Microarray assessment of methylation in individual mouse blastocyst stage embryos shows that in vitro culture may have widespread genomic effects

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BACKGROUND: Although assisted reproductive technology (ART) is reported to result in abnormal genomic imprinting and/or altered genomic methylation, few if any studies have used high-throughput methods to analyze genomic methylation in ART embryos. We hypothesized that a microarray-based assessment of genomic methylation could be used to reveal differences between ART and normal preimplantation embryos.

METHODS: In this pilot study, we performed methylation-sensitive amplification of genomic DNA from preimplantation mouse blastocysts, obtained by natural mating and either maintained in vivo until E3.5 (n = 4) or cultured in vitro (n = 4) from E0.5 until E3.5. An oligonucleotide microarray was then used to perform comparative hybridization of amplified DNA, allowing us to assess relative methylation at ≏16 000 loci on mouse chromosome 7.

RESULTS: We show that for in vivo derived embryos, the methylation/microarray results were strikingly consistent. In contrast, all four in vitro cultured embryos showed evidence of generalized hypermethylation as well as greater locus-to-locus variability, when compared with in vivo derived embryos. Genomic segments that overlapped exons and CpG islands were most likely to be hypomethylated in both normal and experimental blastocysts. Other sequence features, such as repetitive elements, were not associated with the presence of or the degree of methylation.

CONCLUSIONS: We conclude that a general assessment of genomic methylation in blastocyst stage embryos is technically feasible. Data from this small sample suggest that in vitro embryo culture is associated with generalized hypermethylation as well as increased locus-to-locus variability in methylation. However, it is premature to conclude that this is a general property of in vitro cultured blastocysts.

Key words: embryo / assisted reproduction technology / microarray / DNA methylation / chromosome 7

Introduction

Assisted reproduction technology (ART) is widely used to treat human infertility and contributes to the birth of 1–2% of babies in developed countries. Children conceived through ART are at an increased risk of low birthweight and malformations (Williams et al., 2010) and have been said to be at increased risk of epigenetic disorders (Manipalviratn et al., 2009). Multiple recent studies have raised concern about a higher prevalence of imprinting disorders, such as Beckwith–Weidemann syndrome (BWS) and Angelman syndrome (AS), in children conceived via ART (Cox et al., 2002; DeBaun et al., 2003; Gicquel et al., 2003; Maher et al., 2003a,b; Halliday et al., 2004; Chang et al., 2005; Ludwig et al., 2005; Rossignol et al., 2006; Sutcliffe et al., 2006; Doornbos et al., 2007). The increased risk of BWS in children conceived via IVF is estimated to be 3–6-fold (DeBaun et al., 2003; Maher et al., 2003a,b). Importantly, molecular studies demonstrate that ~90% of children with IVF-associated BWS have a molecular defect in an imprinted region, most commonly hypomethylation of the KCNQ1 gene (DeBaun et al., 2003; Gicquel et al., 2003; Maher et al., 2003a,b; Halliday et al., 2004; Chang et al., 2005; Doornbos et al., 2007). In contrast, imprinting defects are detected in only ~50% of sporadic BWS cases in the general population (Owen and Segars, 2009). Furthermore, molecular analysis of IVF-associated AS cases indicates that five of seven reported cases demonstrate a
methylation defect in a known imprinting center (Cox et al., 2002; Orstavik et al., 2003; Ludwig et al., 2003; Sutcliffe et al., 2006); it is improbable that this occurred randomly, given that methylation abnormalities are present in only ~5% of AS patients. This clinical evidence supports the hypothesis that the IVF process results in a higher incidence of methylation abnormalities in imprinted genes. Interestingly, other evidence does not support the idea that such abnormalities are a general finding (Tierling et al., 2010).

