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...
(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 [Angelman 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 and 12 controls (12). DNA samples was analyzed at 1536 CpG sites using a custom-designed platform also contained probes for 182 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 placental 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).

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.
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 \textit{in vivo} or \textit{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 \textit{in vitro} and \textit{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 \textit{in vitro} group and only 81 were less methylated. Of the CpGs that differed in placenta, 154 (63\%) were less methylated in the \textit{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 \textit{in vitro} group. Similarly, of the 1290 additional CpGs assayed in placenta, 722 (56\%) were less methylated in the \textit{in vitro} group.

**Are imprinted loci more susceptible to DNA methylation changes?**

Epidemiological analyses indicate that children conceived \textit{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, 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, \textit{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 \textit{in vitro} conceptions/24 beta values per CpG site versus 10 \textit{in vivo} 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 \textit{in vitro} CpG beta values at the two sites and the \textit{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 \textit{in vitro} individuals have higher average methylation at \textit{CEBP} (Fig. 2A) and \textit{COPG} (Fig. 2B) in cord blood, although they have lower average methylation at \textit{MEST} (Fig. 2C) and \textit{SERPINF} (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 1. Inter-group DNA methylation differences in cord blood and placenta at 1536 CpG sites

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Number of CpGs</th>
<th>Number of CpGs with less methylation</th>
<th>Number of CpGs with more methylation</th>
<th>$P$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cord</td>
<td>358</td>
<td>81 (23%)</td>
<td>277 (77%)</td>
<td>$\leq 0.05$</td>
</tr>
<tr>
<td>Blood</td>
<td>1178</td>
<td>372 (32%)</td>
<td>806 (68%)</td>
<td>$&gt; 0.05$</td>
</tr>
<tr>
<td></td>
<td>1536</td>
<td>453 (29%)</td>
<td>1083 (71%)</td>
<td>Total</td>
</tr>
<tr>
<td>Placenta</td>
<td>246</td>
<td>154 (63%)</td>
<td>92 (37%)</td>
<td>$\leq 0.05$</td>
</tr>
<tr>
<td></td>
<td>1290</td>
<td>722 (56%)</td>
<td>568 (44%)</td>
<td>$&gt; 0.05$</td>
</tr>
<tr>
<td></td>
<td>1536</td>
<td>876 (57%)</td>
<td>660 (43%)</td>
<td>Total</td>
</tr>
</tbody>
</table>

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

In \textit{in vitro} conceived individuals versus \textit{in vivo} conceived individuals.

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

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Number of CpGs representing imprinted/monoallelic genes</th>
<th>Number of CpGs representing non-imprinted genes</th>
<th>$P$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cord</td>
<td>44 (22%)</td>
<td>314 (24%)</td>
<td>$\leq 0.05$</td>
</tr>
<tr>
<td>Blood</td>
<td>205</td>
<td>1331</td>
<td>Total</td>
</tr>
<tr>
<td>Placenta</td>
<td>29 (14%)</td>
<td>216 (16%)</td>
<td>$\leq 0.05$</td>
</tr>
<tr>
<td></td>
<td>205</td>
<td>1331</td>
<td>Total</td>
</tr>
</tbody>
</table>

*Statistical significance was examined by two-way ANOVA and $P \leq 0.05$ was considered significant.
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 ΔΔC_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 group (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.
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.
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 cord blood and higher level in placenta reflect a bias in the modest number of genes present on the array with respect to the potential for

