DNA methylation and gene expression differences in children conceived *in vitro* or *in vivo*

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Epidemiological data indicate that children conceived *in vitro* have a greater relative risk of low birth-weight, major and minor birth defects, and rare disorders involving imprinted genes, suggesting that epigenetic changes may be associated with assisted reproduction. We examined DNA methylation at more than 700 genes (1536 CpG sites) in placenta and cord blood and measured gene expression levels of a subset of genes that differed in methylation levels between children conceived *in vitro* versus *in vivo*. Our results suggest that *in vitro* conception is associated with lower mean methylation at CpG sites in placenta and higher mean methylation at CpG sites in cord blood. We also find that *in vitro* conception-associated DNA methylation differences are associated with gene expression differences at both imprinted and non-imprinted genes. The range of inter-individual variation in gene expression of the *in vitro* and *in vivo* groups overlaps substantially but some individuals from the *in vitro* group differ from the *in vivo* group mean by more than two standard deviations. Several of the genes whose expression differs between the two groups have been implicated in chronic metabolic disorders, such as obesity and type II diabetes. These findings suggest that there may be epigenetic differences in the gametes or early embryos derived from couples undergoing treatment for infertility. Alternatively, assisted reproduction technology may have an effect on global patterns of DNA methylation and gene expression. In either case, these differences or changes may affect long-term patterns of gene expression.

INTRODUCTION

There has been remarkable progress in Assisted Reproductive Technology (ART) with the introduction of new techniques that have improved success rates. Between the years 1996 and 2007, the number of ART cycles performed in the United States more than doubled (from 64 681 cycles in 1996 to 132 745 in 2007) resulting in a steady increase in the number of infants born who were conceived with ART (1). ART now accounts for at least 1%, and perhaps as much as 3%, of all live births in the western world (2). Approximately 3 million babies have been born using ART, to date (3).

Earlier studies reported no, or small, differences in the incidence of major and minor birth defects between children conceived *in vitro* or *in vivo* (4–8). Although the overall rate of congenital anomalies in children conceived by ART is low (4–6%), this rate still represents a significant increase over the background rate of major malformations (3%). When indices of pre- and post-natal development are measured, ART children, as a group, do not differ significantly from their control counterparts, except for having an increased incidence of low birth-weight (6,9–11) and being slightly taller on follow-up studies (12).

Several procedures that may be used in the ART process (hormonal stimulation, egg retrieval, *in vitro* fertilization

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(IVF), intra-cytoplasmic sperm injection (ICSI), micro-manipulation of gametes, exposure to culture medium, pre-implantation genetic diagnosis, *in vitro* oocyte maturation) could subject gametes and early embryos to environmental stress. In addition, a number of imprinted genes are expressed during the human pre-implantation period and expression of imprinted genes is thought to be especially sensitive to disruption by environmental factors (13,14). Despite the many reassuring reports on the safety of ART, there are epidemiological data suggesting that children conceived *in vitro* have a greater relative risk of rare disorders involving imprinted genes [Angelmann Syndrome (AS) (7,15,16) and Beckwith–Wiedemann Syndrome (BWS) (17)]. Recent studies have also shown that ovarian stimulation with gonadotrophins can cause DNA methylation and gene expression differences of some imprinted genes in oocytes from stimulated ovaries (18). Data from animal studies confirm that IVF and some embryo culture media are associated with such 'epimutations' (19,20).

The epigenetic defect in the imprinting disorders found most frequently after ART (AS and BWS) is loss of CpG site methylation at maternal alleles at the respective loci. This observation is consistent with data from animal studies, in which 'large offspring syndrome' is caused by hypomethylation of the maternal *Igf2r* allele (20). However, studies on mouse embryos demonstrate that methylation changes can affect both maternal and paternal alleles (21). Because data from human studies are obtained from infertile couples undergoing treatment with ART, it is difficult to determine whether any of the increased risks observed are an effect of ART or an effect of infertility, *per se*.

In this report, we present an analysis of DNA methylation at a large number of CpG sites, comparing children conceived by ART with a control group who had unassisted conception. We used a custom-designed methylation bead-array platform (GoldenGate Array, Illumina Inc., USA) (22,23) containing probes for 1536 CpG sites located in the promoters of more than 700 genes. We compared DNA methylation at each of these sites in placenta and cord blood taken from 10 children conceived *in vitro* and 13 children conceived *in vivo*, in duplicate. We used the locus-specific methylation differences detected in placenta and cord blood between the *in vitro* and *in vivo* groups as a guide to testing individual loci for differences in steady-state transcript levels in a larger population of *in vitro* or *in vivo* conceived children.

RESULTS

Are DNA methylation differences associated with mode of conception?

Site-specific CpG methylation in cord blood and placenta DNA samples was analyzed at 1536 CpG sites using a bead-array platform (GoldenGate Array, Illumina, Inc., USA) capable of high-throughput and multiplexed measurement with single CpG resolution. The vast majority of the CpG sites are located in the promoters of 736 genes that were selected on the basis of perceived importance in development or cancer (Supplementary Material, Table S1). Our custom-designed platform also contained probes for 182
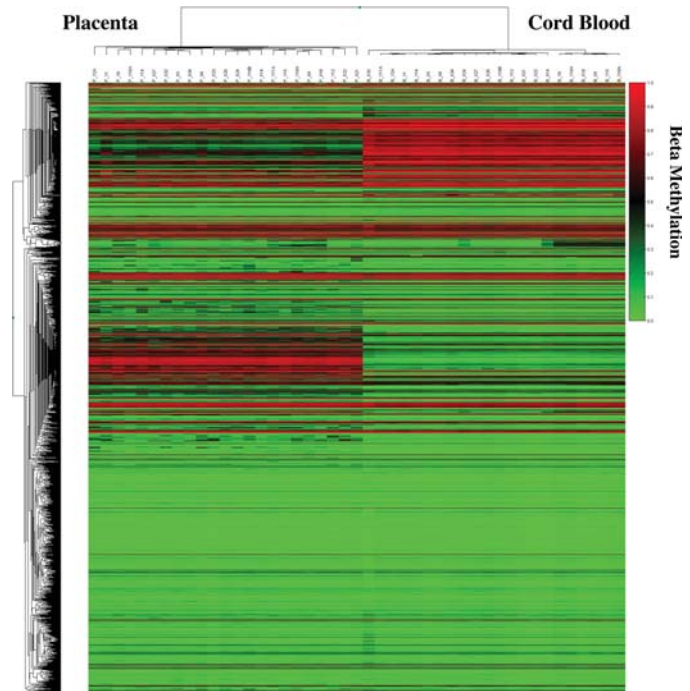
Figure 1. Hierarchical cluster analysis (heat map) of the methylation microarray data, based on all 1536 CpG loci, for 10 *in vitro* and 13 *in vivo* controls (12 for blood). Each sample was assayed in duplicate. Each duplicate clustered together (data not shown) so the mean beta values were used in this figure for ease of representation. Red indicates complete methylation, green indicates absence of methylation and intermediate levels are indicated by intermediate shades. Note that all cord blood samples cluster separately from placental samples.