Animal models also support a link between ART and disordered genomic methylation. In both mice and sheep, altered in vitro embryo culture conditions have been shown to result in altered methylation of specific alleles (Doherty et al., 2000; Khosla et al., 2001; Young et al., 2001; Mann et al., 2004; Li et al., 2005). Other studies have shown that mouse embryos derived from superovulated females also show specific methylation abnormalities at several loci, suggesting that IFV, even without embryo culture, may affect normal processes (Sato et al., 2007; Market-Velker et al., 2010). A recent study has also shown that embryo transfer alone can result in an increased incidence of methylation abnormalities (Rivera et al., 2008), suggesting that methylation of imprinted alleles in the early embryo is highly sensitive to any manipulation.

Although most discussions of methylation abnormalities related to IVF have focused on imprinted loci, it is important to note that the role of cytosine methylation is multifaceted and complex, and that imprinting is only one of the many processes in which DNA methylation plays a vital role in embryonic growth, placental function, carcinogenesis and neurobehavioral processes (Gopalan-krishnan et al., 2008; Mehler, 2008; Novakovic et al., 2008; Shelton et al., 2008). It is plausible that aberrant DNA methylation could have a serious impact on overall health. If ART can result in abnormal methylation that leads to imprinting disorders such as BWS and AS, then it is likely that ART embryos harbor methylation abnormalities at non-imprinted (and therefore silent) loci throughout the genome. In support of this idea, a recent microarray-based study that examined methylation in newborn cord blood and placenta found the evidence of widespread methylation differences between normal and IVF pregnancies (Katari et al., 2009). Based on this reasoning, we have hypothesized that ART conditions may result in hitherto unrecognized, generalized abnormalities in methylation and that these abnormalities might be identifiable through the use of high-throughput genome analysis methods.

Although several tools for the global assessment of genomic methylation have been developed (reviewed in Zilberman and Henikoff, 2007; Bibikova and Fan, 2010), they generally require substantial amounts of input DNA and are thus unsuitable for the investigation of preimplantation embryos. Our laboratory has recently developed and validated a method, based on methylation-sensitive PCR and microarray analysis, that allows the simultaneous assessment of the methylation state of thousands of (CpG) dinucleotides, even when starting with 0.5–1 ng of DNA (Brown et al., 2010). This method provides an opportunity to perform a broad comparative assessment of methylation in blastocyst stage embryos. In this report, we describe the results of a pilot study, using this assay to compare genomic methylation of mouse preimplantation blastocysts that were either maintained in vivo or subjected to in vitro culture.

Materials and Methods

Embryo preparation

C57/BL6 female mice (4–6 weeks old, Jackson Laboratories) were superovulated by injection of 5 U of pregnant mare’s serum gonadotrophin (Calbiochem), followed 48 h later with 5 U hCG (Sigma) and were mated with C3H (Jackson Laboratories) male mice to obtain F1 hybrid embryos. Embryos were flushed from the uterine horns at 3.5 days post coitum (E3.5) using M2 media (Millipore) and were used as control embryos. For ART conditions, C57/BL6 female mice (4–6 weeks old) were superovulated using an identical protocol and were mated with fertile C3H males. E0.5 zygotes were removed from the oviduct and stripped of cumulus cells using hyaluronidase as described (Nagy et al., 2003). Zygotes were cultured at 37°C in synthetic oviducal medium enriched with potassium (KSOm) with amino acids, plus bovine serum albumin (4 mg/ml) in microdrops under light mineral oil in a humidified chamber with 5% CO2. Embryos were assessed visually and embryos that appeared normal were harvested at E3.5. All blastocysts that were subjected to DNA amplification and array analysis appeared to have developed normally.

DNA preparation and amplification

DNA was prepared from individual embryos using standard methods (Sambrook and Russell, 2006). Briefly, embryos were placed in 1.5 ml tubes containing 100 μl of cell lysis buffer containing proteinase K. After incubation at 55°C for several hours followed by phenol/chloroform extraction, DNA was alcohol-precipitated in the presence of 1 μg of transfer RNA. DNAs were digested with the methylation-sensitive restriction enzyme, HpyCh4IV (New England Biolabs), using the manufacturer’s recommended conditions. Following digestion, double-stranded amplification linkers were ligated and then PCR using a primer corresponding to the linker was performed for 24 cycles, as previously described (Brown et al., 2010). Amplified DNA was verified on an agarose gel and then used for labeling.

