# BRIEF COMMUNICATION: DNA methylation patterns in bovine blastocysts suggest that only limited epigenetic reprogramming has occurred in the early embryo

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## INTRODUCTION

Epigenetics is the study of changes in phenotype caused by mechanisms other than changes in the underlying DNA sequence. In agriculture, epigenetics largely resides in the environment component of the equation; Phenotype = Genotype + Environment. Epigenetic mechanisms include but are not limited to, DNA methylation, acetylation and methylation of histone proteins, and non-coding RNA molecules. These epigenetic processes act in an interrelated manner in order to regulate gene expression. Cytosine methylation at CpG dinucleotides is the most common covalent modification of DNA in eukaryotes. There is abundant evidence indicating that it plays a central role in many aspects of biology, including growth, development, genomic imprinting, and X-chromosome inactivation in females. Similarly, aberrant DNA methylation can preclude normal development and lead to disease (Robertson, 2005). Despite the clear importance of DNA methylation, it remains poorly understood how methylation patterns are set during development, maintained, change in response to environmental conditions, and regulate gene expression.

DNA methylation is a dynamic process. Changes in DNA methylation can occur locally (Weaver *et al.*, 2005) to regulate individual genes or on a genome wide scale (Santos & Dean, 2004) to allow complete reprogramming. In mammals, genome wide reprogramming during early embryonic development has been most extensively studied in the mouse. In this model system, epigenetic reprogramming is thought to begin immediately following fertilisation with an active demethylation of the paternal genome followed by a passive demethylation of the maternal genome. Remethylation of both genomes is believed to be complete by the blastocyst stage of development (Santos & Dean, 2004). Although it is assumed that the timing of reprogramming will vary between species, there is little evidence indicating that extrapolation of mouse development is useful for livestock. We have therefore begun to investigate epigenetic reprogramming during early embryonic development in cattle, as a greater understanding of this process will allow the efficiency of somatic cell nuclear transfer (SCNT) to be improved as it has been postulated that incomplete epigenetic reprogramming contributes to the high incidence of embryonic and foetal loss. Understanding basic epigenetic mechanisms will also begin to provide an understanding of how epigenetics contributes to the environmental component of animal phenotype.

## MATERIALS AND METODS

### Embryo production

SCNT and *in vitro* produced (IVP) embryos were generated as previously described (Smith *et al.*, 2010). Donor cells used were obtained from a fibroblast cell line derived from a high genetic merit Friesian bull. Embryos were grouped into pools of 10 for analysis.

### Semen collection

Semen was collected from the bull that provided the somatic donor cell line and from the Control Friesian bulls (n = 3) using an artificial vagina and teaser heifers, according to the commercial practice of Animal Breeding Services, Ltd. (Hamilton, New Zealand).

### DNA extraction

DNA was extracted from approximately 250 µL of semen as described previously (Couldrey *et al.*, 2011). Donor somatic cells were cultured in DMEM/F12 with 10% FCS, as described previously (Oback & Wells, 2003). DNA was extracted from approximately $10^6$ cells using standard proteinase K digestion followed by phenol/chloroform extraction and ethanol precipitation. Pools of blastocysts were lysed and treated briefly with proteinase K using the EZ-DNA methylation direct kit (Zymo, CA, USA) following the manufacturer's instructions.

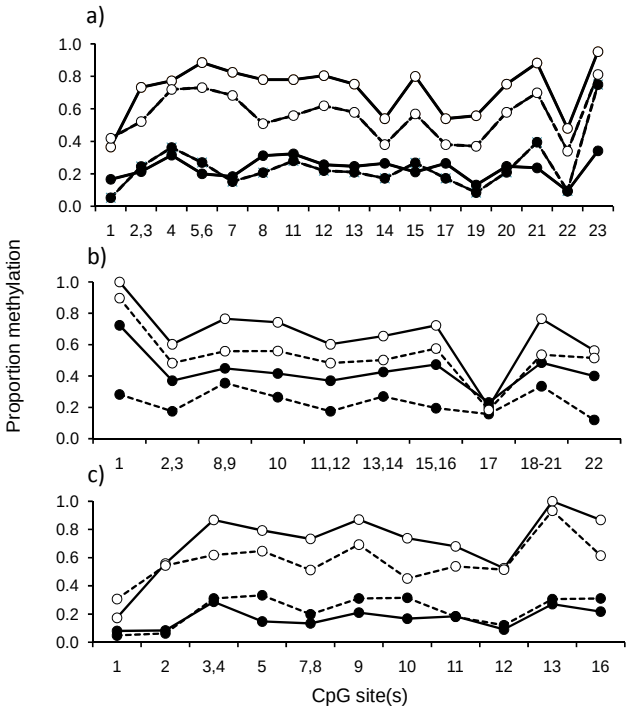
### Primer design

Primers were designed using MethPrimer to amplify CpG islands in repetitive DNA sequences Satellite I, Satellite II, and Satellite alpha, after bisulfite treatment of DNA as described by Couldrey and Lee (2010).

**FIGURE 1:** Mean proportion of DNA methylation at individual CpG sites in genomic DNA sequences in sperm (n=4), donor cells (n=3) and pools of 10 blastocysts, a) Satellite I sequence (IVP n=6 pools, SCNT n=10) b) Satellite II sequence (IVP n=12, SCNT n=14), c) Satellite alpha sequence (IVP n=6, SCNT n=9). Pooled standard error of mean was less that 0.017 for all groups.