CpG sites selected from differentially methylated regions of all genes known or suspected to be imprinted in humans and 23 CpG sites in autosomal genes reported to be expressed from only one allele (24) (Supplementary Material, Table S2).

Sodium bisulfite modified DNA from cord blood and placenta of 10 children conceived *in vitro* and 13 children conceived *in vivo* were assayed, in duplicate, for the fraction of each CpG site methylated in each sample (the 'beta value') using the 'GoldenGate' hybridization/primer extension/ligation and amplification protocol (22,25). The extent of methylation at a given CpG site was determined by comparing the proportion of signal from methylated and unmethylated alleles in the DNA sample (22). One cord blood sample from an *in vivo* conceived child failed internal quality control for completeness of bisulfite modification of unmethylated Cytosine's. The data from this sample were not included in subsequent analyses.

Unsupervised hierarchical clustering of the methylation results (Fig. 1) demonstrates that cord blood samples cluster separately from placenta samples. Thus, tissue types are separated easily on the basis of methylation levels at specific CpG sites, as expected if CpG methylation plays a significant role in the epigenetic identity of cell and tissue type. Validation of the fraction of CpGs methylated at each site was done for 82 CpGs using the HumanMethylation27 DNA Analysis BeadChip (Illumina, Inc., USA, Supplementary Material, Fig. S1 and see Materials and Methods).

Table 1. Inter-group DNA methylation differences in cord blood and placenta at 1536 CpG sites

Tissue	Number of CpGs	Number of CpGs with less methylation[a]	Number of CpGs with more methylation[a]	P*
Cord Blood	358	81 (23%)	277 (77%)	≤0.05
	1178	372 (32%)	806 (68%)	>0.05
	1536	453 (29%)	1083 (71%)	Total
Placenta	246	154 (63%)	92 (37%)	≤0.05
	1290	722 (56%)	568 (44%)	>0.05
	1536	876 (57%)	660 (43%)	Total

*Statistical significance was examined by two-way ANOVA and $P \leq 0.05$ was considered significant.
[a]*In vitro* conceived individuals versus *in vivo* conceived individuals.

The goal of our analysis was to determine whether there was any relationship between mean CpG methylation levels at genes probed by the array and mode of conception. Because there was no obvious clustering of *in vivo* or *in vitro* conceptions within tissues (Fig. 1), we performed two-way analysis of variance (ANOVA) at each CpG site, testing for differences between the two groups. When considering CpGs whose mean methylation levels differed between the *in vitro* and *in vivo* groups with $P \leq 0.05$, 358 CpGs (23%) differed in mean methylation levels in cord blood and 246 CpGs (16%) differed in mean methylation levels in placenta (Table 1). Of the 358 CpGs whose mean methylation level differed between the groups in cord blood, 277 (77%) were more methylated in the *in vitro* group and only 81 were less methylated. Of the CpGs that differed in placenta, 154 (63%) were less methylated in the *in vitro* group. The overall higher level of specific CpG site methylation in cord blood and lower level in placenta does not appear to be a property of only the CpGs with significant overall methylation differences between the groups, but appears to be a characteristic of the probe set, as a whole (Table 1). Of the remaining 1178 CpGs assayed in cord blood, 806 (68%) had higher average methylation (considering only the sign of the difference and not the magnitude) in the *in vitro* group. Similarly, of the 1290 additional CpGs assayed in placenta, 722 (56%) were less methylated in the *in vitro* group.

Are imprinted loci more susceptible to DNA methylation changes?

Epidemiological analyses indicate that children conceived *in vitro* have a higher incidence of rare disorders involving imprinted genes (2) and it has been suggested that epigenetic marks at imprinted genes may be more susceptible to environmental disruption than non-imprinted genes. As a gross test of this hypothesis, we compared the fraction of the 205 CpGs in imprinted genes and genes expressed monoallelically that were significantly different ($P \leq 0.05$) between the two groups (Table 2). Forty-four of the 205 CpGs (22%) were different in cord blood and 29 of the 205 CpGs (14%) were different in placenta. By comparison, 314 of 1331 CpGs (24%) in genes that are not known to be imprinted or expressed monoallelically were different in cord blood,

Table 2. Inter-group DNA methylation differences in cord blood and placenta at CpGs from imprinted or monoallelically expressed genes versus non-imprinted genes

Tissue	Number of CpGs representing imprinted/ monoallelic genes	Number of CpGs representing non-imprinted genes	P*
Cord Blood	44 (22%)	314 (24%)	≤0.05
	205	1331	Total
Placenta	29 (14%)	216 (16%)	≤0.05
	205	1331	Total

*Statistical significance was examined by two-way ANOVA and $P \leq 0.05$ was considered significant.

although 216 of these 1331 CpGs (16%) were different in placenta. Neither the cord blood nor the placenta comparison indicates that CpGs in imprinted and monoallelically expressed genes are more likely to be associated with significant DNA methylation differences, *per se*, than genes transcribed from both alleles.

