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Loss of Methylation at *H19* DMD Is Associated with Biallelic Expression and Reduced Development in Cattle Derived by Somatic Cell Nuclear Transfer[1]

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ABSTRACT

Although cloning of mammals has been achieved successfully, the percentage of live offspring is very low because of reduced fetal size and fewer implantation sites. Recent studies have attributed such pathological conditions to abnormal reprogramming of the donor cell used for cloning. The inability of the oocyte to fully restore the differentiated status of a somatic cell to its pluripotent and undifferentiated state is normally evidenced by aberrant DNA methylation patterns established throughout the genome during development to blastocyst. These aberrant methylation patterns are associated with abnormal expression of imprinted genes, which among other genes are essential for normal embryo development and gestation. We hypothesized that embryo loss and low implantation rates in cattle derived by somatic cell nuclear transfer (SCNT) are caused by abnormal epigenetic reprogramming of imprinted genes. To verify our hypothesis, we analyzed the parental expression and the differentially methylated domain (DMD) methylation status of the *H19* gene. Using a parental-specific analysis, we confirmed for the first time that *H19* biallelic expression is tightly associated with a severe demethylation of the paternal *H19* DMD in SCNT embryos, suggesting that these epigenetic anomalies to the *H19* locus could be directly responsible for the reduced size and low implantation rates of cloned embryos in cattle.

allelic imprinting, cattle, cloning, DNA methylation, early development, embryos, epigenetics, H19 *gene, imprinting, in vitro fertilization, IVF, SCNT*

INTRODUCTION

Cloning of livestock animals by somatic cell nuclear transfer (SCNT) has been successfully achieved in a wide range of mammals, including a number of domestic species [1–4]. Therefore, SCNT has become an important biological tool to introduce and multiply desired alleles into a breeding population without the uncertainty of genetic recombination and to generate transgenic livestock. Even though cloning has proven to be more efficient in cattle than other species studied, certain problems are common, such as low efficiency, pregnancy failure, and gross placental structural and functional abnormalities. In general, about 7%–8% of blastocysts produced by SCNT develop to term [5]. The preimplantation stage accounts for the vast majority of bovine SCNT embryonic loss, and for the embryos that successfully implant, continued pregnancy loss is observed throughout gestation [6]. Hydropsy, enlarged umbilical cords with dilated vessels, enlarged and fewer placentomes, and large offspring syndrome (LOS), often observed in the second and third trimesters of SCNT pregnancies [7, 8], have been attributed to failures in epigenetic reprogramming.

Abnormal patterns of epigenetic marks, such as DNA methylation and histone modifications found in SCNT embryonic and fetal tissue, are often reported as aberrant reprogramming of the donor cell [9, 10]. Epigenetic marks are normally established during the development of primordial germ cells (PGCs). Once the PGCs migrate to the genital ridge, previous DNA methylation marks are erased, and new genomic imprinting is established according to the sex of these cells. This imprinting mechanism is important to determine the parent of origin expression of a particular group of genes called imprinted genes, which will be responsible for extraembryonic and embryonic tissue development. In cloned embryos, a considerable number of studies have reported deficient epigenetic reprogramming, where abnormal patterns of DNA methylation [11–13] and imprinted gene expression [13–16] were found. Because some of these imprinted genes, such as *IGF2* and *H19*, are essential for cell differentiation and tissue growth, this raised the question as to whether epigenetic failure could account for abnormal embryo development and early loss in SCNT pregnancies.

