Targeted DNA Methylation Analysis by High Throughput Sequencing in Porcine Peri-attachment Embryos

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Abstract. The purpose of this experiment was to implement and evaluate the effectiveness of a next-generation sequencing-based method for DNA methylation analysis in porcine embryonic samples. Fourteen discrete genomic regions were amplified by PCR using bisulfite-converted genomic DNA derived from day 14 in vivo-derived (IVV) and parthenogenetic (PA) porcine embryos as template DNA. Resulting PCR products were subjected to high-throughput sequencing using the Illumina Genome Analyzer IIx platform. The average depth of sequencing coverage was 14,611 for IVV and 17,068 for PA. Quantitative analysis of the methylation profiles of both input samples for each genomic locus showed distinct differences in methylation profiles between IVV and PA samples for six of the target loci, and subtle differences in four loci. It was concluded that high throughput sequencing technologies can be effectively applied to provide a powerful, cost-effective approach to targeted DNA methylation analysis of embryonic and other reproductive tissues.

Key words: Bisulfite sequencing, DNA methylation, Illumina, Parthenogenetic embryo

In addition to ‘traditional’ genetic control, epigenetic phenomena are being recognized as playing important roles in directing cellular function and fate. Epigenetics can be defined as the means whereby heritable patterns of gene expression within a population of cells are regulated without alterations in the actual nucleotide sequence of the genome. Epigenetics, broadly defined, can include such processes as post-translational histone modification, chromatin remodeling, RNA-mediated gene silencing (RNAi), and DNA and mRNA methylation [reviewed in 1, 2]. Of these, DNA methylation is the best characterized, and the most extensively studied [see 1, 3 and references therein]. DNA methylation involves the covalent addition of a methyl group (-CH$_3$) at the C5 position of the cytosine nucleotide base. In mammals, this modification is observed primarily at cytosines in a CpG context – that is, when a cytosine is immediately followed by a guanine in the nucleotide sequence. In mammals, 70–80% of CpG dinucleotides are methylated. Regions of higher-than-expected CpG frequency (CpG islands) are associated with most mammalian genes, and the relative preponderance of methylated cytosines within these CpG islands is generally inversely correlated with transcriptional activity of those genes (i.e. high methylation = low gene expression). DNA methylation is carefully regulated within the cell, and the establishment and maintenance of DNA methylation marks are accomplished by a variety of methyltransferase enzymes (DNMT1, DNMT3A, DNMT3B, e.g.). Precisely coordinated methylation and de-methylation of DNA is essential for proper development [4].

Faulty patterns of DNA methylation are associated with myriad disease conditions, including many cancers [5–9].

In livestock animal production systems, application of some assisted reproductive technologies (ART) such as in vitro fertilization (IVF) and somatic cell nuclear transfer (SCNT) is limited because of relatively poor efficiency. In cattle, establishment of pregnancy after transferring embryos derived from IVF into recipient females can be as low as 20% [10, 11]; survival of SCNT embryos after transfer is even lower: 5–10% [reviewed in 12]. Similar – though even less encouraging – data are observed in pigs [13]. Evidence is mounting that embryo and/or gamete manipulations in vitro can adversely impact the delicate constitution of the embryonic epigenome, and that even subtle changes in DNA methylation profiles might provide a mechanistic link between in vitro culture techniques and poor developmental competence.

