in combination with M13 primers and an ABI 3730 DNA Analyser (Applied Biosystems).

In placental samples that were informative (i.e. the parental alleles could be distinguished), the parent-of-origin-specific expression of imprinted genes was determined quantitatively by pyrosequencing of cDNA. All allele-specific expression analyses were performed in triplicate. Regions of interest were amplified using 20 ng of cDNA, and 5 pmol of forward and reverse primer, one of them being biotinylated. Sequences for oligonucleotides for PCR amplification and pyrosequencing are given in Supplementary data, Table SIII. Reaction conditions were 10× HIFI Platinum Taq buffer supplemented with 25 mM $MgCl_2$, 10 mM dNTPs and HIFI Platinum Taq (Life Technologies) in a 25 μl volume. The PCR programme consisted of a denaturing step of 4 min at 95°C followed by 50 cycles of 30 s at 95°C, 30 s at the respective annealing temperature and 15 s at 72°C, with a final extension of 4 min at 72°C. Ten microliters of PCR product was rendered single-stranded as previously described (Tost and Gut, 2007) and 4 pmol of the respective sequencing primer was used for analysis. Quantitative allelic expression analysis was carried out on a PSQ 96MD system with the PyroGold Q96 Reagent Kit (Qiagen) and results were analysed using the PyroMark MD software (V1.0, Qiagen).

Statistical analysis

Statistical analysis was performed using SPSS software for windows version 20.0 (Statistical Package for Social Sciences, USA). To test differences, Student's *t*-test was used for continuous variables and the χ^2 test was used for binary variables. The difference in relative gene expression between both groups, as analysed by qPCR, was tested with the non-parametric Mann–Whitney *U* test. $P < 0.05$ was considered significant.

Microarray data analysis was performed using the R statistical software (http://cran.r-project.org/). A variety of established array specific quality control, visualization, normalization and statistical methods were combined into one workflow at the BiGCaT department (Eijssen *et al.*, 2013). Additionally, we corrected for batch as a possible covariate. Linear modelling using the limma package was conducted to compute the genes that were significantly changed between experimental groups, as defined by a *P*-value <0.05. These genes were mapped to biological pathways using PathVisio (van Iersel *et al.*, 2008; Kelder *et al.*, 2011). Using the statistics function in PathVisio, an ordered list of *Z*-score-ranked pathways was generated based on the over-representation of member genes that were significantly changed between experimental groups. All pathways with a *Z*-score higher than 1.96 were included in the biological interpretation, which is comparable to a significance level of 0.05.

Outlier detection for allele-specific expression was performed by using tests available in the Outliers and the Extreme values packages developed for the R statistical software (http://cran.r-project.org/). Several methods of outlier detection were performed since they all have advantages and disadvantages. The Grubb test (Grubbs, 1969) which is based on the assumption of normality and the Dixon test (Dixon, 1950) which is distribution-free have been used to detect individual outliers. Identified outliers were removed from the data and the test was iterated until no further outliers were detected. However, the iteration process can be prematurely stopped if one outlier masks a second outlier (masking effect). The generalized Extreme Studentized Deviate (gESD) test (Rosner, 1983) and the Loo tests (van der Loo, 2010) are designed to detect one or more outliers simultaneously among data that follow an approximately normal distribution. Samples exhibiting a modified *Z*-score (Iglewicz and Hoaglin, 1993) with an absolute value >3.5 have been labelled as potential outliers, as suggested by the authors.

Results

Sample collection and group comparison

A total of 115 IVF/ICSI and 138 control couples were included in the study after having signed an informed consent (Fig. 1). In the IVF/ICSI group, 34 couples were excluded because either no biopsies were taken ($n = 29$), samples were collected after a stillbirth ($n = 1$) or from patients with pregnancy complications ($n = 2$, hypertension and HELLP syndrome) or a preterm delivery (<37 weeks, $n = 2$), resulting in 81 placenta samples for analysis. The control group contained only term deliveries and no patients with pregnancy complications. However, in 32 cases no biopsies were taken and in one case there was a birth defect (trisomy 18), which resulted in 105 placentas for analysis. In Table I, parental and neonatal characteristics from both groups are shown. With the exception of the age of the parents, all characteristics are similar. During pregnancy, there were two women (one in either group) who used alcohol sporadically and two women in the IVF group who used recreational drugs on one occasion (data not shown).

