Zygote **25** (April), pp. 131–140. © Cambridge University Press 2017
doi:10.1017/S096719941600040X First Published Online 31 January 2017

Satellite DNA methylation status and expression of selected genes in *Bos indicus* blastocysts produced *in vivo* and *in vitro*

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Date submitted: 05.02.2016. Date revised: 02.09.2016. Date accepted: 11.10.2016

Summary

Bovine embryos produced *in vivo* and *in vitro* differ with respect to molecular profiles, including epigenetic marks and gene expression profiles. This study investigated the CpG methylation status in bovine testis satellite I (BTS) and *Bos taurus* alpha satellite I (BTαS) DNA sequences, and concomitantly the relative abundance of transcripts, critically involved in DNA methylation (DNMT1 and DNMT3A), growth and development (IGF2R) and pluripotency (POU5F1) in *Bos indicus* embryos produced *in vitro* or *in vivo*. Results revealed that methylation of BTS were higher ($P < 0.05$) in embryos produced *in vitro* compared with their *in vivo* produced counterparts, while the methylation status of BTαS was similar in both groups. There were no significant differences in transcript abundance for DNMT3A, IGF2R and POU5F1 between blastocysts produced *in vivo* and *in vitro*. However, a significantly lower amount of DNMT1 transcripts was found in the *in vitro* cultured embryos ($P < 0.05$) compared with their *in vivo* derived counterparts. In conclusion, this study reported only minor changes in the expression of developmentally important genes and satellite DNA methylation related to the *in vitro* embryo production system.

Keywords: Bovine embryo, DNA methylation, DNMT1, *In vitro* culture, Satellite DNA

Introduction

In vitro culture systems for early embryo development have contributed substantially to the success of many assisted reproduction technologies (Vajta *et al.*, 2010). Bovine *in vitro* embryo production (IVP) is routinely used to shorten generational intervals and to propagate valuable genetic material among breeding populations. IVP is widely used in commercial cattle breeding systems and 546,628 *in vitro* produced embryos were transferred in 2013, of which 72.7% were produced in South America, especially from *Bos indicus* breeds (Perry, 2014). However, differences between embryos produced *in vivo* with respect to those produced *in vitro* have been reported, which can primarily be associated to abnormal gene expression profiles and aberrant epigenetic marks. These modifications could explain the reported differences in metabolism, cell number, ultrastructure and cryotolerance (see Urrego

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https://doi.org/10.1017/S096719941600040X Published online by Cambridge University Press

et al., 2014). Thus, studying the expression profile of selected genes and epigenetic marks could result in improved oocyte and embryo selection criteria and a better discrimination between viable and non-viable oocytes and embryos (Wrenzycki *et al.*, 2007).

DNA methylation of specific cytosine residues of CpG dinucleotides is a major epigenetic mark catalyzed by DNA methyltransferases (DNMTs) and critically involved in regulation of gene expression during embryonic development and genomic imprinting (Petrussa *et al.*, 2014). Development of the preimplantation mammalian embryo is characterized by dynamic changes in DNA methylation that are critically dependent upon gender and cell lineage (Dobbs *et al.*, 2013). DNA methylation profiles can be significantly altered by assisted reproductive technologies (ARTs), including *in vitro* culture (Niemann *et al.*, 2010) and several studies found altered expression of *DNMT1* (Cirio *et al.*, 2008; Golding *et al.*, 2011) and *DNMT3A* (Sagirkaya *et al.*, 2006; Gómez *et al.*, 2009) in *in vitro* produced embryos. These enzymes are involved in maintenance and *de novo* methylation of DNA, and are thus critical for embryo development. Additionally, aberrant transcript profiles of imprinted genes such as *IGF2R* are particularly critical for inducing developmental disorders (Perecin *et al.*, 2009; Farin *et al.*, 2010; Velker *et al.*, 2012).

Although, *in vitro* embryo production is widely used in *Bos indicus* breeds, little information is known regarding the effect of this biotechnology on the molecular status during preimplantation development. Here, we evaluated the CpG DNA methylation status of bovine testis satellite I (BTS) and *Bos taurus* alpha satellite I (BTαS) sequences, and concomitantly the relative abundance of selected transcripts, critically involved in DNA methylation (*DNMT1* and *DNMT3A*), growth and development (*IGF2R*) and pluripotency (*POU5F1*) in *Bos indicus* embryos produced *in vitro* or *in vivo.*

Materials and methods

In vitro embryo production

In vitro embryo production was performed under conditions similar to commercial *in vitro* production. Therefore, ready-to-use commercially available media were obtained from Nutricell (Nutrientes Celulares Campinas, Sao Paulo, Brazil). Ovaries were collected from *Bos indicus* (Brahman) cows at a local slaughterhouse and maintained at 30°C in sterilized saline solution until use. The method used to produce *in vitro* embryos has been reported recently (Urrego *et al.*, 2015). Briefly, Cumulus–oocyte complexes (COCs) were aspirated from 4 to 8 mm follicles using a 18-gauge needle attached to a 10 ml syringe and handled in Tyrode's Albumin Lactate Pyruvate (TALP)-HEPES medium supplemented with 0.4% bovine serum albumin (BSA) (Sigma Chemical, St Louis, MI, USA).