Microarray design

Microarrays were synthesized by the NimbleGen Corporation according to a custom design. Each array consisted of ~72 000 oligonucleotides, of which ~48 000 represented ~16 000 mouse genomic loci (three separate oligonucleotides per locus). Loci were selected for inclusion on the microarray by first parsing the entire sequence of mouse chromosome 7 into segments bounded by sites for the methylation-sensitive restriction enzyme, HpyCh4IV (recognition sequence ACCTG). This enzyme was chosen because its recognition sequence is 50% AT and therefore does not result in a strong bias for GC-rich sequence. The only other criterion for array representation was fragment length. Although prior reports have suggested that fragments greater than 1000 bp can be efficiently amplified by linker-mediated PCR, we did not include any fragments over 650 bp, and the minimum fragment length was 200 bp. Restriction fragments that did not have suitable sequence for the design of at least three unique oligonucleotides were excluded. In an effort to obtain as unbiased a sample of sequence as possible, no effort was made to select for any given sequence feature. The remainder of the array was occupied by random oligonucleotides (for baseline signal) and by oligonucleotides representing restriction fragments from the prokaryotic Thermus aquaticus genome, which were included as ‘spike-in’ controls for data normalization.

Array loci are alternatively referred to as ‘seqIDs’ throughout this work.
Blastocyst DNA hybridization

Amplified blastocyst DNA was labeled with either Cy3 or Cy5 using a ‘Dual Color Labeling Kit’ from NimbleGen and following the manufacturer’s instructions. The amplified DNA is low-molecular weight, and therefore no shearing was performed prior to labeling or hybridization. Prior to labeling and hybridization, each probe was ‘spiked’ by the addition of a 2.5 ng aliquot of previously amplified *T. aquaticus* DNA. Hybridizations were performed as two-color comparisons according to the NimbleGen protocol in a NimbleGen ‘System 4’ hybridization apparatus. Following washing, slides were scanned with a GenePix 4100A scanner (Molecular Devices), using Gene Pix Pro 6.0 software. Scanned data were extracted using NimbleScan software running on a standard PC.

A total of eight different amplified representations from eight individual blastocysts (four ART and four controls) were used. Each amplified DNA was hybridized to the microarray twice, with dye reversal and each hybridization was performed as a two-color comparison between a control and an *in vitro* cultured embryo. Results from dye-reversed pairs were averaged as a way of correcting for dye bias, a normalization technique that is well-suited in settings where there may be significant overall differences in amplification in the two samples (Dabney and Storey, 2007). Because each seqID was represented by three oligonucleotides, the mean of the three intensities for each seqID was used to summarize the signal associated with it. This resulted in a total of eight color-averaged data sets, each corresponding to one blastocyst and each containing 15 880 intensity values.

Data analysis

All data analysis was performed using the statistical package, ‘R’ (www.bioconductor.org). Following array hybridization, raw microarray data were analyzed as follows: The log2 intensities of each group of three oligonucleotides that represented a single restriction fragment were first averaged, to create a single mean intensity for each locus. Then, in order to normalize data and to correct for dye bias, the data obtained from color-reversed duplicate experiments were averaged at each locus, creating a single composite ‘color-averaged’ data set for each pair of experiments.

Results

Representation of the microarray

A schematic of the coverage of chromosome 7, as seen through the University of California Santa Cruz genome browser (www.genome.ucsc.edu), is shown in Fig. 1. Of the 15 800 loci, 1328 (8%) overlapped coding exons, 448 (3%) overlapped CpG islands, 8092 (51%) overlapped transcribed sequence (introns and untranslated regions), 8996 (57%) overlapped repetitive elements and 2896 (18%) contained no obvious sequence features. Many loci overlapped more than one sequence feature.