Microarray analysis

Ten IVF/ICSI (6 males, 4 females) and 10 control (4 males, 6 females) samples were randomly selected for microarray analysis. All indicators of sample quality, hybridization quality, signal comparability and array correlation met the quality control criteria, so that no array needed to be excluded from the dataset. We corrected for batch as possible covariate. The number of significantly differentially expressed genes (DEGs) between IVF/ICSI and control placentas was calculated. There were 839 (47.5%) up-regulated and 927 (52.5%) down-regulated DEGs, which was comparable. Most DEGs showed a fold change between −1.5 and +1.5. The number of DEGs >1.5-fold up-regulated or <1.5-fold down-regulated was, respectively, 14 and 6.

Pathway analysis of the DEGs in placental tissue from IVF/ICSI and spontaneous pregnancies revealed 13 significantly overrepresented biological pathways, for instance the miRNA Regulation of DNA damage response, cholesterol biosynthesis, one-carbon metabolism and electron transport chain pathway. All 13 pathways and their calculated *Z*-scores are summarized in Table II.

Gene expression analysis

The investigated genes of interest were *H19*, *IGF2*, *PHLDA2*, *CDKN1C*, *MEST* isoform α and β, which are all growth-related genes. After quality control of the qPCR results as described in the methods section, we excluded another 7 samples in the IVF/ICSI group and 13 in the control group (Fig. 1). The relative expression levels of *H19*, *IGF2*, *PHLDA2*, *CDKN1C*, *MEST* isoform α and β were obtained in 166 placental samples (92 in the control group and 74 in the IVF/ICSI group) after standardization against the housekeeping genes *SDHA*, *YWHAZ* and *TBP*. Both *H19* and *PHLDA2* showed a significant increase in expression in the IVF/ICSI versus control group, respectively,