More is known about the role of DNA methylation in development and disease than other epigenetic phenomena, at least in part because robust and simple techniques have been developed that allow for accurate assessment of CpG methylation status, even at single base-pair resolution. The most widely-applied approach to DNA methylation analysis is a technique called bisulfite sequencing. When exposed to bisulfite ions under the proper conditions, methylated cytosines of chromosomal DNA remain intact, while unmethylated cytosines undergo de-amination – thus converting cytosine to uracil. In downstream sequencing analyses of bisulfite-converted DNA, uracil residues behave like thymine, instead of the cytosines they were originally derived from. Thus by comparing DNA sequences ‘before’ and ‘after’ bisulfite conversion, it is possible to ascertain which cytosines within a particular sequence were methylated and which were unmethylated at the time of analysis. Potential drawbacks to traditional bisulfite sequencing include the inconvenience of cloning of
islands are referred to by the name of their nearest gene neighbor. Islands were identified that were putatively differentially methylated epigenetic differences between day 14 IVV and PA embryos, CpG susceptible to insult in response to the [17, 18]. It has been suggested that imprinted genes are particularly maternal and paternal alleles is at the heart of genomic imprinting phenomenon of parent-of-origin genomic imprinting. Genomic imprinting epigenetic determinants of successful embryogenesis and the phe- parthenogenetic oocyte activation (PA) could yield insight into the; IVV) and in vivo embryos derived from artificial insemination (on-line only). A high throughput sequencing approach was taken to evaluate the methylation status of these 14 CpG islands in IVV and PA embryos. Genomic DNA from a single intact day 14 embryo from each production method (IVV and PA) was collected and subjected to bisulfite conversion, which provides a mark to differentiate unmethylated from methylated cytosine bases within the nucleotide sequences evaluated. A single PCR amplicon from each of fourteen distinct CpG islands was generated using bisulfite-converted DNA from both embryo types. The average amplicon length was 359 base pairs (bp; range 249–485 bp), covering an average of 27.1 CpG dinucleotides per amplicon (range 19–40 CpG sites). These bisulfite PCR products were subjected to high throughput sequencing using the Genome Analyzer IIx platform from Illumina, and the short (80 bp) reads produced by the instrument were mapped to the reference sequences for each CpG island used for downstream analyses.

Sequence information and chromosomal coordinates for these CpG islands can be found in Suppl Table 1 (on-line only). A high throughput sequencing approach was taken to evaluate the methylation status of these 14 CpG islands in IVV and PA embryos. Genomic DNA from a single intact day 14 embryo from each production method (IVV and PA) was collected and subjected to bisulfite conversion, which provides a mark to differentiate unmethylated from methylated cytosine bases within the nucleotide sequences evaluated. A single PCR amplicon from each of fourteen distinct CpG islands was generated using bisulfite-converted DNA from both embryo types. The average amplicon length was 359 base pairs (bp; range 249–485 bp), covering an average of 27.1 CpG dinucleotides per amplicon (range 19–40 CpG sites). These bisulfite PCR products were subjected to high throughput sequencing using the Genome Analyzer IIx platform from Illumina, and the short (80 bp) reads produced by the instrument were mapped to the reference sequences for each individual amplicon.

A total of 2,297,348 sequencing reads were aligned, with a slight bias overall towards reads from the PA sample. Table 1 provides summary statistics regarding the number of reads aligned and used for downstream methylation analyses for each CpG island queried. In addition to the tendency for more reads from the PA sample, the number of aligned reads per amplicon varied greatly within a sample. Likewise, the distribution of reads across the amplicons was not uniform. Figure 1 shows representative traces for four PCR products, plotting depth of coverage (Y-axis) against nucleotide position (X-axis). Read distribution bias was so extreme for one amplicon (NDUFB11; not shown) that large gaps in coverage were
observed, and this genomic locus was not evaluated further. While the absolute depth-of-coverage values differed between samples, the shapes of the read distribution traces for a given PCR product were virtually indistinguishable between samples. Notwithstanding these peculiarities associated with read generation and alignment, the average depth of coverage across all nucleotide positions in all amplicons was 14,611 for IVV and 17,068 for PA, with a maximum of 77,944 at nucleotide position 55 of the RRM1-IVV amplicon and a minimum of 718 at nucleotide position 279 of the FLT1-PA amplicon.

Using a sophisticated single nucleotide polymorphism identification algorithm, we were able to precisely determine the proportion of cytosines in CpG context that retained their identity after bisulfite sequencing (i.e. methylated in original template) relative to those that were deaminated by the conversion process (unmethylated in original sample). Data from the comparative methylation analysis are summarized in Fig. 2. We saw clear differences in overall methylation levels (more than 30% difference) between IVV and PA embryos in six of the thirteen amplicons analyzed, while overall percent methylation was not obvious in the remaining seven amplicons. It was interesting to note, however, that subtle differences (greater than 1%, but less than 5% difference) in overall methylation patterns were suggested between sample types for three of the amplicons (BMP7, 4.5% difference; LHX4, 2.9% difference; and RUNX1T1, 4.4% difference).