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

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Gene</th>
<th>% Increase or decrease in methylation</th>
<th>No. individuals (in vitro/in vivo)</th>
<th>Fold changeb</th>
<th>P*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cord Blood</td>
<td>COPEG2</td>
<td>+23.6, 16.0</td>
<td>73/85</td>
<td>2.05</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>CEBPA</td>
<td>21.5, 11.8</td>
<td>70/93</td>
<td>1.77</td>
<td>0.050</td>
<td></td>
</tr>
<tr>
<td>RPL7A</td>
<td>13.4, 9.3</td>
<td>73/100</td>
<td>0.83</td>
<td>0.121</td>
<td></td>
</tr>
<tr>
<td>EGFR</td>
<td>13.8, −8.4</td>
<td>22/33</td>
<td>0.70</td>
<td>0.445</td>
<td></td>
</tr>
<tr>
<td>MEST</td>
<td>30.1, 13.5</td>
<td>Low expression</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SERPINF1</td>
<td>4.1, 3.8</td>
<td>60/71</td>
<td>1.61</td>
<td>0.190</td>
<td></td>
</tr>
<tr>
<td>EGF</td>
<td>−3.3, −0.8</td>
<td>Low expression</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PEG3</td>
<td>9.5, 3.8</td>
<td>67/67</td>
<td>0.99</td>
<td>0.908</td>
<td></td>
</tr>
<tr>
<td>SLC22A2</td>
<td>−7.6, −6.9</td>
<td>67/67</td>
<td>0.99</td>
<td>0.908</td>
<td></td>
</tr>
<tr>
<td>MEST</td>
<td>−2.6, −1.2</td>
<td>69/69</td>
<td>2.09</td>
<td>&lt;0.001</td>
<td></td>
</tr>
</tbody>
</table>

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

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Gene</th>
<th>% Increase or decrease in methylation</th>
<th>No. individuals (in vitro/in vivo)</th>
<th>Fold changeb</th>
<th>P*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cord Blood</td>
<td>SERPINF1</td>
<td>−1.6, −0.1</td>
<td>58/60</td>
<td>1.15</td>
<td>0.192</td>
</tr>
<tr>
<td>EGF</td>
<td>0.3, 0.4</td>
<td>50/52</td>
<td>1.02</td>
<td>0.837</td>
<td></td>
</tr>
<tr>
<td>Placenta</td>
<td>COPEG2</td>
<td>4.3, 3.0</td>
<td>84/117</td>
<td>0.63</td>
<td>0.024</td>
</tr>
<tr>
<td>RPL7A</td>
<td>4.3, 1.2</td>
<td>62/62</td>
<td>0.82</td>
<td>0.049</td>
<td></td>
</tr>
<tr>
<td>CEBPA</td>
<td>4.1, −0.3</td>
<td>70/71</td>
<td>1.72</td>
<td>0.025</td>
<td></td>
</tr>
<tr>
<td>EGFR</td>
<td>4.2, −5.9</td>
<td>70/71</td>
<td>0.89</td>
<td>0.568</td>
<td></td>
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<tr>
<td>PEG3</td>
<td>−4.6, −1.2</td>
<td>80/112</td>
<td>0.84</td>
<td>0.106</td>
<td></td>
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<tr>
<td>SERPINF1</td>
<td>−2.8, −2.6</td>
<td>62/62</td>
<td>1.03</td>
<td>0.909</td>
<td></td>
</tr>
<tr>
<td>NNAT</td>
<td>0.3, 0.2</td>
<td>82/111</td>
<td>0.55</td>
<td>&lt;0.001</td>
<td></td>
</tr>
</tbody>
</table>

### References

1. Student’s t-test (P ≤ 0.05).
2. % Increase or decrease in methylation in the in vitro group, at two CpGs for each gene.
3. Fold change in in vitro conceived individuals compared with in vivo conceived individuals, obtained using the ΔΔC_T method (28).
4. Gene known/suspected of being transcribed from one allele.
5. Genes known/suspected to be imprinted.
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 H2O, 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™ (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 locusspecific 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 LightCycler), comparing BstUI cleaved versus uncleaved DNA in the same cord blood samples shown in Fig. 2A. (Data not shown.) BstUI is a methylation-sensitive restriction endonuclease that recognizes the same CEBPA CpG site assayed on the GoldenGate 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™ 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™ III RT buffer (10 ×), 1 μl DTT (0.1 M), 1 μl RNaseOUT™ Recombinant RNase inhibitor (40 U/μl; Invitrogen Corporation, USA) and 1 μl SuperScript™ 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 dH2O) 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 dH2O] 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 ΔΔCt 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 ≤ 0.08). Data from the real time RT–PCR experiments were analyzed with Student’s unpaired or paired t-tests where appropriate. P ≤ 0.05 were considered significant.

SUPPLEMENTARY MATERIAL

Supplementary Material is available at HMG online.

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