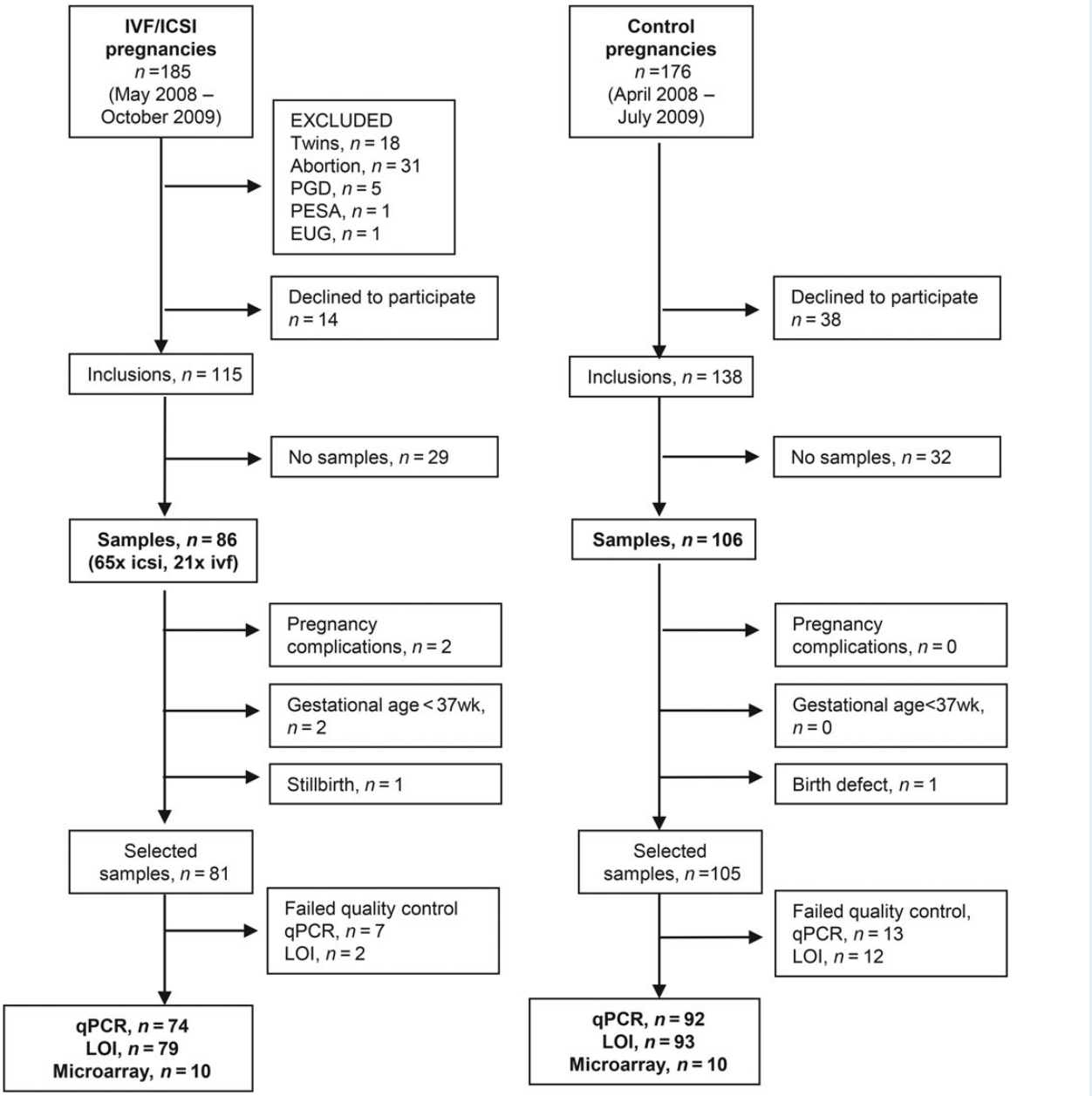


Figure 1 Flowchart.

1.3-fold ($P = 0.033$) and 1.5-fold ($P = 0.002$) (Fig. 2). Additionally, we performed a linear regression analysis per imprinted gene with group (IVF/control), maternal and paternal age as possible covariates. Only group was significantly associated with gene expression of *H19* and *PHLDA2*, while parental age was not.

Allele-specific expression analysis

Next, we investigated whether the significantly increased expression of *H19* and *PHLDA2* in the IVF/ICSI placental samples was the result of LOI leading to expression of the silent paternal allele. Pyrosequencing has a detection limit of 5% (Ogino *et al.*, 2005). Therefore, we considered any result below 5% as technical noise. For SNP rs10840159, the observed background signal was higher and the threshold was set to 10%.

For the investigation of the imprinting status of *PHLDA2*, genomic DNA from 172 placental samples was tested for heterozygosity at SNP rs1056819 (A/G); 46 samples were found to be informative. Saliva samples from the corresponding 46 parent-couples were tested of which 21 appeared informative (9 control and 12 IVF/ICSI). Quantitative allele-specific expression analysis of the cDNA from 21 placental samples revealed LOI in 11 placental samples (52.4%), varying from 6 to 13.6% paternal expression (Fig. 3A and C). The mean rate of paternal expression in the control group ($n = 5$) was 10.1% compared with 11.2% in the IVF group ($n = 6$), which was not significant (Mann–Whitney

U test). According to the LOO I outlier detection method, two samples (242 and 103) displayed abnormal paternal expression (Supplementary data, Table SIV). Both samples belong to the IVF group.

For the *H19* LOI analysis, the placental genomic DNA was tested for heterozygosity at four SNPs (C/G, rs2839701; G/T, rs2839702; A/G, rs10840159; C/T, rs217727), 90 samples were found to be informative for at least one SNP. Saliva samples from 180 parents were tested for heterozygosity and 52 sample-couples were informative. Quantitative allele-specific expression analysis revealed differences among the investigated SNPs (Fig. 3B and C). LOI was observed in 18/23 (78.3%) samples at SNP rs2839702 (5.1–36.4% paternal expression) and in 30/33 (90.9%) samples at SNP rs10840159 (11.5–30.9% paternal expression). LOI was less common at SNP rs217727 (12/26, 46.2%) and rs2839701 (16/34, 47.1%), with paternal expression rates ranging from 5.1 to 23.5% and 5.8 to 34.2%, respectively. Although the mean rate of paternal expression appeared slightly higher in the IVF group at three of the five investigated SNPs, the difference was not significant (Fig. 3C). Supplementary data, Table SIV displays the summary of the outlier search using univariate methods. Most tests defined sample 185 (IVF) and 214 (control) as outlying samples. It is interesting to note, that sample 185 was defined as an outlier at all informative SNPs (2/2) and 214 at two out of three SNPs (Fig. 3B). In the third SNP (rs2839702) of sample 214, the paternal expression was also among the highest. Sample 218 (control) was only informative at one SNP, but was found in several outlier tests. The characteristics of the LOI outliers were also compared with the characteristics of the non-outliers (Table III), which revealed no differences. Altogether, there is no evidence that there is more LOI in IVF placental samples.