The physiological significance of these subtle differences is under investigation, and is more appropriately debated in another venue. The prevailing dogma, though, is that major changes in the overall density of methyl-cytosine bases across large regions of chromosomal DNA alters the conformation of chromatin, which can impact gene expression, and cell physiology. By this definition, these subtle differences in DNA methylation between IVV and PA embryos would not be expected to have any physiological relevance. However, there is no clear threshold of DNA methylation that is required to elicit biological change. As little as 10% change in promoter methylation can cause phenotypic variation in the viable yellow agouti mouse [23], and constantly-emerging evidence suggests that even very minor changes in DNA methylation patterns might have distinct effects on cellular and organismal physiology [see references 24–26, e.g.]. Thus the importance of being able to confidently detect such subtle changes in DNA methylation patterns cannot be overstated.

Traditional bisulfite sequencing experiments typically report methylation statistics for 10–20 DNA strands for each target sequence and for each sample tested. Figure 3 depicts the relationship between the number of DNA strands queried and the resulting statistical power for different magnitudes of discrepancies in percent methylation between two samples. While a traditional bisulfite sequencing experiment utilizing 20 sequences per amplicon would have sufficient statistical power to confidently detect a 50% difference in methylation frequency between sample types (as might be expected in classic instances of genomic imprinting), more subtle differences (≤ 10%) between samples would not be reliably detected with even 100 sequences per amplicon (Fig. 3). Herein lies one major benefit of
**Fig. 2.** Comparative analysis of DNA methylation patterns of *in vivo*-produced (IVV) and parthenogenetic (PA) porcine embryos. The percentages of methylated cytosines at each potential methylation site for each of thirteen distinct PCR products are depicted. Deep red colors indicate very high percent methylation; pale blue indicates very low methylation. Locus = gene nearest the CpG island queried.

**Fig. 4.** Manual methylation analysis of FLT1 sequencing reads from high throughput and traditional bisulfite sequencing techniques. A) The FLT1 locus queried in these experiments corresponds to a CpG island that maps to the 3’ end (exon 29 and flanking intron sequences) of the FLT1 gene. The long horizontal line represents the full 864 base-pair CpG island. Each vertical line represents a potential methylation site within the island. The hashed bar marks the approximate location of the full PCR amplicon generated for these experiments, while the green shaded box shows the relative position of exon 29 of the FLT1 coding sequence. The CpG sites marked with red lines are those described in further detail in this figure (CpG dinucleotides A–H in Panels B and C below), which correspond to CpG dinucleotide numbers 15–22 (in reverse order) in Fig. 2. Primer sequences and precise chromosomal coordinates of this and other genomic sequences analyzed in these experiments can be found in Suppl Table S1 (on-line only). B) Short Illumina reads corresponding to the FLT1 locus (n = 54 reads for PA; n = 52 reads for IVV) were aligned to the reference sequence and the methylation status of each DNA fragment was evaluated. Each row in the figure represents a short sequencing read. Each column represents a potential methylation site. Methylated CpG sites are indicated by red boxes, whereas unmethylated sites are colored blue. For this specific segment of the FLT1 locus, 82.1% of the DNA fragments analyzed were either fully methylated or completely unmethylated, while only 17.9% of the clones were partially methylated. These data illustrate the utility of a manual analysis of short reads for evaluating the distribution pattern of methylated vs. unmethylated cytosines. C) Methylation analysis by traditional bisulfite sequencing of the same eight CpG dinucleotides within the FLT1 locus that were evaluated in Panel B.
### Methods

#### Collection of embryonic tissue

Embryos derived from parthenogenetic activation of *in vitro* matured porcine oocytes were produced, transferred into recipient females, and harvested on day 14 of gestation as described elsewhere [27]. The IVV embryos were produced by artificial insemination.
according to standard industry practices, and were harvested by uterine flushing on day 14 of gestation as well. Upon collection, embryos were snap frozen in liquid nitrogen and stored at –80°C until use.