Are differences in DNA methylation associated with transcription differences?

We wished to determine whether the CpG methylation differences observed predict differences in gene expression between the two groups. Because only a small number of individuals/observations were compared to assess methylation differences between the two groups (12 *in vivo* conceptions/24 beta values per CpG site versus 10 *in vitro* conceptions/20 beta values per CpG site), we assumed that there could be many false-positive and false-negative results at individual CpG sites. As a way of limiting false-positive methylation differences in candidate genes between the two groups, we applied the selection criterion that any candidate gene should be represented by at least two CpGs with mean methylation differences between the two groups. As a way of decreasing false-negative differences, we considered all CpG mean methylation differences with $P \leq 0.08$, rather than 0.05. This two-step selection process yielded 78 genes (163 CpGs) with methylation differences in cord blood and 40 genes (86 CpGs) with methylation differences in placenta (Table 3, Supplementary Material, Table S3).

We note that the character of the methylation differences between the two groups are such that the distributions of the *in vitro* CpG beta values at the two sites and the *in vivo* CpG beta values at the two sites have substantial overlap. The methylation fractions of two CpG sites in four genes whose beta value distributions were among the most disparate between the two groups are shown in Fig. 2.

The *in vitro* individuals have higher average methylation at *CEBPA* (Fig. 2A) and *COPG*2 (Fig. 2B) in cord blood, although they have lower average methylation at *MEST* (Fig. 2C) and *SERPINF1* (Fig. 2D) in placenta. The higher average methylation inter-group differences in cord blood (Fig. 2A and B) and lower average methylation inter-group differences in placenta (Fig. 2C and D) appear representative of the probe set inter-group differences, as a whole.

Table 3. Genes with methylation differences at two or more CpGs between *in vitro* and *in vivo* conceived individuals

Tissue	Gene*							
Cord Blood	*ACBD4*	*COPG2*	*FGFR2*	*HIST1H2AD*	*LYPLA2*	*PDPK1*	*RAET1L*	*TBX20*
	ACVR1B	*CPA4*	*FOXD3*	*HNRPA0*	*MAPK14*	*PEG10*	*RANBP1*	*TJP1*
	APOE	*CXCL12*	*FUBP1*	*HSPA1L*	*MEOX1*	*PEG3*	*RPL7A*	*TJP3*
	BMI1	*CYP26C1*	*GNAS*	*IGF2AS*	*MEST*	*PIK3CA*	*RPS15*	*TRIM8*
	CARHSP1	*DES*	*GPR176*	*IRX3*	*MYH11*	*PMAIP1*	*RPS18*	*UBE2I*
	CEBPA	*DGCR8*	*GPR19*	*KLF7*	*NASP*	*PRDX3*	*SEPHS1*	*USF1*
	CHEK2	*DLG7*	*GRB10*	*KRT5*	*NNAT*	*PRKCI*	*SHC1*	*UTF1*
	CLDN6	*EFEMP2*	*GTL2*	*LBX2*	*ORC1L*	*PSMB6*	*SHC3*	*WNT5A*
	COL3A1	*EGFR*	*GYLTL1B*	*LEFTY2*	*OTX2*	*PSMC3*	*SLC22A2*	
	COL6A3	*FGF5*	*H1FNT*	*LRRC47*	*PABPC4*	*PTK2*	*SNAI2*	
Placenta	*ARNT*	*DLX1*	*ESRRB*	*IL6*	*MGC3101*	*PAX4*	*SHC2*	*TAT*
	BCAN	*DLX2*	*FURIN*	*LAMC1*	*MUC3B*	*PEG3*	*SLC22A2*	*TDGF1*
	BMP1	*DPF3*	*GRB10*	*MCM5*	*MYL2*	*PYY*	*SOX1*	*TNNI1*
	COL6A3	*DPPA4*	*GYLTL1B*	*MDH1*	*NODAL*	*RPE65*	*SOX14*	*TTR*
	DES	*EGF*	*IDH1*	*MEST*	*ONECUT1*	*SERPINF1*	*STAT3*	*ZFP42*

Genes in bold have two CpGs with $P \leq 0.05$ and the remainder have one CpG with $P \leq 0.05$ and $P \leq 0.08$, except OTX2 which has two CpGs with $P \leq 0.08$.
*Statistical significance was examined by two-way ANOVA for repeated measures with an arbitrary threshold of $P \leq 0.08$.

Because CpG methylation levels are correlated inversely with steady-state transcript levels at some loci (26,27), we used the gene-specific mean methylation differences found between *in vitro* and *in vivo* conceived individuals as a guide to select candidate genes for comparison of transcript levels in 22–84 individuals conceived *in vitro* and 29–117 individuals conceived *in vivo*.

We selected 10 genes (*CEBPA*, *COPG2*, *EGFR, GNAS, MEST, NNAT, PEG3, PEG10, RPL7A* and *SLC22A2*) from the 78 genes with methylation differences (at two CpG sites) in cord blood, and five genes (*EGF, MEST, PEG3, SERPINF1* and *SLC22A2*) from the 40 genes with methylation differences (at two CpG sites) in placenta (Table 3, Supplementary Material, Table S3). Three of the genes (*MEST, PEG3* and *SLC22A2*) were selected because methylation differences were present in both cord blood and placenta. Two of the genes (*EGF* and *PEG10*) were selected because methylation was different at two CpG sites in one tissue and at one of the same CpG sites in the other tissue. Six genes (*CEBPA, COPG2, EGFR, GNAS, NNAT* and *RPL7A*) exhibited methylation differences in cord blood but not placenta and one gene (*SERPINF1*) exhibited methylation differences in placenta but not cord blood. Because approximately twice as many genes showed methylation differences in cord blood as placenta, we selected twice as many genes for analysis by real time RT–PCR based on methylation differences in cord blood as in placenta.