The *H19* gene is probably one of most studied imprinted genes in mammals because it belongs to the same cluster and shares regulation with *IGF2*, a gene that functions as a growth-promoting hormone controlling fetal growth during gestation. Their imprinted expression is controlled by the imprinting control region (ICR), which is also referred to as the differentially methylated domain (DMD) [17] and whose

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mechanism of control seems to be well conserved in mammals [18–20]. The DMD is preferentially methylated on the paternal allele and controls the activity of the enhancer-blocking protein CTCF, whose ability to bind to DNA is abolished by the presence of CpG methylation of its recognition motif, the CTCF binding site [21]. On the maternal allele, however, demethylated CTCF binding sites allow the CTCF protein to promote exclusive expression of the maternal *H19* gene allele. In the bovine species, even though many studies have shown that abnormal regulation of the *H19* gene is often reported in failed SCNT pregnancies, either by abnormal methylation patterns [22] or aberrant *H19* expression [23], the mechanism underlying these abnormalities remains misunderstood. Because single-nucleotide polymorphisms (SNPs) have not been reported so far in the bovine *H19* locus, a comprehensive analysis of the parental DMD methylation patterns remains compromised by the fact that the paternal and maternal alleles cannot be distinguished, and methods to determine allelic expression are mainly qualitative. We hypothesized that paternal methylation of *H19* DMD is abnormally established in SCNT embryos and that this failure in cell reprogramming accounts for aberrant imprinted gene expression and pregnancy failures in cattle. Our results showed that the CTCF binding site is severely demethylated in the paternal allele of most SCNT embryos at Day 17 and that methylation was inversely proportional to paternal expression of the *H19* gene. In the postimplantation period, from Days 30 to 50, methylation levels and paternal expression of the *H19* gene in SCNT embryos were similar to controls, suggesting that embryos with widespread demethylation of the *H19* DMD are unable to implant. To our knowledge, this study has indicated for the first time a close association between the paternal expression of *H19* and the methylation levels on its DMD in cattle.

MATERIALS AND METHODS

All procedures were performed in compliance with the Guide for the Care and Use of Agricultural Animals in Research and Training, approved by the animal experimentation committee of the Université de Montréal, and sanctioned by the Canadian Council on Animal Care.

Nuclear Donor Cells

Fetal fibroblast cell cultures were established from a 60-day-old crossbred fetus produced by artificial insemination of a *Bos taurus* heifer with semen from a *Bos indicus* bull. A different fibroblast line with a reciprocal genetic background was produced by crossing a *B. indicus* heifer to a *B. taurus* bull. Tissues were minced manually and digested with 0.25% trypsin and 0.02% ethylenediaminetetraacetic acid (Gibco, Burlington, ON) at 37°C for 10 min. Isolated cells were washed and cultured for approximately 4 days in Dulbecco modified Eagle medium (Gibco) supplemented with 10% fetal bovine serum (FBS; Gibco) and 0.5% antibiotics (penicillin 10 000 U/ml and streptomycin 10 000 μg/ml; Gibco) at 37°C in 5% CO_2. When the cultures were confluent, primary passage fibroblast cells were frozen in culture media supplemented with 10% dimethyl sulfoxide and stored in liquid nitrogen. Donor fibroblasts were thawed at 37°C for 1 min and cultured to confluence for a maximum of five passages before use for SCNT.

Host Oocytes

Cattle ovaries were collected from a local abattoir and transported to the laboratory in saline at 30°C–35°C within approximately 2 h after slaughter. Follicles with diameters between 2 and 10 mm were punctured with an 18-gauge needle, and cumulus oocyte-complexes (COCs) with approximately four to six layers of cumulus cells and homogeneous oocyte cytoplasm were washed in Hepes-buffered tissue culture medium (TCM-199; Gibco) supplemented with 10% (vol/vol) FBS. Groups of 20 COCs were placed in 100 μl of bicarbonate-buffered TCM-199 supplemented with 10% FBS, 50 μg/ml luteinizing hormone (Ayerst, London, ON), 0.5 μg/ml follicle-stimulating hormone (FSH; Folltropin-V; Vetrepharm, St-Laurent, PQ), 1 μg/ml estradiol 17-β (Sigma-Aldrich, St. Louis, MO), 22 μg/ml pyruvate (Sigma-Aldrich), and 50 μg/ml gentamicin (Sigma-Aldrich). After 19 to 20 h of in vitro maturation, cumulus cells were removed from the COCs by vortexing for 2 min in PBS and 0.2% hyaluronidase (Sigma-Aldrich). Only oocytes with homogeneous cytoplasm and intact cell membrane were selected for micromanipulation.