Reliability of procedure

Although linker-mediated DNA amplification has been validated by us as well as others, we were concerned that a blastocyst containing 50–100 cells might not provide enough DNA for reproducible amplification. To this end, we first sought to assess the reproducibility of the aspects of the procedure that did not depend on DNA amplification by comparing the results obtained by labeling and hybridizing the same DNA sample twice (technical replicates). Because we performed color-reversal, a total of eight such replicate hybridizations were performed. The correlation coefficients for these eight comparisons ranged from 0.975 to 0.99, attesting to the extremely good technical reproducibility of the labeling, hybridization and scanning processes.

The ideal way to test the reliability of the DNA amplification process would be to perform two separate amplifications on the DNA from a single blastocyst; however, we considered ~300 pg to be insufficient for two separate amplifications. Therefore, as an alternate means of assessing the reliability of the amplification process, we performed pair-wise comparisons of the results obtained from the four control blastocysts, reasoning that any differences between them must represent either technical artifact or true biologic variation. Figure 2A provides a representative scatter plot showing that two

![Figure 1](link_to_image)

**Figure 1** Approximate chromosomal locations of the ~16 000 array loci, viewed as a custom track within the UCSC browser.
ART embryos show consistent results. Because we had data from four control embryos, a total of six pair-wise comparisons between them could be performed. Correlation coefficients varied from 0.95 to 0.98, which is very close to the technical reproducibility of the labeling and hybridization procedures, as noted earlier. This result demonstrates the reproducibility of the amplification process itself and the consistency of methylation among blastocysts.

Increased variation with in vitro culture

Figure 2B and C are scatter plots comparing representative control and ART blastocysts as well as two ART blastocysts. Correlation coefficients of the six pair-wise comparisons among the four experimental blastocysts ranged from 0.62 to 0.85, reflecting a much larger degree of variability than that seen in in vivo derived blastocysts. Given the consistency, we found among control blastocysts that the variation seen with in vitro cultured blastocysts is striking.

MA plots, where the log₂ intensity difference at each locus (\(M\)) is plotted against the log₂ mean intensity (\(A\)), provide a convenient way to summarize two-color microarray data. In this format, a comparison between two identical samples is expected to produce a linear cloud of points approximating \(y = 0\), with any deviations representing technical artifact. Figure 3 shows the \(M\) ‘A’ plots from the four comparisons of control and ART blastocysts. In each plot, \(M\) is defined as log₂ (control/experimental), so that positive y-axis values reflect greater amplification in the control blastocysts. The most striking feature of all four plots is the generalized higher degree of amplification from the control blastocysts, presumably reflecting an overall tendency toward relative hypermethylation in the ART blastocysts. Importantly, the array data with the lowest A values (corresponding to seqIDs where there was little or no amplification) have M values that are approximately centered on \(y = 0\). This, along with the spike-in control data (also approximately centered on \(y = 0\)), indicates that the normalization process resulted in a reasonable correction for dye bias, and this in turn implies that the overall differences are not likely to be related to technical artifacts. The mean M values for the four comparisons ranged from 0.5 to 1.2, while mean M values derived from comparisons between control blastocysts ranged from \(-0.3\) to 0.2.

Methylation differences appear to be non-random

Although the majority of loci showed evidence of relative hypermethylation among ART embryos, all four data sets also contained loci showing relative hypomethylation. In order to test whether these relatively hypomethylated loci occurred at random, we selected the 1500 loci (\(\sim 10\%\)) with the lowest intensity ratio (i.e. most hypomethylated in vitro cultured embryos) from each of the four hybridizations. We then determined which of these loci were common to all four hybridizations. A total of 344 (23%) were common to all four sets of 1500, whereas, under the assumption that methylation differences between embryos occur at random, one would expect that the four sets of the 1500 most hypomethylated loci would contain few, if any, members in common. The fact that such a large proportion of hypomethylated loci were common to all four comparisons, strongly suggested that in vitro culture resulted in non-random differences.

In order to understand what underlying sequence features might be associated with hypomethylation, we used the UCSC genome browser in conjunction with the Galaxy website (Goecks et al., 2010) to test for the presence of various features in the set of 344 loci that were commonly hypomethylated. We found that 233 (67%) overlapped exons, which is strikingly different from the \(\sim 34\) (\(\sim 10\%\)) that would have been expected at random. Likewise, 77 of the 344 (22%) overlapped with CpG islands, as opposed to the \(\sim 10\) (3%) that would have been expected. The presence of other sequence features, such as repetitive elements or those showing strong evolutionary conservation, showed no association with relative hypomethylation.