We analyzed steady-state transcript levels by real time RT–PCR and mRNA levels (relative to a housekeeping gene that was expressed at comparable levels—*GAPDH, GUSB, HPRT, TBP* or *TFRC*) were obtained using the $\Delta\Delta C_T$ method (28).

Of the 10 genes that exhibited methylation differences in cord blood (Table 4), transcripts from three of the genes (*MEST, PEG3* and *SLC22A2*) were present at very low levels; therefore, we did not attempt to make comparisons between the groups for these genes. Of the seven remaining genes, two (*CEBPA* and *COPG2*) showed significant differences in mean transcript levels between the *in vitro* and *in vivo* groups. Both showed higher average transcript levels in the *in vitro* group (1.77- and 2.05-fold increase, respectively). We note that, with the exception of *MEST*, which was not expressed in cord blood, *CEBPA* and *COPG2* also showed the biggest differences in CpG methylation between the two groups, with higher methylation observed in the *in vitro* population (23.6 and 16% for *COPG2* and 21.5 and 11.8% for *CEBPA*). The real time RT–PCR results in cord blood also showed that non-imprinted genes were predominantly more methylated in the *in vitro* group, whereas, with the exception of *MEST*, imprinted/monoallelic genes were less methylated. We did not observe a greater incidence of significant differences in transcript levels between the *in vitro* and *in vivo* populations in the imprinted/monoallelic gene group.

Of the five genes that exhibited methylation differences in placenta (Table 4), *EGF* transcripts were present at very low levels; therefore, we did not attempt to make comparisons between the groups for this gene. Of the four remaining genes, two (*SERPINF1* and *MEST*) showed significant differences in mean transcript levels between the *in vitro* and *in vivo* groups. Both showed higher average transcript levels in the *in vitro* group (1.81- and 2.09-fold increase, respectively). *SERPINF1* also showed the biggest difference in CpG methylation between the two groups, with lower methylation observed in the *in vitro* population (−23.6 and −12.1%). Although *MEST* showed significant differences in mean transcript levels, the difference in CpG methylation between the two groups were small (−2.6 and −1.2%), contrary to expectations. With the exception of *PEG3*, genes were predominantly less methylated in the *in vitro* group.

We also analyzed steady-state transcript levels in cord blood, of two genes (*EGF* and *SERPINF1*), which were initially selected on the basis of differences in methylation between the *in vitro* and *in vivo* groups in placenta
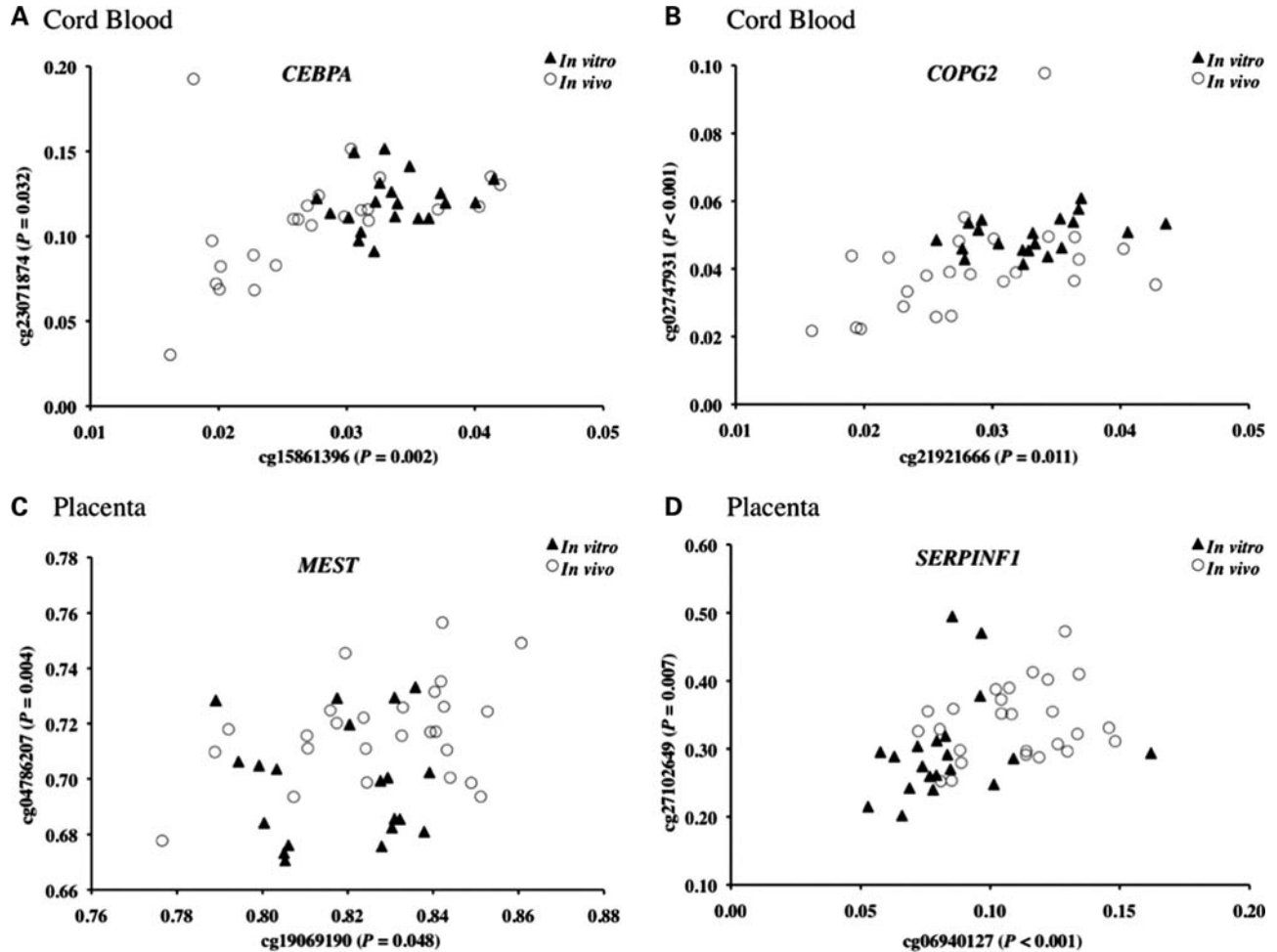
Figure 2. Beta values (fraction of CpG methylated at the indicated site) of CpG sites at which mean methylation differences were different at two sites within the same gene (two-way ANOVA, $P \leq 0.08$). (**A**) *CEBPA* in cord blood, (**B**) *COPG2* in cord blood, (**C**) *MEST* in placenta and (**D**) *SERPINF1* in placenta. The points represent data from 12 *in vivo* conceptions/24 beta values per CpG site versus 10 *in vitro* conceptions/20 beta values per CpG site. The *in vitro* individuals have slightly higher average methylation at the two *CEBPA* and *COPG2* sites in cord blood and slightly lower average methylation at *MEST* and *SERPINF1* CpG sites in placenta.