**Genomic DNA preparation and bisulfite PCR**

Genomic DNA was isolated from the IVV and PA embryos using the AllPrep DNA/RNA Micro kit from Qiagen (Valencia, CA, USA), and was stored at –20°C until use. Genomic DNA (500 ng) from both embryo types was subjected to bisulfite conversion using the EZ DNA Methylation-Gold kit from Zymo (Irvine, CA, USA), according to the manufacturer’s directions. The EpiDesigner software from Sequenom was used to design primers for bisulfite PCR (Suppl Table S1: on-line only). The HotStarTaq Plus Master Mix kit from Qiagen was utilized to generate PCR amplicons from bisulfite-converted genomic DNA. PCR reaction mixes included 20.0 μl 2× MasterMix, 10.0 μl water, 4.0 μl CoraLoad loading reagent, 2.0 μl bisulfite-converted gDNA (at 20 ng/μl), and 4.0 μl forward/reverse primer mix (final concentration of 1 μM each). Samples were then subjected to thermal cycling for target amplification. Cycling parameters included a 5 min initial enzyme activation cycle followed by 40 cycles of 95°C/15 sec – 55°C/30 sec – 72°C/30 sec and then a single 5 min extension termination step of 72°C. Resulting PCR products were purified using the QiAquick PCR Purification kit from Qiagen and pooled by sample type in equimolar concentrations, then sheared to approximately 100–150 bp fragments using the BioRuptor from Diagenode (Liege, Belgium). Sequencing library preparation proceeded according to standard single-end sequencing protocols provided by Illumina, and both final libraries (IVV and PA) were loaded onto a single lane of the Genome Analyzer IIx flow cell at a final combined concentration of 7 pM. Sequencing consisted of 2×40 bp cycles, resulting in a final read length of 80 bp.

**Sequencing read alignment and methylation analysis**

Sequencing reads were aligned to reference sequences using the bisulfite sequencing algorithm of the Novoalign software from Novocraft (Selangor, Malaysia). Aligned reads were uploaded into Geneious, a sequence manipulation software product from Biomatters (Auckland, New Zealand), and the SNP detection algorithm was used to determine the frequencies of methylated versus unmethylated cytosines in CpG context in the distinct samples for each PCR reaction.
product. To compare with the results of traditional sequencing (see below), the methylation status of the FLT1 locus was determined by selecting short reads that aligned perfectly to the target sequence (n = 54 reads for PA; n = 52 reads for IVV), and evaluating the methylation status of each read using BISMA, a web-based DNA methylation analysis platform [28].

Traditional E. coli-based bisulfite sequencing

Traditional bisulfite sequencing to validate the deep sequencing data was performed by subcloning four of the same PCR products used for Illumina sequencing (BCOR, CDC42BPB, CDX2, FLT1) in to the pGEM T-Easy vector from Promega (Madison, WI, USA), transforming subcloned plasmids into E. coli. cloni 10G chemically-
competent bacterial cells (Lucigen; Middleton, WI, USA), and sequencing the resulting plasmid preparations. A minimum of seven unique plasmid preparations were sequenced for each sample/amplicon combination. Methylation frequencies of subcloned PCR products were determined using BISMA as described above.

Power analysis calculations

The power analysis calculations were performed using software written and made freely available online by Dr. Russ V. Lenth at the University of Iowa [29]. Repeated power tests to compare two proportions were performed, changing sample sizes and expected differences between samples with each iteration. Alpha (α) at 0.05 and equal sample sizes per treatment group were set for each permutation. The null hypothesis to be tested in each instance was that the two proportions (% methylated) were equal (p1 = p2). For each set of hypothetical experimental parameters, a power statistic was generated and recorded to create the table of statistical power in Fig. 3.

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