Cumulus–oocyte complexes were classified morphologically according to the oocyte cytoplasm status and the morphology of cumulus cell layers (Khurana & Niemann, 2000). Only COCs with a compact cumulus and homogenous (grade I) or slightly heterogeneous (grade II) cytoplasm were used for this study. Groups of 10 COCs were matured in 50 µl drops of maturation medium (Nutricell), supplemented with 10% fetal bovine serum (FBS; Gibco, Life Technologies, Grand Island, NY, USA), covered with mineral oil (Sigma). *In vitro* maturation was performed for 24 h in a humidified environment of 5% CO_2 in air at 38.5 °C.

In vitro fertilization was performed using commercial frozen sperm from a single Brahman bull with proven fertility. Straws were thawed in a water bath at 37°C for 60 s. Spermatozoa were obtained after centrifugation at 700 *g* for 10 min in a Percoll (Sigma) discontinuous density gradient (45–90%). After maturation, oocytes were washed three times in 100 µl drops of *in vitro* fertilization (IVF) medium (Nutricell), supplemented with penicillamine, hypotaurine, epinephrine (Nutricell) and heparin (10 µl/ml) and then transferred to 50 µl IVF medium drops under oil. Spermatozoa were added to reach a final concentration of 2×10^6/ml and co-incubated with approximately 10 COCs *in vitro* matured for 18–21 h at 38.5°C and 5% CO_2 in air.

Presumptive zygotes were partially denuded by mechanical pipetting in TALP–HEPES medium and after washing three times in 100 µl medium drops. Zygotes were cultured in groups of 15–20 in 50 µl drops of synthetic oviduct fluid with amino acids (SOFaa) medium (Nutricell), supplemented with 5% FBS. Embryos were cultured in 5% CO_2, 5% O_2 and a humidified atmosphere at 38.5°C in air. Half of the culture medium was replaced after 72 h post-insemination (hpi) with fresh SOFaa medium, when cleavage rates were determined. Blastocysts rates were assessed at 162 hpi (D7). Grade 1, 2 or 3 blastocysts (Gordon, 2003) were stored at −80°C in pools of five embryos for gene expression and 10 embryos for DNA methylation analysis in 70 µl Trizol® reagent (Ambion, Life Technologies, Carlsbad, CA, USA).

In vivo embryo production

All procedures involving animals were carried out with the approval from the Committee for Ethical Care and Use of Animals of the University of Antioquia (Colombia). Twelve non-pregnant adult Brahman female donors from a commercial herd were selected according to sanitary and reproductive status.

https://doi.org/10.1017/S096719941600040X Published online by Cambridge University Press

Reproductive organs were examined by transrectal palpation and ultrasonography using an Aloka SSD 500 ultrasound system and an 5 MHz linear transducer (Aloka, Inc., Tokyo, Japan). Only cycling animals confirmed by the presence of a functional *corpus luteum* and with a body condition score of 3 ± 0.5 units were included (scale of 1 to 5, where 1 indicates emaciated and 5 obese).

Cows were synchronized and superstimulated with the following protocol: D0: placement of an intravaginal device containing 1 g progesterone (DIB, Syntex S.A., Buenos Aires, Argentina) and injection of 2.0 mg estradiol benzoate *im* (Ric-Be, Syntex S.A.); D4 to D7: superstimulation with eight equal doses of 250 IU FSH at 12 h intervals (Pluset®, Calier, Spain); D6: 0.150 mg cloprostenol *im* (Prolise®, Tecnopec, São Paulo, Brazil); D6.5: removal of the intravaginal device; D8.5 and D9: Two artificial inseminations were performed, with sperm from the same bull used for IVF with a 12 h interval; D15: embryos were non-surgically collected from the uterine horns as previously described (Neto *et al.*, 2005). Briefly, to perform uterine flushings, the catheter was gently inserted into the uterus, the balloon was inflated with air and the uterus was flushed three to four times with a total of 1 litre Dulbecco's phosphate-buffered saline (DPBS; Nutricell). For the second flushing, 80–150 ml of DPBS was infused through the catheter and the plunger was closed with a disposable 5 ml syringe. The same experienced operator performed all procedures within this study. Retrieved embryos were evaluated according to developmental stage and quality (Gordon, 2003). Grade 1, 2 or 3 blastocysts were collected, washed and finally stored at −80°C in pools of five and 10 embryos in 70 µl Trizol® reagent (Ambion, Life Technologies) for further molecular analyses as described above for *in vitro* produced embryos.