(Table 5). No significant changes in transcript levels of *EGF* and *SERPINF1* were observed in cord blood.

We then analyzed steady-state transcript levels in placenta, of seven genes (*CEBPA*, *COPG2*, *EGFR*, *GNAS*, *NNAT*, *PEG10* and *RPL7A*), which were initially selected on the basis of differences in methylation between the *in vitro* and *in vivo* groups in blood (Table 5). Unexpectedly, four of these genes (*CEBPA*, *COPG2*, *NNAT* and *RPL7A*) showed significant changes in transcript levels between the *in vitro* and *in vivo* groups.

As observed for the between-group differences in CpG methylation (Fig. 2), the genes for which significant differences were observed in mean transcript levels also showed substantial overlap between the *in vitro* and *in vivo* groups. The distribution of individual relative transcript levels is shown (Fig. 3) for four of the eight between-group comparisons that differed significantly in mean transcript levels (Table 4). Although the level of *CEBPA* transcripts observed in cord blood of the *in vitro* group is largely within the range of the *in vivo* group (Fig. 3A), 8 of the 70 individuals

assayed in the *in vitro* group are outside two standard deviations of the *in vivo* mean and two individuals are further than three standard deviations from the *in vivo* mean. *COPG2* levels were highly variable and a small number of *in vitro* (two), as well as *in vivo* (four), individuals were found to be more than two standard deviations from the *in vivo* mean (Fig. 3B). Although the mean level of *MEST* transcripts in placenta (Fig. 3C) differs significantly between the two groups, only two of the *MEST* observations in the *in vitro* group lie outside two standard deviations of the *in vivo* mean. However, *SERPINF1* transcript levels are outside two standard deviations from the *in vivo* mean for 13 of the 68 individuals assayed in the *in vitro* group (Fig. 3D).

DISCUSSION

We report the first comprehensive analysis of differences in DNA methylation at a large number of CpG sites, including sites in all imprinted genes, between children conceived using ART and a control group who had natural conception.

Table 4. Differences in transcript level between *in vitro* and *in vivo* groups in genes with methylation differences at two or more CpGs

Tissue	Gene	% Increase or decrease in methylation[a]	No. individuals (*in vitro/in vivo*)	Fold change[b]	P*
Cord Blood	*COPG2*	23.6, 16.0	73/85	2.05	<0.001
	CEBPA	21.5, 11.8	70/93	1.77	0.050
	RPL7A	13.4, 9.3	73/100	0.83	0.121
	EGFR[c]	13.8, −8.4	22/33	0.70	0.445
	MEST[d]	30.1, 13.5	Low expression		
	SLC22A2[d]	−5.7, −0.2	Low expression		
	PEG10[d]	−5.6, −2.8	24/34	0.60	0.233
	PEG3[d]	−5.4, −5.0	Low expression		
	GNAS[d]	−4.7, −1.4	66/63	0.90	0.879
	NNAT[d]	−1.7, −1.5	25/29	1.00	0.675
Placenta	*SERPINF1*	−23.6, −12.1	68/68	1.81	<0.001
	EGF	−3.3, −0.8	Low expression		
	PEG3[d]	9.5, 3.8	60/71	1.61	0.190
	SLC22A2[d]	−7.6, −6.9	67/67	0.99	0.908
	MEST[d]	−2.6, −1.2	69/69	2.09	<0.001

*Student's *t*-test ($P \leq 0.05$).
[a]% Increase or decrease in methylation in the *in vitro* group, at two CpGs for each gene.
[b]Fold change in *in vitro* conceived individuals compared with *in vivo* conceived individuals, obtained using the $\Delta\Delta C_T$ method (28).
[c]Gene known/suspected of being transcribed from one allele.
[d]Genes known/suspected to be imprinted.

Table 5. Differences in transcript level between *in vitro* and *in vivo* groups in genes without significant methylation differences at two or more CpGs

Tissue	Gene	% Increase or decrease in methylation[a]	No. individuals (*in vitro/in vivo*)	Fold change[b]	P*
Cord Blood	*SERPINF1*	−1.6, −0.1	58/60	1.15	0.192
	EGF	0.3, 0.4	50/52	1.02	0.837
Placenta	*COPG2*	4.3, 3.0	84/117	0.63	0.024
	RPL7A	4.3, 1.2	62/62	0.82	0.049
	CEBPA	4.1, −0.3	70/71	1.72	0.025
	EGFR[c]	4.2, −5.9	70/71	0.89	0.568
	PEG10[d]	−4.6, −1.2	80/112	0.84	0.106
	GNAS[d]	−2.8, −2.6	62/62	1.03	0.909
	NNAT[d]	0.3, 0.2	82/111	0.55	<0.001

*Student's *t*-test ($P \leq 0.05$).
[a]% Increase or decrease in methylation in the *in vitro* group, at two CpGs for each gene.
[b]Fold change in *in vitro* conceived individuals compared with *in vivo* conceived individuals, obtained using the $\Delta\Delta C_T$ method (28).
[c]Gene known/suspected of being transcribed from one allele.
[d]Genes known/suspected to be imprinted.