Table I Neonatal and parental characteristics.

	Control (n = 105)	IVF/ICSI (n = 81)	*P*-value
Birthweight (g)	3501 ± 445	3460 ± 576	NS
Gestational age (weeks)	39.7 ± 0.9	40.0 ± 1.3	NS
Z-score −0.002 ± 0.95	−0.12 ± 1.11	NS	
Boys	54 (51.4)	42 (51.9)	NS
Mother			
Age (years)	31.1 ± 4.6	33.9 ± 4.1	<0.001
BMI	24.1 ± 4.4	23.9 ± 3.3	NS
Primipara	55 (52.4)	54 (66.7)	NS
Smoking during pregnancy	11 (10.5)	7 (8.6)	NS
0–9 cigarettes/day	9 (8.6)	4 (4.9)	
≥10 cigarettes/day	2 (1.9)	3 (3.7)	
Father			
Age (years)	33.5 ± 5.1	36.3 ± 5.8	0.002
BMI	25.2 ± 3.7	25.8 ± 3.6	NS

Data are presented as numbers (%) or mean ± SD. NS; not significant. Student's *t*-test.

Discussion

Genome-wide mRNA expression analysis in placentas from IVF/ICSI derived pregnancies and spontaneously conceived pregnancies revealed 13 significantly overrepresented biological pathways. These pathways can be roughly clustered according to their function into metabolism, immune response, transmembrane signalling and cell cycle control as indicated by the cancer pathways. In the RB in Cancer pathway, the *BRCA1*, *CDK1* and *CCNA2* genes were all up-regulated in the placental samples from the IVF/ICSI group. These genes play a role in maintaining genomic stability and cell cycle control. Also in the Gastric Cancer Network, DNA replication and miRNA Regulation of DNA damage response pathway, all DEGs were up-regulated and again important for cell cycle control. This could imply that ART manipulations or the parental subfertility lead to enhanced cell cycle control mechanisms, although the cell cycle pathway itself was not significantly up-regulated according to the PathVisio analysis (10 out of the 100 genes in this pathway were

Table II Pathways significantly overrepresented in the DEGs list.

Pathway	Positive (r)	Measured (n)	Total	%	*Z*-score
RB in cancer	25	98	104	25.51	6.58
Gastric cancer network 1	8	26	29	30.77	4.37
One-carbon metabolism	6	24	51	25.00	3.14
Heme biosynthesis	3	8	28	37.50	3.13
DNA replication	8	40	50	20.00	2.88
Electron transport chain	15	96	118	15.63	2.87
Oxidative phosphorylation	10	56	69	17.86	2.81
Type III interferon signalling	3	10	11	30.00	2.61
Statin pathway	6	29	46	20.69	2.59
Cholesterol biosynthesis	4	16	33	25.00	2.56
miRNA regulation of DNA damage response	12	80	106	15.00	2.41
Fluoropyrimidine activity	6	31	44	19.35	2.40
TFs Regulate miRNAs related to cardiac hypertrophy	3	12	16	25.00	2.22

miRNA; microRNA; RB; retinoblastoma protein; TF; transcription factors.