RNA extraction and quantitative real-time-polymerase chain reaction (RT-qPCR)

Pools of five blastocysts (produced *in vitro* and *in vivo*) were processed for total RNA extraction using Trizol® reagent protocol, according to the manufacturer's instructions. Briefly, 430 µl of Trizol (Ambion, Life Technologies), was added to the tubes containing embryos to adjust the volume up to 500 µl. Immediately thereafter, 10 µg of RNase-free glycogen (Ambion, Life Technologies) and 200 µl of chloroform (Merck, Darmstadt, Germany) were added to the tubes. Samples were vigorously shaken, incubated at room temperature for 2 min, and centrifuged at 12,000 *g* for 15 min at 4°C. The upper aqueous phase was removed, and 500 µl cold isopropanol (Merck) was added to the pellet. RNA was precipitated overnight at −20°C, followed by centrifugation at 12,000 *g*, for 10 min at 4°C. After supernatant removal, RNA pellets were washed with 500 µl 75% ethanol (Merck), air-dried, and resuspended in 30 µl RNase-free water.

The recovered total RNA was immediately transcribed into cDNA. Reverse transcription was performed with total RNA using the Superscript™ III first strand synthesis kit (Invitrogen, Life Technologies) with random hexamer primers. The temperatures and times were 25°C for 10 min, 42°C for 50 min, and 85°C for 5 min. Then, 2 IU of *E. coli* RNase H was added to each tube and the samples were incubated at 37°C for 20 min.

Primers for RT-qPCR were designed using Primer Premier 5 software (Premier Biosoft International, Palo Alto, CA, USA). All primers were designed to span exon–intron boundaries to rule out genomic DNA amplification (Table 1). RNA relative quantification was performed in three biological replicates and three technical replicates and RT-qPCR was performed on a Rotor-Gene™ 6000 Real-Time PCR instrument (Corbett Life Science, Australia). Quantitative assessment was performed by QuantiTec SYBR PCR kit, (Qiagen, Valencia, CA, USA). Reactions were performed in a total volume of 20 µl using cDNA equivalents of 0.2 embryos and gene specific primers. The polymerase chain reaction (PCR) parameters were 95°C for 5 min for denaturation, 50 cycles of 95°C for 15 s at 60°C for 20 s, 72°C for 30 s and for final extension of 72°C for 5 min. After each PCR run, a melting curve analysis was performed for each sample to confirm that a single specific product had been generated. A negative reverse transcription control was performed to check for genomic DNA contamination. Gene expression levels were calculated by efficiently corrected $^{\Delta\Delta}$Ct method, using *GAPDH* for data normalization. Primer efficiency was calculated using the program LinRegPCR (Ramakers *et al.*, 2003) for each reaction. The selected transcripts are related to DNA methylation (*DNMT1* and *DNMT3A*), growth and development (*IGF2R*, imprinted gene) and reprogramming (*POU5F1*).

Analysis of CpG methylation

Bos indicus genomic DNA from pools of 10 expanded blastocysts produced *in vitro* or *in vivo* was subjected to bisulfite conversion, to study the methylation status of a set of CpG sequences from the BTS and *Bos taurus* alpha satellite I (BTαS) (*Bos taurus* genome). The *Bos taurus* sequences were used due to limited annotation in the *Bos indicus* genome when these experiments were performed. However, analysis using the *Bos indicus* databased at the Basic Local Alignment Search Tool (BLAST) from the National Center for Biotechnology Information (NCBI), revealed hits with more than 96% of identity and E-values lower than

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Table 1 Primer sequences used for gene expression analysis by real-time PCR

Gene name	Gene symbol	Accession number	Primer sequence (5′→3′)	Fragment size (bp)
DNA (cytosine-5-)-methyltransferase 1	*DNMT1*	NM_182651.2	F: AGTGGGGGACTGTGTTTCTG; R: TGCTGTGGATGTACGAGAGC	218
DNA (cytosine-5-)-methyltransferase 3 alpha	*DNMT3A*	NM_001206502.1	F: GGGGTCTTCATTCCCAATTT; R: AAAACTGCAGCCTTTGGAGA	266
Insulin-like growth factor 2 receptor	*IGF2R*	NM_174352.2	F: GTCGTGCAGATCAGTCCTCA; R: GTCGTTCTGGAGCTGAAAGG	153
POU class 5 homeobox 1	*OCT-4*	NM_174580.2	F: AGTGAGAGGCAACCTGAAGA; R: ACACTCGGACCACGTCTTTC	110
Glyceraldehyde-3-phosphate dehydrogenase[a]	*GAPDH*	NM_001034034	F: TGCTGGTGCTGAGTATGTGGT; R: AGTCTTCTGGGTGGCAGTGAT	295

[a]Denotes the endogenous reference gene.