A similar analysis of the 1500 loci showing the strongest hypermethylation among ART embryos revealed substantially similar results. Greater than 300 (21%) were common to all four data sets, and \(\sim 90\%\) of these overlapped either exons or CpG islands. This again indicates that methylation differences between the two types of embryos do not appear to be random.

Imprinted loci

Published reports have suggested that IVF is associated with an increased risk of imprinting disorders that have generally resulted...
from hypomethylation. Therefore, we sought to determine whether the 344 loci which were most hypomethylated in all four comparisons (as defined above) occurred in imprinted regions of the genome with higher than expected frequency. We used the imprinting map maintained by the Medical Research Council-Harwell (Williamson et al., 2011) to find imprinted loci on chromosome 7, and we tested whether more than the expected number of hypomethylated loci mapped to any given imprinted locus. Overall, we found no evidence to support the idea that hypomethylation was more common near imprinted loci. However, we note that given the small size of most imprinted regions and the fact that we had four data sets, our study was underpowered to detect any but the most striking effects. Interestingly, we found that among the 61 array loci that fell within the 200 kb region surrounding the H19 imprinted locus, five loci were strongly hypomethylated in all four comparisons (Fig. 4). While it is entirely possible that this arose by chance, it does show that the

Figure 3 MA plots of results from the four comparisons of ART and control blastocysts. In all four panels, M is defined as control/ART, so that positive values indicate higher intensity or relative hypomethylation in the control embryo. The black points represent mouse loci and the red points represent the ‘spike-in’ control data.

Figure 4 Cartoon showing the H19 region of mouse chromosome 7. The region contains 61 array loci, and the five vertical black bars indicate the approximate location of the five array loci that showed extreme hypomethylation in all four normal embryos. Genes and CpG islands are as marked.
overall non-randomness of hypomethylation could easily result in the appearance of an association between imprinting and methylation changes associated with in vitro culture.

**Association of array intensity with underlying sequence features**

The fact that loci containing exons and CpG islands were more likely to be relatively hypomethylated among ART embryos led us to conduct a more systematic evaluation of associations between sequence features and methylation. To this end, we separated the color-averaged mean intensities of the ~16 K array loci into 15 equally sized bins according to intensity and we then used the UCSC genome browser table function, in conjunction with tools available through the Galaxy website, to look for associations between array signal and various genomic features. By sequentially testing for the overlap between a given sequence feature and the seqIDs within each of the 15 bins, we were able to determine whether there was an association between the presence of that feature and degree of genomic methylation.

Although methylation has been associated with transposons (Meissner et al., 2008), we found no evidence of a strong relationship between the presence of repetitive elements and array signal strength (data not shown). On the other hand, we found a clear relationship between the presence of a coding exon and array signal strength. Figure 5 shows these results for both control and experimental blastocysts. Clearly, seqIDs that overlapped coding exons were much more likely to be relatively hypomethylated, implying that sequences within or near exons are generally hypomethylated. This relationship was true for both ART as well as control blastocysts. Interestingly, we found no evidence that the overlap of seqIDs with non-coding transcribed sequence (introns and non-coding exons) was associated with array signal intensity (data not shown).

CpG islands are generally hypomethylated and are thus expected to be hypomethylated in early embryos as well. We tested whether overlap with a CpG island was associated with relative hypomethylation, by looking for the number of overlaps with CpG islands, using the same strategy as described already. The ~14 000 non-exon containing loci were divided into 10 bins according to array intensity, and the number of loci within each bin that overlap a CpG island were plotted in Fig. 6A. Clearly, seqIDs that overlapped with CpG islands were generally (although not universally) hypomethylated. Given the fact that the definition of CpG island is somewhat arbitrary, we sought to determine whether there was a general relationship between GC content and methylation. To this end, we divided mouse chromosome 7 into ~152 000 sequential segments of 1000 bp each and calculated the GC content of each segment. We then plotted the GC percentage of the 1000 bp genomic segments that overlapped each of the ~15 000 array loci versus the intensity of each locus. This analysis indicates that increasing GC content is generally associated with decreasing methylation (Fig. 6B), an observation that is in keeping with other efforts at global methylation assessment (Zhang et al., 2009).