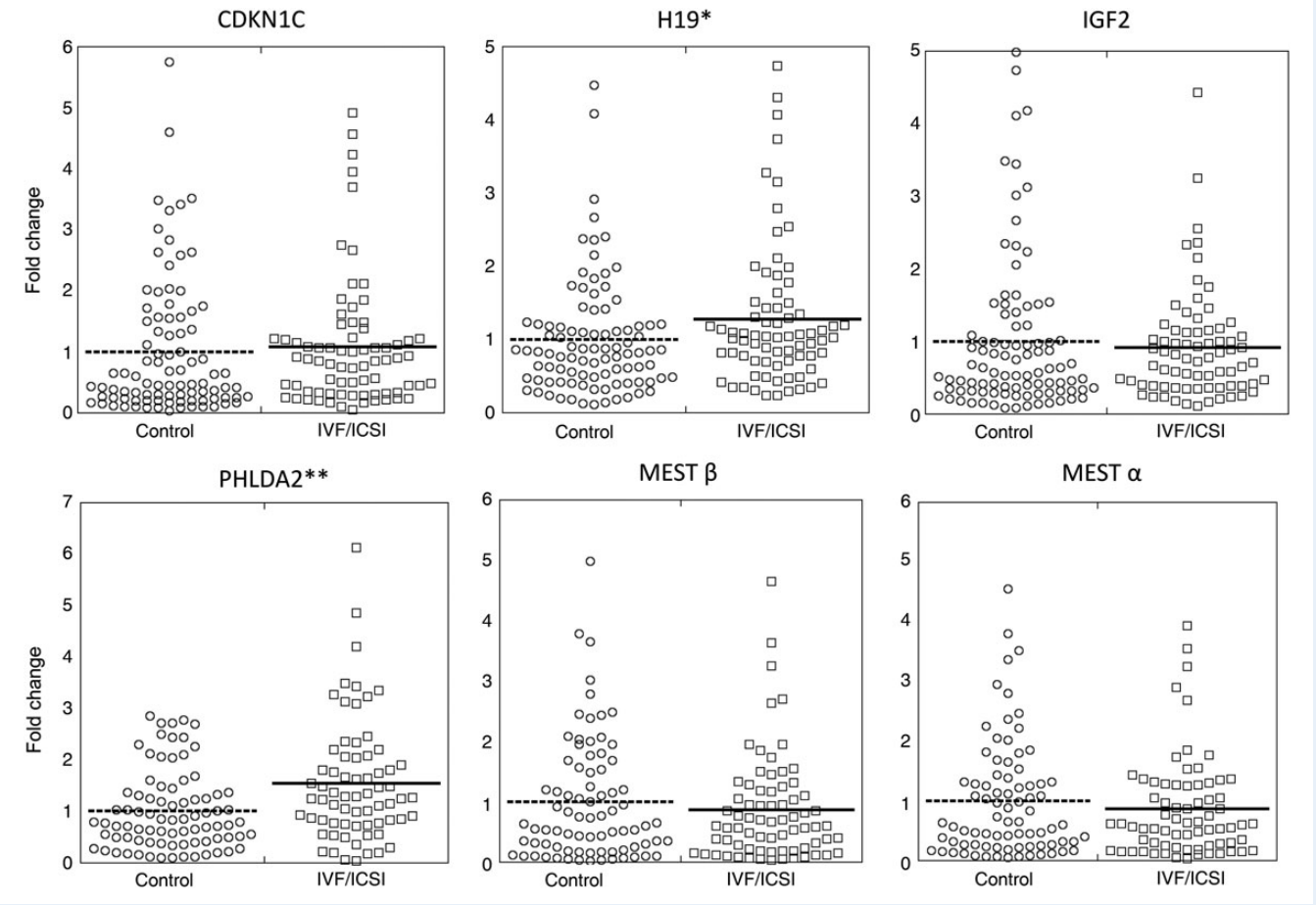
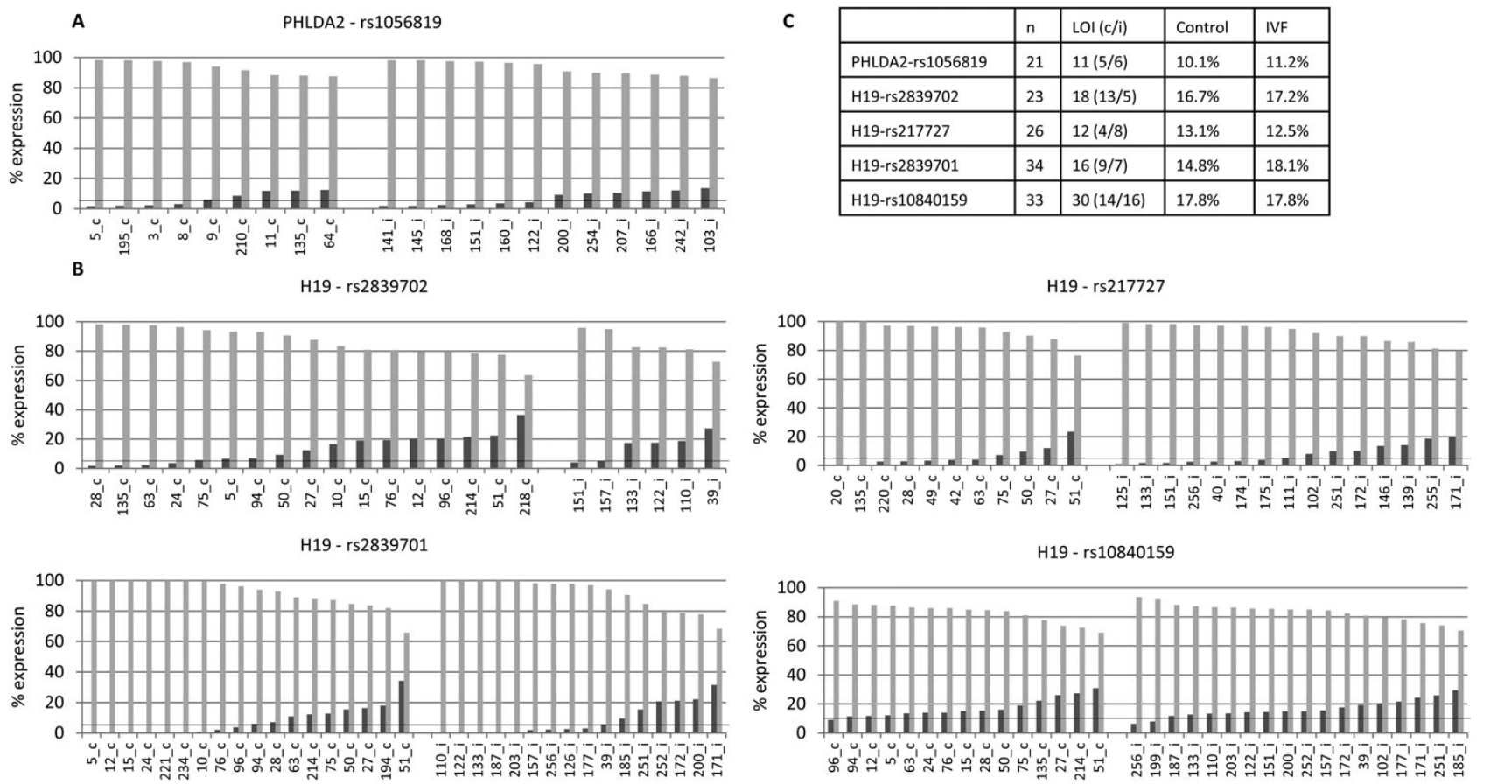