$3e^{-61}$, located in different chromosomes for the studied sequences, indicating conserved homology. For DNA extraction, DNA/RNA from *in vitro* and *in vivo* produced blastocysts were isolated simultaneously using Trizol® reagent (Invitrogen) according to the manufacturer's instructions. After complete removal of the aqueous phase, as described above for the RNA isolation protocol, DNA in the interphase was isolated by removing the remaining aqueous phase, and precipitating the DNA with ethanol. For initial homogenization, 150 μl ethanol (Merck) were added, and samples were mixed by inversion. Next, samples were stored at room temperature for 3 min and DNA was precipitated at 2000 *g* for 5 min at 4°C. The supernatant was removed and DNA pellets were washed twice in a solution containing 0.1 M sodium citrate (Merck) in 10% ethanol (Merck). At each wash, DNA pellets were stored in the washing solution for 30 min at 15 to 30°C (with periodic mixing). After the second wash, DNA was centrifuged at 2000 *g* for 5 min at 2 to 8°C, and the pellet was resuspended in 750 μl of 75% ethanol (Merck). DNA was incubated for further 15 min at room temperature (with periodic mixing) and centrifuged at 2000 *g* for 5 min at 4°C. Finally, cleaned DNA samples were dissolved in 50 μL 8 mM NaOH (Merck), frozen and stored at −20°C until further analysis.

Bisulfite conversion was performed using the EZ DNA Methylation-Direct™ Kit (Zymo Research, Freiburg, Germany) according to the manufacturer's instructions as described recently (Bernal *et al.*, 2015). Purified extracted DNA was converted using the CT Conversion Reagent provided by the kit, and incubated at 98°C for 8 min followed by 64°C for 3.5 h in a thermal cycler. After DNA conversion, unmethylated cytosines are transformed into uracil, while methylated cytosines remain unchanged. Converted DNA was washed and cleaned using the Zymo-Spin™ IC column and dissolved in 10 μl M-Elution Buffer.

Bovine testis satellite I (BTS) and *Bos taurus* alpha satellite I (BTαS) sequences were amplified using specific primers (Table 2) (Kang *et al.*, 2005; Diederich *et al.*, 2012). BTS contains 12 highly conserved CpG sites in a 211-bp fragment. For the BTαS sequence, a fragment of 154 bp containing nine CpG sites was analyzed (Kang *et al.*, 2005). Satellite sequence-specific PCR fragments were amplified and successful amplification was confirmed by agarose gel electrophoresis. PCR products were cleaned using the Invisorb® Fragment Cleanup System (Stratec Molecular GmbH, Berlin, Germany) according to manufacturer's instructions. PCR products were ligated into the pGEM-T easy vector (Promega Corporation, Madison, USA) and the plasmids were transformed into *Escherichia coli* XL10-Gold cells (Stratagene, Santa Clara, CA, USA). Clones were screened for successful ligation and transformation; therefore colonies were picked and directly used for PCR amplification of the insert using the universal T7 and SP6 primers (Table 2). Positive clones were submitted to DNA sequencing using the same universal primers on a 3730xl DNA Analyzer (Applied Biosystems, Foster City, CA, USA). Sequences were analyzed using the BiQ Analyzer program (Bock *et al.*, 2005). The specific genomic sequence from the bovine genome for each studied satellite was used for comparison and CpG identification on sample sequences. Clone sequences with a conversion rate lower than 90% or with a high number of sequencing errors in the alignment were excluded from the analysis. The methylation profiles for each satellite were evaluated by counting the methylated CpG sites of the total number of analyzed CpG.

Statistical analysis

Relative expression software tool (REST) was used to compare mRNA abundances in each group. The mathematical model used in REST software is based on PCR

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Table 2 Primer sequences used for analysis of satellite sequences

Repeat/binding site	GenBank accession no.	Primer sequences (5→3)	Fragment size (bp)	References
Bovine testis satellite I (BTS)	J00032.1	F: AATACCTCTAATTTCAAACT; R: TTTGTGAATGTAGTTAATA	211	Kang *et al.*, 2005
Bos taurus alpha satellite I (BTαS)	AJ293510.1	F: GATGTTTTYGGGGAGAGAGG; R: CCRATCCCCTCTTAATAAAAACC	154	Kang *et al.*, 2005
T7		ACTCACTATAGGGCGAATTG		
SP6		ATTTAGGTGACACTATAGAATACTC		