In Vivo-Derived and In Vitro-Derived Embryos

Production of embryos and fetuses for in vivo (AI) and in vitro (IVF) controls, as well as donor cells were conducted as described previously [12]. Follicular waves were monitored by ultrasound, and heifers were superovulated by intramuscular injection of porcine FSH (Folltropin-V) given every 12 h in decreasing doses starting at Day 9–11 of the estrous cycle. On Day 12, heifers received an injection of 500 μg of cloprostenol (Estrumate; Schering-Plough Animal Health, Pointe-Claire, QC) and were artificially inseminated at 48 h after the injection of cloprostenol [13].

To obtain IVF embryos with reciprocal genetic background, in vitro-matured oocytes of either *B. indicus* or *B. taurus* origin were fertilized in vitro with semen from the opposite breed using standard protocols [12]. Briefly, 20–25 COCs were placed in 100-μl drops of Tyrode medium supplemented with 0.6% bovine serum albumin (BSA; fraction V; Sigma-Aldrich), lactate, pyruvate, gentamicin, and heparin (10 μg/ml). Frozen-thawed spermatozoa were washed and centrifuged through a Percoll (Sigma) gradient and diluted to 10^6 live spermatozoa per milliliter. At 20 h following the start of incubation with spermatozoa, COCs were denuded of cumulus cells by brief shaking, and the putative zygotes were transferred to 25-μl drops of synthetic oviduct fluid (SOF) medium [24] and cultured for 8 days with an additional 25 μl of SOF medium under the same conditions used for the SCNT embryos.

Somatic Cell Nuclear Transfer

Bovine oocytes were obtained by aspiration of small antral follicles (2–8 mm in diameter). Immature COCs were cultured in TCM-199 supplemented with 10% FBS, 0.2 mM pyruvate, 50 μg/ml gentamicin, 0.5 μg/ml FSH, and 150 μg/ml luteinizing hormone. Approximately 20 h after the start of maturation, cumulus cells were completely removed from the oocyte by manual pipetting in the presence of 0.2% hyaluronidase, and oocytes with an extruded first polar body were selected for enucleation. The oocytes were labeled with 10 μg/ml DNA fluorochrome (Hoechst 33342) for 10 min at room temperature in SOF containing 0.2 mM pyruvate and 3 mg/ml BSA, and were washed and transferred in a manipulation drop of SOF-HEPES [24] supplemented with 5.0 μg/ml cytochalasin B. The first polar body and metaphase II (MII) plates were removed by aspiration with a 15-μm inner diameter enucleation pipette. To ensure that oocyte chromatin was properly removed, aspirated cytoplasm was exposed to ultraviolet light and examined to confirm the absence of the polar body and metaphase plate.

A single donor cell was placed into the perivitelline space of each enucleated oocyte. The SCNT couplets were then placed into a fusion chamber filled with 0.27 M mannitol. One pulse of alternating current (5 msec, 50 V/cm) and two pulses of direct current (60 μsec each, 1.2 kV/cm) were applied to promote fusion. Successfully reconstructed SCNT oocytes (i.e., fused couplets) were activated by ionomycin [13]. Twenty-six hours after the start of maturation, SCNT oocytes were placed in 5 μM ionomycin in TCM-199-Hepes medium (supplemented with 1 mg/ml fatty acid-free BSA) for 5 min. The SCNT oocytes were then moved into TCM-199-Hepes medium (supplemented with 30 mg/ml BSA) for 5 min, followed by incubation in SOF medium supplemented with 6-dimethylpurine for 3 h. At the end of incubation, SCNT oocytes were washed in SOF medium and placed into in vitro culture medium drops. Cleavage and blastocyst rates were evaluated on the second and seventh days, respectively.