Figure 2 Relative gene expression of imprinted genes in placental tissue from spontaneous (control; $n = 92$) and IVF/ICSI pregnancies ($n = 74$). Control group is set at one. * $P < 0.05$, ** $P < 0.01$.

significantly altered between IVF and control placentas, Z-score = 0.81). Other affected pathways play a role in metabolism. In the cholesterol biosynthesis and heme biosynthesis pathways, all DEGs were up-regulated in placental samples from the IVF/ICSI group. Cholesterol is of vital importance for fetal development as a key constituent of cell membranes, precursor of steroid hormones and metabolic regulators. The placenta regulates maternal–fetal cholesterol transport and a disturbed regulation can lead to atherogenic programming in the offspring (Palinski, 2009). Hemes are involved in oxygen transport, detoxification and signal transduction processes (Ryter and Tyrrell, 2000). Most of the DEGs in the electron transport chain pathway (drives ATP synthesis) and in the one-carbon metabolism pathway are also up-regulated: the latter is important for the production of purines and pyrimidines (required for synthesis of DNA and RNA) as well as *S*-adenosylmethionine (substrate, for example, DNA methylation). Furthermore, the statin pathway and oxidative phosphorylation pathway have DEGs that are both up- and down-regulated. According to all these altered metabolism pathways, an altered metabolic activity in placentas of IVF/ICSI pregnancies might be an adaptive mechanism to ART-imposed stress or a subfertility-induced deregulation, which potentially leads to a poorer outcome in ART pregnancies. Our results are, however, still too preliminary to elucidate these questions.

A small study by Zhang *et al.* (2010) investigated global gene expression in three term placentas from IVF pregnancies compared with three placentas from spontaneous pregnancies. They found 18 DEGs and classified them according to their role in biological processes in immune response, transmembrane transport, metabolism, oxidative stress, cell differentiation and other processes. These pathways are strikingly comparable to those found in our study. Furthermore, an extensive study on the placental transcriptome after ART in mice also revealed comparable results. IVF techniques in mice triggered the induction of genes involved in cellular proliferation and cell cycle pathways and an alteration of gene expression involved in apoptotic pathways (Fauque *et al.*, 2010). They also observed a decrease in expression of genes involved in the angiogenic signalling and immune response.