Table 3 Embryo production

In vivo embryo production in *Bos indicus* cows (% mean ± SEM)				
Items	Total structures	Fertilization rate *n* (%)[a]	Transferable embryos *n* (%)[b]	Degenerated embryos *n* (%)[c]
Cows ($n = 12$)	127	113 (88.8 ± 3.6)	101 (79.4 ± 8.7)	12 (10.6 ± 8.3)
Embryo developmental rates *in vitro* of *Bos indicus* cows (% mean ± SEM)				
No. of IVM oocytes		Cleavage *n* (%)		Blastocyst *n* (%)
880[d]		713 (81.1 ± 1.4)		284 (39.9 ± 0.5)

[a]Fertilization rate: proportion of recovered grade 1, 2, 3 or 4 embryos divided by total ova.
[b]Transferable embryos rate: proportion of recovered grade 1, 2 or 3 embryos divided by total ova.
[c]Degenerated embryos rate: proportion of grade 4 embryos divided by total ova.
[d]Six replicates. The blastocyst rate is based on cleaved embryos.
IVM, *in vitro* matured.

efficiencies and the crossing point deviation between samples (Pfaffl, 2002). For each group there were three biological and three technical replicates. Methylation profiles were analyzed using the Pearson's chi-squared test from R software (R Development Core Team, 2011). The level of significance for all tests was set at $P \leq 0.05$. Data from *in vivo* and *in vitro* embryo production are presented descriptively.

Results

In vivo and *in vitro* production of bovine embryos

To determine the effects of *in vitro* embryo production on DNA methylation profiles and the expression of genes involved in epigenetic reprogramming during early embryo development in *Bos indicus*, we produced *in vivo* and *in vitro* bovine blastocysts. All donors submitted to multiple ovulations (MO) responded to the stimulation protocol, and two or more corpora lutea (CL) were visualized by ovarian ultrasound examination. In total, 101 embryos with grade 1, 2 or 3 were recovered after uterine flushing for molecular analysis. Cleavage rates were calculated as the proportion of all recovered embryos divided by the number of total obtained ova (88.8 ± 3.6%). The rate of transferable embryos, measured as the proportion of grade 1, 2, and 3 recovered embryos divided by the number of total ova, was 79.4 ± 8.7% (Table 3). After *in vitro* embryo production, 284 embryos were obtained for this study. Six replicates of the *in vitro* embryo production, using COCs obtained from slaughterhouse material, were performed. On average, the cleavage rate was 81.1 ± 1.4% at 72 hpi and after 7 days of *in vitro* embryo culture 39.9 ± 0.5% of the cleaved embryos had developed to the blastocyst stage (blastocysts/cleaved embryos).

mRNA expression analysis of *DNMT1*, *DNMT3A*, *IGF2R* and *POU5F1* genes in bovine blastocysts produced *in vitro* or *in vivo*

Expression of *GAPDH* (glyceraldehyde-3-phosphate dehydrogenase) was used as the internal control. DNA methyltransferase 1 (*DNMT1*) transcript levels were significantly reduced in *in vitro* produced blastocysts ($P < 0.05$) compared to their *in vivo* derived counterparts. DNA methyltransferase 3A (*DNMT3A*), *IGF2R* (Insulin-like growth factor 2 receptor) and *POU5F1* (POU class 5 homeobox 1) mRNA abundance were not significantly different between *in vivo* and *in vitro* derived blastocysts (Fig. 1).

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Table 4 Methylation pattern of bovine testis satellite I (BTS) and *Bos taurus* alpha satellite I (BTαS) sequences.

Satellite	Protocol	Total CpGs evaluated (*n*)	Total CpGs methylated (*n*)	Methylation level (%)[c]
Bovine testis satellite I (BTS)	*In vivo*	518	68	13.1[a]
	In vitro	588	110	18.7[b]
Bos taurus alpha satellite I (BTαS)	*In vivo*	374	134	35.8
	In vitro	397	129	32.5

[a,b]Rows with different superscript letters per satellite are significantly different ($P < 0.05$).
[c]Per cent methylation levels are the proportion of methylated CpG sites relative to the total number.

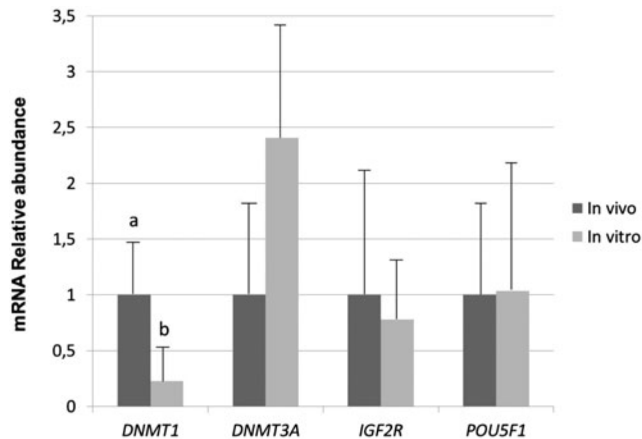

Figure 1 Transcript levels [(mean ± standard error of the mean (SEM)] for DNMT1, DNMT3A, IGF2R, and POU5F1, analyzed by RT-qPCR in *Bos indicus* embryos produced *in vivo* (black columns) and *in vitro* (grey columns). Each group was analyzed using three biological replicates and three technical replicates. Each biological replicate consisted of a pool of five embryos. [a,b]Different letters in the bars indicate different values ($P < 0.05$).