Day 17 Elongating Embryos and Day 30–50 Fetuses

The estrous cycles of recipient Holstein heifers were synchronized by an injection of 500 μg of the prostaglandin F2α analogue, cloprostenol (Estrumate; Schering Canada Inc.). Six to eight days after standing heat, Day 8 blastocysts were washed with TCM-199 Hepes-buffered medium supplemented with 10% FBS, loaded into a 250-μl straw, and transferred to the uterine horn ipsilateral to the corpus luteum. In general, heifers received between 10 and 15 Day 8 in vitro-produced or SCNT embryos, and development was allowed for another 9 days in the uterine horn. Day 17 elongated embryos were nonsurgically recovered in the IVF, SCNT, and AI groups by flushing the uterus of heifers with PBS using a Foley catheter. Embryos were removed from the flushing media and inspected; only embryos that were recovered intact were used for the experiments. After selection, embryos were washed three times in PBS, frozen, and stored individually at −70°C. For postimplantation collection, groups of heifers carrying

TABLE 1. PCR primers used for the semi-quantitative and parental expression analysis.

Primer	Sequence
bGAPDHUPL-F	5′-TCTGGCAAAGTGGACATCG-3′
bGAPDHUPL-R	5′-GACCATGTAGTGAAGGTCAATGAA-3′
GAPDHUPL#147	5′-GCCATCAA-3′
ACTB-UPL-F	5′-CGCGACAGGATGCAGAAA-3′
ACTB-UPL-R	5′-ACGGAGTACTTGCGCTCAG-3′
H2AFZ-UPL-F	5′-CGTGGAGATGAAGAATTGGAC-3′
H2AFZ-UPL-R	5′-AGATTTGTGGATGTGTGGAATG-3′
bH19UPL-F	5′-CTCCAGTGGGGTGTTGGT-3′
bH19UPL-R	5′-GACCATATCATATCCCTCTGTGC-3′
H19UPL#162	5′-GGCCAGGA-3′
bH19FRET-F	5′-TATGATATGGTCCGGTGTGAT-3′
bH19FRET-R	5′-CAGGCATGAGCTGGGTA-3′
bH19LCRed	5′-LCRed640-CGTCGATATC[C]GGGCTCCC-Phosphate-3′
bH19fluo	5′-TGTCTGCTCCGTCAGGAGACTAAAGG-fluo-3′
H19F1	5′-AGTGGGAGGGGCATTGGACTT-3′
H19EX4R2	5′-CTTCCAGAGCTGATTCCTGAG-3′
U-H19-F1	5′-ACACCTTAAAAAACTCAAATAAATACC-3′
U-H19-R1	5′-TATTTTAGATAGGGTTGAGAGGTTG-3′

one or two blastocysts each were allowed to continue gestation to Days 30–50. Recipients carrying fetuses with a normal heartbeat were slaughtered at the local slaughterhouse and transported within approximately 1 h.

DNA and RNA Extraction, and RT Reactions

DNA was isolated from Day 17 and Day 30–50 fetuses and placenta using a DNAeasy extraction kit (Qiagen). Total RNA was extracted using an RNeasy Extraction kit (Qiagen) with a DNase treatment. Reverse transcription was performed using an Omniscript RT kit (Qiagen) with 1 μg of total RNA and random primers (3 μg). The RT reactions were then purified with a MinElute Reaction cleanup kit (Qiagen) and resuspended in a final 50-μl volume of elution buffer. For Day 30–50 fetuses and placenta samples, 1 μg of total DNAse-treated RNA was reversed transcripted with Improm-II Reverse Transcription System (Promega) with random primers (3 μg; Invitrogen) following the manufacturer's instructions. The cDNA was purified with a MinElute Reaction cleanup kit and resuspended in 50 μl of elution buffer.

Gene Expression Analysis

Five microliters of purified cDNA was quantified by real-time PCR in duplicate using the RotorGene instrument and software (Qiagen). Following the company specifications, a comparative analysis was performed using three housekeeping genes: glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*), β-actin (*ACTB*), and the H2A histone family, member Z (*H2AFZ*; see Table 1 for primers and probe sequences). Reactions consisted of an initial denaturation of 5 min at 95°C and 40 subsequent cycles of 5 sec at 95°C, 10 sec at 60°C, and 1 sec at 72°C. A normalization factor was obtained using the three housekeeping genes (GeNorm VBA version 3.5 software) in order to calculate percentage values of *H19* and *IGF2* transcripts.