Subsequently, we analysed the gene expression of several placental imprinted genes (*H19*, *IGF2*, *PHLDA2*, *CDKN1C*, *MEST* isoform α and β) in a larger sample size ($n = 166$) by qPCR. We chose to investigate these particular genes as they are all growth-related genes and IVF children are known to have a significantly lower birthweight (Pandey *et al.*, 2012). *MEST* and *IGF2* are paternally expressed genes, which favour placental and fetal development. In contrast, *H19*, *PHLDA2* and *CDKN1C* are maternally expressed and are growth inhibitory genes (Tycko and Morison, 2002; Tycko, 2006). Both *H19* (a non-coding RNA) and



	n	LOI (c/i)	Control	IVF
PHLDA2-rs1056819	21	11 (5/6)	10.1%	11.2%
H19-rs2839702	23	18 (13/5)	16.7%	17.2%
H19-rs217727	26	12 (4/8)	13.1%	12.5%
H19-rs2839701	34	16 (9/7)	14.8%	18.1%
H19-rs10840159	33	30 (14/16)	17.8%	17.8%

Figure 3 Allele-specific expression of *PHLDA2* and *H19* in term placental samples. The *x*-axis indicates the placental samples; percentage expression from each allele is indicated on the *y*-axis. Light grey bar indicates percentage of maternal expression; dark grey bar represents percentage of paternal-specific expression. *PHLDA2* (**A**) was investigated at the SNP (rs1056819) and *H19* (**B**) at four SNPs (rs2839701, rs2839702, rs10840159 and rs217727). Black line represents the detection limit of 10% at SNP H19-rs10840159 and 5% at the other SNPs. C, controls; i, IVF/ICSI. In the table (**C**), the total number of informative samples, samples with LOI and the mean percentages of paternal expression are shown (not significant, Mann–Whitney *U* test).

Table III Characteristics of LOI outliers in comparison with the non-outliers.

	Outliers	Non-outliers	*P*-value
Birthweight (g)	3296 ± 442	3507 ± 517	NS
Range	2650–3860	2360–5185	
Gestational age (weeks)	39.5 ± 0.9	39.8 ± 1.1	NS
Range	38.1–40.9	37.0–41.9	
Z-score	−0.40 ± 0.81	−0.01 ± 1.04	NS
Range	−1.440–0.598	−2.576–2.576	
Mother			
Age (years)	34.3 ± 6.1	32.0 ± 5.2	NS
Range	22–39	21–42	
BMI	22.6 ± 2.3	24.0 ± 4.0	NS
Range	20–26	18–43	
Father			
Age (years)	34.0 ± 5.5	34.6 ± 5.6	NS
Range	25–38	20–58	
BMI	24.7 ± 2.1	25.5 ± 3.6	NS
Range	22–29	18–39	

Data are presented as mean ± SD. NS; not significant. Student's *t*-test.

PHLDA2 (the pleckstrin homology-like domain, family A, member 2 gene) showed a significant increase, respectively, of 1.3-fold and 1.5-fold, in expression in the IVF/ICSI group. *PHLDA2* expression was also up-regulated according to our microarray analysis (fold change 1.2; data not shown). Not entirely unexpected, two growth inhibitory genes were up-regulated in placental samples from IVF/ICSI derived pregnancies. In human studies, an increased (1.3–2.8-fold) *PHLDA2* expression was found in the placenta of intrauterine growth restricted (IUGR) babies (McMinn *et al.*, 2006; Diplas *et al.*, 2009; Kumar *et al.*, 2012), and an association between elevated placental *PHLDA2* expression levels and low birthweight was described (Apostolidou *et al.*, 2007). However, it is important to note that we did not find any significant differences in birthweight between the groups. Additionally, Turan *et al.* (2010) found significantly lower transcripts of both *IGF2* and *H19* in placentas from the *in vitro* group .

Several studies on epigenetic deregulation in placentas after IVF/ICSI treatments focused on DNA methylation as a possible cause for altered gene expression of imprinted genes. In mice, a loss of *H19* methylation and/or an increase in the biallelic expression of *H19* was found specifically in the placenta after ovulation induction (Fortier *et al.*, 2008) or embryo culture (Mann *et al.*, 2004; Rivera *et al.*, 2008). Katari *et al.* (2009) investigated genes with methylation differences in placental samples of *in vitro* or *in vivo* conceived children and found that *MEST* and *SERPINF1* showed a higher average transcript level in the *in vitro* group. In our previous study, we found a reduced DNA methylation level at the *H19* and *MEST* DMRs, and an increased *H19* expression in placentas from pregnancies conceived by IVF/ICSI (Nelissen *et al.*, 2013). A correlation between *H19* hypomethylation and increased expression was also reported by Rancourt *et al.* (2012) in placentas from pregnancies achieved with ovulation induction and/or IVF.