One goal of our analysis was to determine whether there was any relationship between CpG methylation levels at specific sites and mode of conception.

Our methylation results demonstrated that embryonic and extraembryonic tissue types are separated easily on the basis of methylation levels at specific CpG sites, as expected if CpG methylation plays a significant role in the epigenetic identity of cell and tissue type.

Because the site-specific CpG methylation levels showed no obvious clustering of *in vivo* or *in vitro* conceptions within tissues, we performed two-way ANOVA at each CpG site, testing for differences between the two groups. We observed an overall lower level of specific CpG site methylation in placenta and higher level in cord blood. If these differences are characteristic of differences in embryonic versus extraembryonic tissues, it may suggest differences in the way 'outer' and 'inner' blastomeres of pre-implantation embryos (29,30) respond to *in vitro* culture. Methylation of normally unmethylated CpGs at imprinted genes has been reported to occur sporadically in mouse embryos that have been cultured *in vitro* (31). However, we are unaware of any other reports of widespread increases in CpG methylation (18% of all probes on the array) in embryonic tissues from human or animal embryos cultured *in vitro*. The overall higher level of specific CpG site methylation observed in cord blood and lower level observed in placenta does not appear to be a property of only the CpGs that differ significantly but appears to be a characteristic of the probe set, as a whole. The overall lower level of methylation we observed in placenta in the *in vitro* group is consistent with observations for several imprinted genes in placentae of mouse embryos subjected to prior culture *in vitro* (19,30). The most likely alternative explanation is that the increases in CpG methylation in cord blood reflect a bias in the modest number of genes present on the array with respect to the potential for
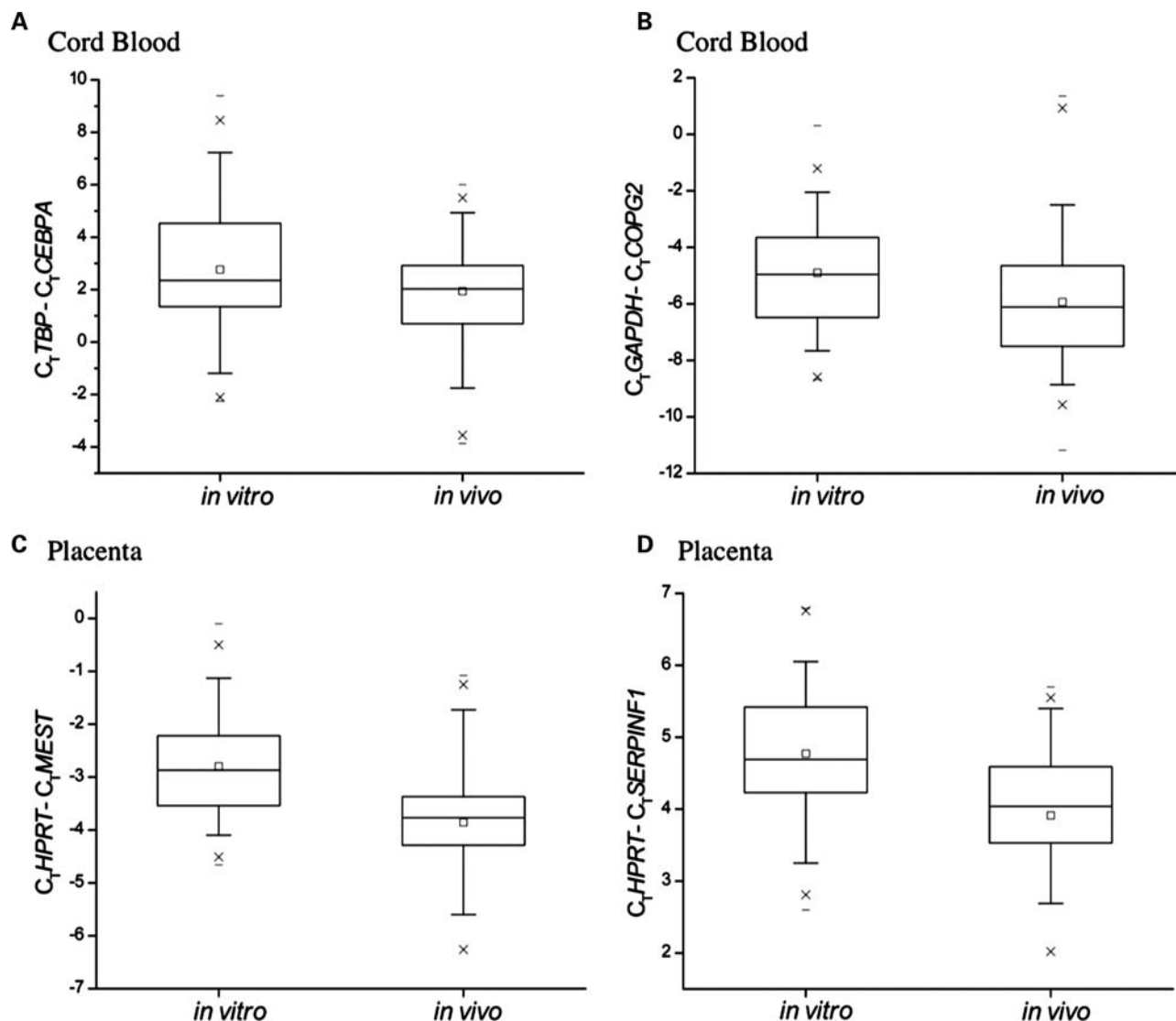
Figure 3. Boxplots with whiskers showing transcript levels for four genes in which mean transcript levels differ significantly (Student's *t* test, $P \leq 0.05$) between *in vitro* and *in vivo* populations (**A**) *CEBPA* in cord blood, (**B**) *COPG2* in cord blood, (**C**) *MEST* in placenta and (**D**) *SERPINF1* in placenta. The plots represent data from 93, 85, 69 and 68 *in vivo* conceptions for *CEBPA*, *COPG2*, *MEST* and *SERPINF1*, respectively, versus 70, 73, 69 and 68 *in vitro* conceptions for *CEBPA*, *COPG2*, *MEST* and *SERPINF1*, respectively. Boxplot interpretation: the box contains the middle 50% of the data. The upper edge of the box indicates the 75th percentile of the data set, and the lower edge indicates the 25th percentile (the range of the middle two quartiles is known as the inter-quartile range). The mean (open square) is shown and the line in the box indicates the median value with the ends of the vertical lines or 'whiskers' indicating the 95th and 5th percentiles of the data set. The symbol (x) depicts the 99th and 1st percentiles of the data set, and (-) depicts the maximum data range.

increases in methylation versus decreases in methylation; i.e. if the 'normal' state of most CpGs on the array in cord blood DNA is unmethylated/undermethylated, as appears to be the case by inspection of Fig. 1, then the only change possible to detect is to become more methylated.