Allelic H19 *Gene Expression*

Two microliters of purified cDNA was mixed with 1 unit of Taq DNA Polymerase (Invitrogen), 0.2 μM H19F1 primer, 0.2 μM H19EX4R2 primer, 0.2 mM mixed dinucleotide triphosphates (dNTPs), 1.5 mM $MgCl_2$, and 1× reaction buffer in a final volume of 50 μl. The PCR reactions were performed using an initial step at 94°C for 2 min, followed by 25 cycles of 30 sec at 94°C, 30 sec at 60°C, and 30 sec at 72°C, and a final extension of 5 min at 72°C.

To assess allelic expression, fluorescence resonance energy transfer (FRET) technique was used with the Light Cycler 1.2 instrument (Roche Applied Science). The PCR reactions were diluted 250 times. 5 μl of PCR product was mixed with 0.5 μM bH19FRET-F, 0.1 μM bH19FRET-R, 0.1 μM bH19LCRed, 0.1 μM bH19fluo, 0.15 mM $MgCl_2$, and 2 μl of LightCycler DNA Master HybProbe 10× in a final volume of 20 μl (see Table 1 for primers and probe sequences). The amplification program consisted of an initial denaturation of 10 min at 94°C followed by 40 subsequent cycles of 10 sec at 95°C, 5 sec at 58°C, and 8 sec at 72°C, superseded by a melting program consisting of 0 sec at 95°C, 30 sec at 55°C, and 0 sec at 95°C (slope = 0.1°C/sec). Data were acquired using the area under the curve in the melting program.

Search for the Bovine H19 *DMD*

Genome walking was used to identify the position of the DMD in the bovine *H19* gene. Total genomic DNA was extracted from fibroblasts of an adult *B. indicus* using a DNeasy Tissue Kit (Qiagen). DNA libraries were obtained using the Universal Genome Walker Kit (Clontech), which requires two nested PCR reactions per library. Briefly, the primers for the PCR reaction consisted of adaptor primers provided with the kit and gene-specific primers. The *H19* DMD is localized approximately 2.0 kb upstream of the *H19* promoter region in most species analyzed to date [21, 25]. Therefore, we designed our gene-specific primers in exon 1 using sequences obtained from GenBank (AF087017, AF049091) and moved in the 5′ direction. The protocol produced a series of four fragments (2.1, 2.0, 0.9, and 0.8 kb) that covered 2.2 kb upstream of the promoter region. Each PCR fragment was cloned (pGEM-T easy) and sequenced for analysis. The cloned nucleotide sequence matches sequence data that are currently available for the *B. taurus* chromosome 29 on public databanks (GenBank accession no. NW_001494548).

Bisulfite Sequencing

Approximately 400–500 ng of total genomic DNA was used for a bisulfite treatment reaction using the EpiTect Bisulfite kit (Qiagen). Primers specific for bisulfite-converted DNA were designed within the *H19*-ICR region to amplify a 660-bp fragment, spanning from −3327 to −2675 bp downstream of exon 1 (GenBank accession no. NW_001494547). Each PCR reaction was performed in triplicate using the primers U-H19-F1 and U-H19-R1. The PCR reaction was carried out in a final volume of 50 μl containing 1–2 μl of bisulfite-treated DNA, 0.2 μM each primer, 0.3 mM mixed dNTP, 1× PCR buffer, 1.5 mM $MgCl_2$, and 2.5 units of Platinum Taq DNA polymerase (Invitrogen). The reactions were performed using an initial 2-min step at 94°C followed by 50 cycles of 30 sec at 94°C, 30 sec at 53°C, 1 min at 72°C, and a final 3-min step at 72°C. The PCR products were resolved in 1.2% agarose gels, followed by purification using the QIAquick Gel Extraction kit (Qiagen). Purified fragments were subcloned in pGEM-T Easy Vector (Promega), and cell transformation protocol was performed using competent *Escherichia coli* DH5α cells (Invitrogen). To ensure that reliable data were collected, a total of 16–40 clones (at least 10 clones per allele) for each sample were picked and sequenced.