The earlier-mentioned results indicate that seqIDs overlapping exons or CpG islands had a strong tendency to be hypomethylated;
Discussion

Our goal in these studies was to perform a general or global comparison of methylation between ART and control embryos. In fact, the array that we have used assesses ∼16,000 loci distributed along mouse chromosome 7, which is only a small proportion of the entire genome. However, we believe that our results are likely to be generally true for the entire genome, since methylation is not known to vary markedly between chromosomes or large chromosomal regions. Clearly, a formal proof of this would require the use of a larger microarray with broader genomic coverage.

Although the quantity of DNA from a single blastocyst stage embryo is small (∼300–600 pg), our results indicate that it is possible to perform methylation-sensitive amplification and to assess the degree of amplification by microarray hybridization. The reliability and consistency of the amplification procedure is evidenced by the fact that variation between normal embryos was very small and is close to that seen when the same DNA sample was labeled and hybridized twice. This degree of consistency attests both to the technical reproducibility of the assay and to the biological consistency seen among in vivo derived E3.5 embryos.

Because all of the DNA from an embryo is used for a single experiment, it is not possible to perform an independent validation study to verify that the degree of amplification is indeed a reflection of underlying methylation; however, several lines of evidence point to the conclusion that our results are indeed a reflection of underlying genomic methylation. First, our previously reported validation study using the same basic procedure was performed on similarly small amounts of DNA and shows that array signal, in general, is proportional to methylation (Brown et al., 2010). Likewise, other studies using similar methodology have shown that amplification is dependent on the degree of methylation (Khulan et al., 2006; Suzuki and Greally, 2010). Importantly, the technique has been shown to work reliably, even with very small quantities of DNA (Oda et al., 2009). A key piece of evidence indicating that array signal intensity is indeed dependent on underlying genomic methylation is the fact that array signal intensity correlates well with sequence features. As is shown in Figures 5–7, overlap of a CpG island or coding exon with a seqID is clearly associated with strong amplification. The only plausible explanation for these findings is that amplification efficiency depends on underlying genomic methylation.

An array-based method such as the one we have employed is expected to result in at least some ‘false discoveries’, where the array reports methylation differences that cannot be verified by an independent method. In the absence of a detailed validation study, we are not able to provide an estimate of the magnitude of false discovery associated with our method. In general, array-based assessments of methylation have proved to have low rates of false discovery (Khulan et al., 2006). Importantly, we note that if even a large proportion (e.g. 10%) of all array results were, in fact, false discoveries, it would not change any of our basic findings or conclusions.

A variety of studies have shown that in vitro embryo culture is likely to result in alterations of normal methylation in blastocyst stage embryos (Mann et al., 2003; Sato et al., 2007; Market-Velker et al., 2010), but we do not know of other studies that have attempted to assess the overall or general effects of in vitro embryo culture on methylation. The four comparisons of ART and control embryos that we report show that, in all cases, the in vitro cultured embryos showed much greater variability in amplification, suggesting that methylation is generally less consistent than in control embryos. Given the very low level of variation that we found among normal embryos, it is improbable that the much larger degree of variation that we found among in vitro cultured embryos is caused by technical artifacts. However, it is premature to conclude that this is a general property of in vitro cultured blastocysts, since it is of course possible that the four ART embryos that we studied showed more variation simply by chance. This possibility can only be addressed by performing more experiments.