Overall, our results indicate that conception *in vitro* is associated with small, but statistically significant, differences in methylation of CpG sites compared with individuals conceived *in vivo*. Whether this association is a result of the ART process itself, or a characteristic of the patient population served by ART, cannot be determined at this time.

We also investigated whether the CpG methylation differences observed predict differences in gene expression between the two groups. Our results suggest that a fraction

of the observed differences in methylation are associated with differences in transcription of adjacent genes, however, neither the magnitude nor the direction of the change between groups can be predicted from the difference in CpG methylation at many of the sites examined. The magnitude of the effect on transcription appears to be relatively small for most genes, although a fraction of *in vitro* conceived individuals have transcript levels at individual genes that are outside the range found in the *in vivo* conceived population. Of note, in the cases for which a significant between-group difference is observed, is that the entire ART population appears to be shifted with respect to the control population in both methylation (Fig. 2) and steady state transcript levels of individual genes (Fig. 3). This relative shift in the

population mean suggests that any effect of ART is relatively constant, and in the same direction for each individual.

We note that 6 of the 12 genes tested for differences in steady-state transcript level between the two groups (Table 4) are transcriptionally imprinted (32) and one (*EGFR*) may be transcribed predominately from one allele (24). One might have predicted, a priori, that imprinted genes are more likely to be influenced by environmental factors because only one allele need be activated or silenced to have a significant effect on transcription. In fact, a large fraction of the imprinted genes that have been tested in the extraembryonic tissues of mouse embryos subject to *in vitro* culture do show aberrant expression of the normally silenced allele (21). Although we observe that two of the six imprinted genes examined show significant differences in steady state transcript level, we do not observe enrichment for imprinted genes among those with significant differences because three of the six genes that are not known to be imprinted in Tables 4 and 5 also show differences in mean transcript levels in placenta. Whether this circumstance indicates that the genome of extraembryonic cells is especially sensitive to disruption by environmental factors or biological factors that occur in conjunction with infertility is a question that bears further investigation. In this regard, it is noteworthy that significant methylation differences were observed at only two of the six loci that showed significant transcript level differences in placenta (Tables 4 and 5). It is possible that this discrepancy is the result of meso-scale intra-individual placental mosaicism in the fraction of cells that contain a methylated CpG site or that express a particular allele (33,34). All of the placental biopsies used in this study were taken from directly behind the umbilical cord insertion but adjacent samples could differ in some epigenetic parameters (35). It is also likely that our failure to detect methylation differences at some loci reflects our inability to discriminate true methylation differences between the groups because of the small number of individuals assayed using the array. In addition, transcript levels of some genes may be more or less sensitive to gene methylation differences in placenta than in cord blood.

At this juncture, the differences observed in transcript levels are of unclear phenotypic significance. However, it is notable that one of the two genes exhibiting a significant difference in mean transcript level in blood (*CEBPA*) and four of the six genes with significant differences in placenta (*CEBPA*, *MEST*, *NNAT* and *SERPINF1*) have been linked to adipocyte development and differentiation (36,37), insulin signaling and/or obesity (38,39). Whether these gene regulatory abnormalities observed at birth might be connected to birth weight, obesity, type II diabetes or hypertension later in life is a significant concern. Such differences have the potential to affect many aspects of embryonic development and fetal growth, as well as influence long-term patterns of gene expression that might be associated with increased risk of many human diseases.

In conclusion, we have shown that *in vitro* conception is associated with quantitative differences in DNA methylation and that some of these differences may have a significant effect on gene expression. There seem to be only two likely explanations for these differences between children conceived by ART and children conceived *in vivo*; either infertile

couples, themselves, are more likely to have gametes that bear abnormal epigenetic marks or some aspect of the ART process causes the epigenetic differences we observe. Determination of which of these possibilities is most likely is an important question that may be approached by comparing the offspring of couples who are infertile as a result of physical barriers to fertilization, such as tubal ligation, with those who are infertile for other reasons. If ART is causal of the methylation and gene expression differences we observe, ART procedures may be modified to minimize these effects after testing in a suitable animal model.

MATERIALS AND METHODS

Patient recruitment and sample collection

An attempt was made to match *in vivo* and *in vitro* cases with regards to maternal age, race and gestational age. The *in vivo* group had no prior history of infertility and the index pregnancy was achieved spontaneously, without the use of any infertility medications or treatments. We have provided the demographic data showing maternal and paternal age, race, suspected cause of infertility, gestational age, fetal sex and weights at delivery for the individuals in the GoldenGate Assay in an additional supplemental table (Supplementary Material, Table S4). All the patients were stimulated with the standard ovulation induction drugs. Although three patients had mild male factor infertility, successful IVF occurred with conventional IVF and did not necessitate ICSI. The embryo culture medium and the incubation parameters were the same for all the samples. Embryo transfer was performed on Day 3 for seven out of the ten individuals, and frozen embryo transfer was performed on Day 2 for the remaining three individuals. Cord blood and placenta samples were collected from each *in vitro* and *in vivo* newborn. Written, informed consent was obtained in advance from the mother of each newborn (University of Pennsylvania I.R.B. approved protocol no. 804530).