Donor cells ○—○ SCNT○---○

Sperm ●—● IVP ●---●




**DNA methylation analysis**

DNA samples were analysed using Sequenom MassARRAY technology as previously described (Coolen *et al.*, 2007; Couldrey & Lee, 2010; Ehrich *et al.*, 2005). Briefly, the EZ-DNA methylation direct kit was used to produce methylation-dependent sequence variations of C to T and regions of interest amplified using T7 tagged PCR primers. *In vitro* amplification and transcription was performed on the reverse strand with simultaneous U specific cleavage by RNAseA. Samples were subject to mass spectrometry to provide high-resolution DNA methylation analysis, quantitative to 5% methylation for informative CpG dinucleotides (Coolen *et al.*, 2007).

Data were not normally distributed; therefore statistical comparisons for individual CpG sites were performed using a ranked t-test. Storey-Tibshirani adjusted q was used to estimate true nulls (false discovery rate) (Storey & Tibshirani, 2003).

## RESULTS AND DISCUSSION

We assessed the extent of reprogramming in bovine embryos by comparing DNA methylation patterns of three repetitive DNA sequences in pools of blastocysts generated by IVP and SCNT. These methylation levels were further compared with methylation in sperm from adult bulls and nuclear donor somatic cells.

The DNA methylation patterns for each of the genomic regions are shown in Figure 1. For each of the regions, not all CpG sites or groups of sites could be analyzed by this method; the number of CpG sites able to be analysed are listed in Table 1. Where a fragment contained two or more CpG sites, or where two fragments could not be distinguished from each other, the methylation levels were averaged across the CpG sites or fragments respectively (Table 1).

Individual CpG sites or groups of CpG sites in the donor cells were methylated to varying levels in each region, giving distinctive methylation profiles (Figure 1). In each of the three repeat regions analysed, donor cells exhibited the highest consistent methylation. In contrast, sperm and IVP blastocysts were consistently the least methylated.

Methylation at 34 out of 38 (34/38) CpG sites examined were significantly ($P < 0.05$; with 14/38 $P < 0.001$) hyper-methylated in pools of SCNT compared with IVP blastocysts, with one CpG site showing hypo-methylation and three showing no significant differences. This comparison highlights that embryos generated by SCNT do not display DNA methylation patterns appropriate for their developmental stage. However, we have previously shown that SCNT embryos able to survive to mid-gestation display methylation patterns in three selected organs that are indistinguishable from those

**TABLE 1:** Analyzable CpG sites in each of the three satellite sequences investigated. The table shows the total number of CpG sites present in each amplicon, the number of CpG sites that were analyzed and the CpG sites that were contained within fragments that could not be resolved because they had identical mass/charge ratios. At these sites, the proportion of methylation is the averaged values from all these fragments.

| Amplicon | GenBank accession number | CpGs in amplicon | CpGs analyzed | CpGs in fragments with identical mass/charge ratios |
|---|---|---|---|---|
| Satellite I | J00032 | 23 | 20 | 13 and 20 |
| Satellite II | X03116 | 22 | 18 | 8-9 and 11-12 |
| Satellite alpha | AJ293510 | 16 | 11 | 3-4 and 16 |

of foetuses generated by artificial insemination (Couldrey & Lee, 2010). These conflicting results suggest that either epigenetic reprogramming following SCNT has not occurred appropriately by the blastocyst stage, or that reprogramming occurs later in the bovine embryo than in other species.

A comparison of IVP blastocysts with sperm indicated that methylation patterns in these cell types were remarkably similar. Although 11/38 CpG sites showed significant differences ($P < 0.05$) in methylation between sperm and IVP blastocysts, the magnitude of the differences were smaller than between IVP and SCNT blastocysts, especially in Satellite I and Satellite alpha sequences. The similarity of DNA methylation patterns of sperm and IVP blastocysts suggests that reprogramming in the bovine embryo has not occurred by the blastocyst stage. Similarly, analysis of methylation patterns in SCNT blastocysts and the nuclear donor cells used for their generation identified only 8/38 CpG sites at which methylation levels were significantly different ($P < 0.05$) between the groups. Again the magnitudes of differences in methylation levels were small in this comparison.

During the seven day period from SCNT to the blastocyst stage, changes in DNA methylation levels were of a magnitude that could be accounted for by passive demethylation similar to that seen in the maternal genome following fertilization (Santos & Dean, 2004). Given that neither the somatic donor cell DNA in SCNT, nor the oocyte DNA in fertilization contain any protamines, it seems possible that these DNA sources would both be passively demethylated. This is in contrast to DNA derived from sperm that is tightly packaged around protamines and actively demethylated immediately following fertilization. Taken together the DNA methylation data presented here supports the hypothesis that there is no driving force for genome wide demethylation until after the blastocyst stage in bovine development.

These data, together with recently published morphological and molecular data (Berg *et al.*, 2011) provide evidence that reprogramming, previously described to occur between the two-cell and blastocyst stages of development in mice, occurs at a later developmental stage in the bovine embryo, possibly playing a role in the highly selective nature of the second week of development (Berg *et al.*, 2010). Consequently, using both SCNT and IVP embryos is potentially a useful model to assist in understanding the process and function of epigenetic reprogramming and epigenetic control of gene expression in cattle. Such an understanding will lead to the ability to not only select superior animals based on their genetics, but also identify animals that have epigenetic profiles allowing them to fulfil their genetic potential by minimising the contribution of the environment to the phenotype thereby allowing more efficient production of meat, milk and wool in the future.

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