Methylation profiles of two satellite DNA sequences

In total, 184 clones, including 1887 CpGs, were evaluated to determine the *Bos indicus* blastocyst methylation status of the ovine testis satellite I (BTS) and *Bos taurus* alpha satellite I (BTαS) sequences (Table 4). For *in vivo* produced embryos, the methylation percentage of BTS was 13.1%, whereas the methylation profile of *in vitro* produced embryos was significantly higher (18.7%, $P < 0.05$). The CpG methylation level for the BTαS sequence did not differ significantly between embryos produced *in vivo* (35.8%) and embryos produced *in vitro* (32.5%).

Discussion

In vitro embryo production has emerged as a useful alternative to conventional embryo transfer and the technology is being used commercially in several countries around the globe. In South America, a significant proportion of cattle embryos has been produced by IVP since 2004, primarily in *Bos indicus* animals (Camargo *et al.*, 2010), but little information is known about the effects on embryo quality in this species. Furthermore, numerous studies have indicated that the consequences of exposing embryos to *in vitro* culture conditions include alterations in gene expression and aberrant DNA methylation (Wrenzycki *et al.*, 2002; Niemann *et al.*, 2010; Urrego *et al.*, 2014). Here, we investigated the effects of *in vitro* embryo production on satellite DNA CpG methylation status and relative mRNA abundance of four developmentally important genes in expanded *Bos indicus* blastocysts produced *in vivo* and *in vitro*.

In the present study, blastocyst rates using commercial media were consistent with previous reports, in which culture was carried out under similar conditions (Camargo *et al.*, 2011; Urrego *et al.*, 2015). *In vivo* blastocyst production was as expected in *Bos indicus* animals. It is known that *Bos indicus* cattle differ from their *Bos taurus* counterparts in several details of reproductive physiology (Baruselli *et al.*, 2011). For instance, *Bos indicus* have a greater sensitivity to gonadotropins, a shorter duration of estrus, and more often express estrus during the night (Bó *et al.*, 2003). It has been also reported that *Bos indicus* cows have a smaller diameter of the dominant follicle and more ovarian follicles recruited per follicular wave than *Bos taurus*, which in turn may increase embryo production (Sartori *et al.*, 2010).

Development of bovine embryos subjected to *in vitro* culture has been associated with an increased frequency of pre- and postnatal abnormalities that are thought to be the result of changes in epigenetic marks and gene expression profiles (Wrenzycki *et al.*, 2005; Rodriguez-Osorio *et al.*, 2012; Urrego *et al.*, 2014). In the present study, we found that *DNMT1* expression was significantly reduced in *in vitro* produced blastocysts compared with *in vivo* blastocysts (Fig. 1), whereas no significant differences were found with regard to the relative mRNA abundance for *DNMT3A*, *IGF2R* and *POU5F1* between *in vitro* and *in vivo* derived embryos.

DNMT1 plays a critical role in the maintenance of DNA methylation by restoring the methylation pattern

https://doi.org/10.1017/S096719941600040X Published online by Cambridge University Press

of newly synthesized hemi-methylated DNA strands during replication (Bestor *et al.*, 1992; Pradhan *et al.*, 1999). Previous studies had indicated a pattern of aberrant expression profile for *DNMT1.* In human embryos, *DNMT1* expression was lower in poor quality embryos compared to the reference group (Petrussa *et al.*, 2014). Transcript levels of *DNMT1* were lower in somatic cell nuclear transfer (SCNT) derived bovine embryos, suggesting that epigenetic programming by *DNMT1* is critical for regular bovine preimplantation development (Golding *et al.*, 2011). Likewise, it has been demonstrated that vitrification decreased the mRNA abundance of *Dnmt1o* in mouse oocytes, probably as an effect of altered epigenetic marks (Zhao *et al.*, 2013). Therefore, the lower mRNA abundance observed in the *in vitro* produced blastocysts is probably associated with decreased blastocyst quality.