DNA and RNA isolation

Samples for DNA isolation were frozen and stored at $-80°C$ before DNA extraction using standard phenol-chloroform extraction methods. DNA was dissolved in 10 mM TrisCl, pH 8.0, quantified using a spectrophotometer and stored at $-80°C$ until use. Samples for RNA isolation were pre-treated with RNAlater® (Applied Biosystems/Ambion, USA), according to the manufacturer's guidelines, then stored at $-80°C$ before RNA extraction. Total cellular RNA was extracted from each sample using Trizol (Gibco BRL, USA), according to the manufacturer's instructions. RNA was dissolved in distilled H_2O, quantified using a spectrophotometer and stored at $-80°C$ until use.

Bisulphite conversion and methylation assay

Unmethylated cytosine in genomic DNA (500 ng) was converted to uracil by treatment with sodium bisulfite using the EZ DNA Methylation Kit[TM] (Zymo Research Corp., USA), according to the manufacturer's recommendations.

Site-specific CpG methylation was analyzed in the converted DNA template (5 µl at 50 ng/µl) using the GoldenGate Assay (Illumina, Inc., USA) on a bead array-based platform capable of high-throughput and multiplexed measurement with single CpG resolution. Bisulfite-converted genomic DNA from one conversion was assayed in duplicate. Bisulfite-treated genomic DNA was mixed with assay oligonucleotides. Each allele specific oligonucleotide is complimentary to either the converted U (unmethylated site) or the protected C (methylated site). Single-stranded PCR products were prepared by denaturation, and then hybridized to a Sentrix Array Matrix (40). The array hybridization was conducted under a temperature gradient program. Following hybridization, primers were extended and ligated to locus specific oligonucleotides, creating a template for universal PCR. Fluorescently Cy3/Cy5 labeled PCR primers were used to create a detectable product and individual assays localized to specific bead types by hybridization of the address sequences. The arrays were imaged using a BeadArray Reader scanner (41). Image processing and intensity data extraction software was as described previously (42). The extent of methylation at a given CpG site was determined by comparing the proportion of signal from methylated and unmethylated alleles in the DNA sample (20). Validation of fraction of CpGs methylated at each site was done for 82 CpGs using the Illumina Infinium array (Supplementary Material, Fig. S1). We also validated two of the same individual CpGs in the *SNURF/SNURPN* locus by bisulfite pyrosequencing of the same samples analyzed on the GoldenGate array. (Data not shown.) Although the absolute values of methylation found by the two assays were different, the ranges were similar and positively correlated. In addition, we validated the difference between mean methylation in *in vitro* and *in vivo* groups at *CEBPA* by quantitative Real time PCR (Roche LightCyler), comparing *BstU*I cleaved versus uncleaved DNA in the same cord blood samples shown in Fig. 2A. (Data not shown.) *BstU*I is a methylation-sensitive restriction endonuclease that recognizes the same *CEBPA* CpG site assayed on the Golden-Gate array. Both assays distinguished the *in vitro* from the *in vivo* group and the between group means were significantly different.

Quantitative real time RT–PCR

First-strand cDNA was obtained using SuperScript[TM] III Reverse Transcriptase (RT) (Invitrogen Corporation, USA). To produce cDNA from total RNA, a mixture containing 1 µg extracted total RNA, 0.5 µg oligo(dT)$_{18}$ primer and 1 µl dNTP mix (10 mM each) in final 13 µl of solution was heated to 65°C for 5 min, cooled down on ice for 2 min, and then added to a 7 µl of reaction mixture (4 µl SuperScript[TM] III RT buffer (10×), 1 µl DTT (0.1 M), 1 µl RNaseOUT[TM] Recombinant RNase inhibitor (40 U/µl; Invitrogen Corporation, USA) and 1 µl SuperScript[TM] III M-MLV RT (200 U/µl), for reverse transcription at 50°C for 60 min. Reactions were terminated at 70°C for 15 min. RT products were stored at −20°C until use. Quantitative real-time RT–PCR assays were carried out using a 7700 Sequence Detector (Applied Biosystems, USA). All probes spanned exon/intron boundaries to prevent genomic DNA amplification. Gene-specific primer sequences and annealing temperatures and Taqman probe information are listed in Supplementary Material, Table S5.

Steady state mRNA levels were measured using gene-specific TaqMan probes (Applied Biosystems, USA). Taqman PCR reactions were performed by mixing 1 µl of cDNA (50 ng/µl) with 19 µl of reaction mixture (10 µl Taqman Master Mix (2×), 1 µl Taqman primer (20×) and 8 µl nuclease free dH$_2$O) and amplified under the following conditions: 50°C for 2 min, 95°C for 10 min, followed by 45 cycles of 95°C for 15 s and 60°C for 60 s.

Steady state mRNA levels of *COPG2* and the housekeeping gene *GAPDH* were measured using gene-specific primers and QuantiFast SYBR Green PCR Master Mix (Qiagen, USA). PCR reactions were performed by mixing 1 µl of cDNA (50 ng/µl) with 24 µl of reaction mixture [10 µl QuantiFast SYBR Green PCR Master Mix (2×), 2.5 µl forward primer (10 µM), 2.5 µl reverse primer (10 µM) and 6.5 µl nuclease free dH$_2$O] and amplified under the following conditions: 95°C for 5 min, followed by 45 cycles of 95°C for 10 s and 60°C for 30 s. A melting curve analysis of the PCR products was performed to verify their specificity and identity. Relative gene expression levels were obtained using the $\Delta\Delta C_T$ method (28).

Statistical analysis

The statistical significance of the methylation datasets representing the *in vivo* and *in vitro* group was examined with two-way ANOVA for repeated measures. A less stringent selection criterion was used for selection of genes in Table 3 and Supplementary Material, Table S3 ($P \leq 0.08$). Data from the real time RT–PCR experiments were analyzed with Student's unpaired or paired *t*-tests where appropriate. $P \leq 0.05$ were considered significant.

SUPPLEMENTARY MATERIAL

Supplementary Material is available at *HMG* online.

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