Perhaps even more striking than the increased variability of methylation among ART embryos was our finding of overall diminished amplification among ART embryos. The most obvious explanation for this
findings is generalized increased methylation in ART embryos; however, it is important to consider other possible explanations. By their nature, microarrays are good at detecting localized differences in amplification between two complex samples but are less efficient for the detection of overall or global differences. In fact, commonly used data normalization methods, such as LOESS or quantile, both make the assumption that there are no overall differences in signal between two samples, and their use tends to minimize such differences. For this reason we did not use these approaches for data normalization. Rather, we performed hybridizations in duplicate, with color reversal, and then used color averaging as a means to correct for dye bias without minimizing overall or global differences between samples (Dabney and Storey, 2007). The validity of this approach is evidenced by the fact that the normalized intensity ratios of the random sequence controls were centered on \( y = 0 \), as would be expected. Likewise, the intensity ratios of the ‘spike-in’ control data approximated \( y = 0 \) over a broad range of mean intensity values. Given this evidence for appropriate data normalization, it seems unlikely that the large differences we detected between ART and control blastocysts were related to technical artifacts.

The fact that we found evidence of overall hypermethylation among ART embryos was somewhat surprising, since existing reports have generally found hypomethylation of imprinted loci in the context of ART (Owen and Segars, 2009). However, we note that one recent report did find evidence of generalized hypermethylation in tissues derived from ART pregnancies (Katari et al., 2009). We also note that the overall tendency toward hypermethylation that we found among ART embryos does not preclude the possibility that some loci are routinely hypomethylated. In fact the evidence we present suggests that this may be so.

The broad patterns of methylation seen among in vitro cultured embryos were generally similar to those seen in normal embryos, as the correlations between methylation and sequence features (exons and CpG islands) were at least as strong and sometimes stronger in the context of in vitro culture. Likewise, fully methylated (non-amplifying) segments tended to be similar between the two groups of embryos. In keeping with this idea, exons and CpG islands were overwhelmingly over-represented among those segments showing the most striking methylation differences between ART and control embryos. These observations argue that the genomic effects of embryo culture are broad and general and are most significant in sequence that is normally less methylated, such as exons and CpG islands. This would make it unlikely that imprinted regions or other localized genomic regions are specifically affected by in vitro culture. Our limited data support this idea.

Our study cannot specifically address the mechanism(s) by which in vitro culture might result in abnormal methylation; however, our findings indicate that there are broad, general effects. During preimplantation development, there is a generalized wave of demethylation that results in a relatively low overall level of methylation immediately prior to implantation (Rouger et al., 1998). A delay or general disorganization in the timing of this wave, perhaps caused by altered substrate availability or some other subtlety of culture conditions, could result in the differences that we see. Future studies will determine whether the effects we see in this pilot study are consistent and predictable, and whether the methylation abnormalities seen at the blastocyst stage persist into later gestation or into post-natal life. Furthermore, an independent embryo set should be investigated to determine the biological relevance of the observed methylation changes in embryos cultured in vitro. The availability of the methylation assay that we describe will also make it possible to test various in vitro culture conditions in order to determine which culture parameters have the most effect on methylation. It is at least possible that, by minimizing the effects of embryo culture on the genome, the outcome of ART may be improved.

**Authors’ roles**

K.W. is a clinical fellow in Reproductive Endocrinology at the University of Vermont. Her role in this project was to perform mouse matings, harvest and culture embryos and to perform DNA amplifications. In addition, she prepared an initial draft of this manuscript. L.B. is a Postdoctoral Fellow in Dr Brown’s laboratory. She worked out the many technical details of performing methylation-sensitive amplifications on blastocyst stage embryos. In addition, she instructed and supervised Dr Wright in laboratory and mouse husbandry techniques. G.B. is an expert in programming and computer techniques. He was responsible for the majority of the bioinformatics aspects of this work, including array design and data analysis. S.B. is the Principal Investigator of the laboratory. He initially proposed the experiments described in this work and conceived of the idea of performing array-based methylation analysis using the approach described. In addition, he assisted in data analysis and is responsible for preparing the final version of this manuscript. P.C. is the director of Reproductive Endocrinology at the University of Vermont. He provided critical discussions about experimental design and execution. In addition, he provided critical reviews of data and of this manuscript.

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**References**