The *DNMT3A* protein is a *de novo* DNA methyltransferase, which acts upon hemi-methylated and non-methylated DNA with equal efficiency during early embryonic development (Okano *et al.*, 1999). In contrast with *DNMT1*, transcript levels of *DNMT3A* were not affected by *in vitro* culture in the present study. These findings differ from the results reported by Hoffmann *et al.* (2006), in which the amount of *DNMT3A* mRNA was affected by *in vitro* culture. Various protocols are presently employed to generate bovine IVF embryos, and protocol-specific differences therefore might explain discrepancies in results.

Transcript abundance for *IGF2R* did not differ in IVP embryos compared with those of the control group. However, loss of *IGF2R* expression in bovine *in vitro* produced embryos was associated with excessive fetal and placental growth (Farin *et al.*, 2006, 2010; Farmer *et al.*, 2013). Aberrant expression of *IGF2R* directly correlated with the large offspring syndrome (LOS) in sheep (Young *et al.*, 2001). In general, *SNRPN*, *H19/IGF2*, and *IGF2R* imprinted genes are extensively hypomethylated in early stage embryos derived from SCNT and to a lesser extent in IVP embryos, indicating that reprogramming of the chromatin and the *in vitro* culture of oocytes and/or embryos may be associated with epigenetic erasure of imprinted loci (Smith *et al.*, 2015).

POU5F1 (also called *OCT4*) is a member of the *POU* transcription factor family with a germ line-specific expression profile. It is widely used to identify pluripotent cells in different species and plays a critical role in bovine preimplantation development (Kirchhof *et al.*, 2000; Herrmann *et al.*, 2013). This study did not find differences in the level of *POU5F1* expression. However, it has been shown that the mean transcript level of *POU5F1* was significantly higher in KSOMaa cultured blastocysts than in blastocysts produced in SOFaa medium or *in vivo*, clearly indicating sensitivity of OCT-4 expression to culture environment (Purpera *et al.*, 2009).

Epigenetic reprogramming of the mammalian genome after fertilization creates the methylation patterns needed for normal development by activation and silencing of specific genes (Reik *et al.*, 2001; Haaf, 2006). The global methylation of the bovine genome declines to a nadir at the 6–8-cell stage and increases thereafter (Dobbs *et al.*, 2013), rendering early embryos specifically vulnerable to ART-related epigenetic defects (El Hajj & Haaf, 2013). Genome-wide abnormalities in DNA methylation patterns or cytosine methylation levels after IVP have been observed in bovine embryos (Hou *et al.*, 2007; Suzuki *et al.*, 2009; Niemann *et al.*, 2010). Our results revealed significant hypermethylation for BTS in IVP embryos compared with their *in vivo* derived counterparts, while no significant difference was observed for BTαS between the two groups of embryos.

Satellite DNA is found in centromeric and pericentromeric regions and is highly conserved in mammals (Enukashvily and Ponomartsev, 2013). Considered as 'junk DNA' for many years, it is known that satellite DNAs have significant impact on genomic functions including chromosome organization and segregation. Furthermore, their transcripts have functional roles on formation and maintenance of heterochromatin structure (Plohl *et al.*, 2012), and have been related to stress, embryogenesis, mitosis, senescence and carcinogenesis (Enukashvily & Ponomartsev, 2013). Some DNA satellites sequences have been successfully analyzed for monitoring epigenetic changes in early embryos related to the origin of oocytes and embryos (Suzuki *et al.*, 2009; Sawai *et al.*, 2011; Bernal *et al.*, 2015). The hypermethylation observed in the BTS satellite sequence in the *in vitro* generated embryos compared with their *in vivo* produced counterparts, could indicate perturbation of demethylation of specific sequences. The decreased *DNMT1* expression detected in the *in vitro* produced embryos could be the result of a compensation mechanism to control global DNA hypermethylation. However, future studies are necessary to confirm these observations.

Similar to our results, increased DNA methylation levels of BTS have been reported previously for blastocysts produced *in vitro* (Bernal *et al.*, 2015). Satellite DNA hypermethylation has been observed in aborted cloned fetuses (Zhang *et al.*, 2014) and abnormal placenta (Perrin *et al.*, 2007). Furthermore, highly methylated DNA may be associated with gene silencing. The bisulfite sequencing approach in the present study does not allow discrimination between 5-methylcytosine (5-mC) and 5-hydroxymethylcytosine (5-hmC), and the identification of non-CpG methylation sites, but it provides a general overview of