Next, we investigated whether the significantly increased expression of *H19* and *PHLDA2* in the IVF/ICSI placental samples was the result of LOI leading to expression of the silent paternal allele. To the best of our knowledge, LOI has never been investigated in human IVF/ICSI placental samples before. LOI was common at two SNPs of H19 (90.9 and 78.3% of placental samples showing LOI) and less common at the other SNPs of H19 and PHLDA2 (46.2–52.4%). Although the mean LOI appeared slightly higher in the IVF group at three of the five investigated SNPs, there was no difference in mean LOI between the control group and the IVF group (non-parametric test). Our study shows that LOI is common in placental samples (whether or not conceived via IVF) which is in agreement with two previous reports (Lambertini *et al.*, 2008; Rancourt *et al.*, 2013). Lambertini *et al.* found a wide range of LOI in human placenta varying from 0 to 96% (with 25% of the informative polymorphisms showing LOI>3%), depending on the gene (i.e. *H19*, *IGF2*, *MEST*) and additionally the type of pregnancy complications (i.e. pre-eclampsia or IUGR). Rancourt *et al.* (2013) found LOI of *H19* in placentas of phenotypically healthy individuals, while complete maintenance of *IGF2* mono-allelelic expression was observed in all these placental samples. Although there was a slightly higher mean LOI in the IVF/ICSI placental samples, there are currently no indications that the significantly increased expression of *H19* and *PHLDA2* in the IVF/ICSI placental samples is related to a higher expression of the silent paternal allele. Possibly, the smaller sample size in our LOI analysis, being dependant on the number of informative heterozygous SNPs, could have influenced the significance of the results. Certainly, this needs further investigation. The outlier analysis showed no difference between the IVF/ICSI group and the control group.

How the differences in gene expression are regulated, what exactly causes these differences and what the clinical relevance is, remains so far unknown. The molecular mechanisms that underlie placental adaptations *in vivo* are mostly unknown and are a challenge to study given the complexity of the maternal–placental–fetal interactions. Placental adaptations may either affect fetal development directly, or indirectly by changing the maternal gestational physiology, for instance through an effect on maternal appetite or maternal disease such as gestational diabetes and pre-eclampsia (Sandovici *et al.*, 2012). Even though we analysed the fetal side of the placenta that is derived from trophoblast cells which have surrounded the *in vitro* exposed embryonic cells, our study cannot distinguish whether the reported gene expression differences in the IVF/ICSI versus control group are exclusively attributable to the IVF/ICSI technique itself or to the underlying subfertility of the patients.

A shortcoming of our study is that only a subset of samples was found to be fully informative for LOI analysis. Although comparable to another study (Apostolidou *et al.*, 2007), this implies less samples for LOI analysis and therefore less conclusive power. Another issue is that there was no difference in birthweight between IVF/ICSI children and spontaneously conceived children, and that placental-related problems, i.e. pre-eclampsia and hypertension, were excluded from our study. This could possibly explain the subtle differences between groups and perhaps we have missed more severe differences with this selection.

In conclusion, an altered gene expression occurs in placental tissue of IVF/ICSI patients when compared with placental tissue of spontaneous pregnancies in both non-imprinted and imprinted genes: the latter was not due to LOI. Furthermore, several biological pathways were significantly overrepresented and mostly up-regulated in the IVF/ICSI placental samples. Whether these are adaptations or deregulations, and whether

these are connected to the adverse perinatal outcome after IVF remains unknown. However, it is imaginable that these differences have the potential to affect fetal development either directly or indirectly by changing the maternal gestational physiology. Altogether, these changes may lead to long-term patterns of fetal gene expression that might be associated with an increased risk for (late onset) diseases.

Supplementary data

Supplementary data are available at http://humrep.oxfordjournals.org/.

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Authors' roles

E.C.M.N. and A.P.A.M. initiated and designed the study and played a role in recruitment of patients and sample collection. E.C.M.N., F.B., J.T. and A.P.A.M. contributed towards acquisition of the data. L.M.E., E.C.M.N. and A.P.A.M. were involved in analysis and interpretation of the data. E.C.M.N. and A.P.A.M. drafted the manuscript. All authors contributed to the revision of the manuscript and approval of the final draft.

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Conflict of interest

None declared.

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