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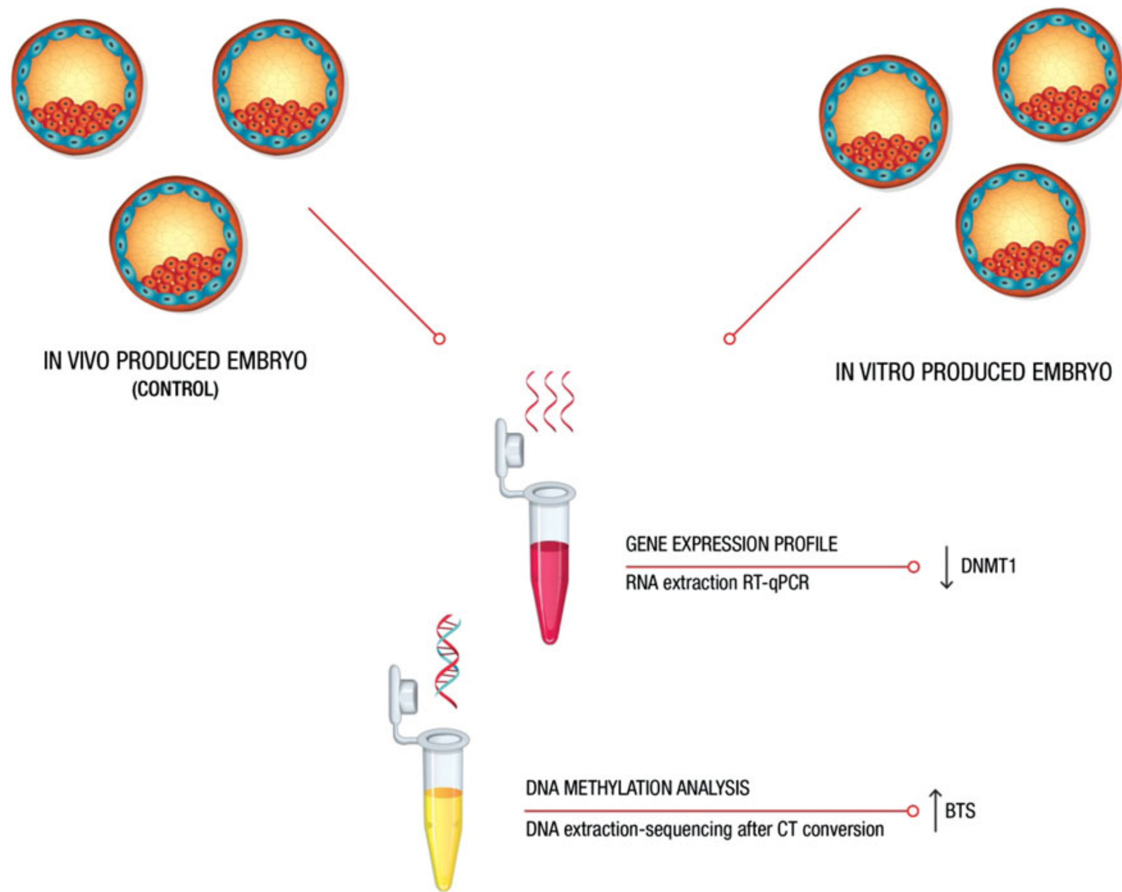

Figure 2 Influence of IVP on epigenetic profiles and gene expression in *Bos indicus* embryos. Relative transcript abundance for genes was compared in embryos produced *in vivo* (control) vs. embryos produced *in vitro*. *In vitro* produced embryos had significantly lower amounts of DNMT1 ($P < 0.05$), while DNA methylation was significantly higher ($P < 0.05$) in the bovine testis satellite I (BTS) sequence in embryos produced *in vitro* compared with that of *in vivo* produced embryos.

CpG DNA methylation status, indicating that *in vitro* embryo production in *Bos indicus* cattle affects embryo quality at the molecular level as reported previously in other cattle species (Hou *et al.*, 2007). When interpreting these findings, one has to take into account that *in vivo* derived embryos used for these experiments, were collected after superovulatory treatment of the donors. Although superovulation can affect embryonic gene expression (Mundim *et al.*, 2009; Diederich *et al.*, 2012; Urrego *et al.*, 2014), it is the only way to produce sufficient *in vivo* derived embryos for research purposes.

In conclusion, our study reports changes in gene expression profiles and aberrant methylation patterns of satellite DNA in *Bos indicus* blastocysts produced *in vitro* (Fig. 2). The present results are consistent with previous findings, in which bovine embryos respond to alterations in their environment by modifying DNA methylation and transcription, thus confirming the impact of ARTs on epigenetic marks during embryo development in different species. Further research in *Bos indicus* cattle is needed to clarify the effects of ARTs on *in vitro* production to improve the quantitative and qualitative efficiency of the process.

Acknowledgments

The authors thank Administrative Department of Science, Technology and Innovation (COLCIENCIAS) for providing a scholarship for the first author. COLCIENCIAS COD 122852128473, CES University and the Sustainability Strategy 2013–2014 from CODI (Universidad de Antioquia), supported this work. We thank Jose Tamayo for assistance in figure design.

Conflicts of interests

None of the authors has any conflict of interest to declare.

https://doi.org/10.1017/S096719941600040X Published online by Cambridge University Press

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