Statistical Analyses

Data were analyzed using ANOVA with JMP software (SAS Institute Inc., Cary, NC), and log transformation was performed when necessary to remove heterogeneity of variance. The arcsine of percentage value of paternal alleles over the total expression analyzed by FRET PCR was used for allelic expression, and the arcsine of the ratio of the number of methylated sites over the total number of sites was used for methylation studies. For the data from preimplantation embryos (Day 17), one-way ANOVA was performed, and individual means were compared using the Tukey-Kramer HSD test. For the data from postimplantation embryos, a two-way ANCOVA was performed with tissue type (placenta, fetus) and group (AI, IVF, and NT) as main effects and a tissue × group interaction. Because samples were collected on different days of

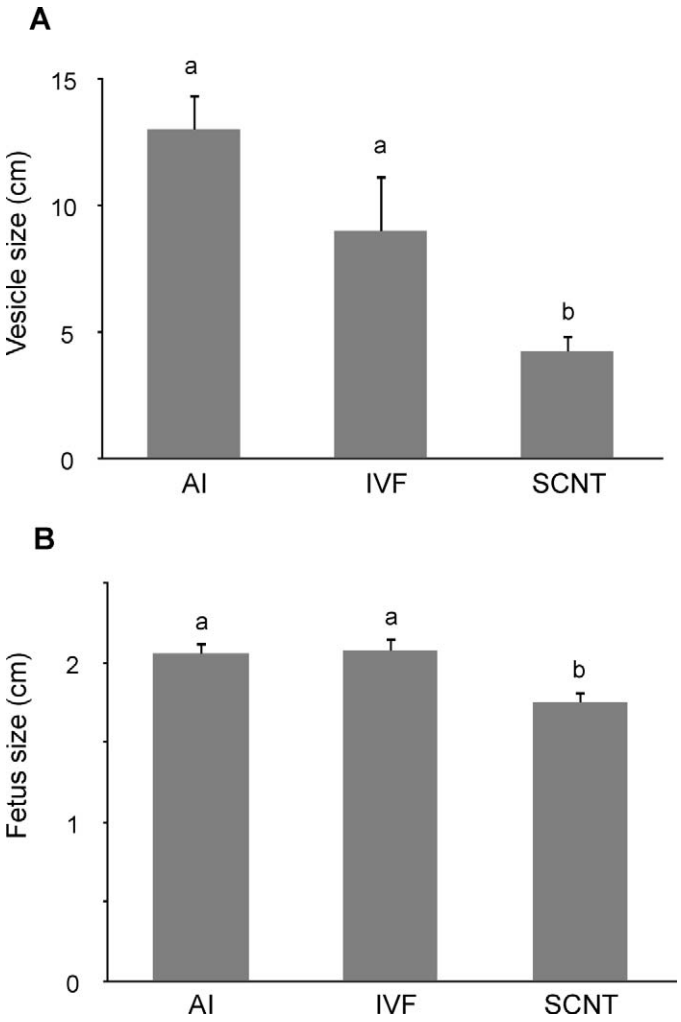

FIG. 1. Size of Day 17 and Day 35–45 embryos produced by AI, IVF, and SCNT. Average size of embryos at Day 17 produced by SCNT (n = 8), IVF (n = 6), and AI (n = 3), and fetus size between Days 35 and 45 in SCNT (n = 9), IVF (n = 8), and AI (n = 12). Embryos and fetuses were collected and visualized under a stereomicroscope and measured in centimeters. For Day 17 embryos, only intact embryos were measured. Data are mean ± SEM; different letters represent significant differences ($P < 0.05$).

pregnancy (30–50), day of pregnancy was used as a covariant. Individual means were compared using the Tukey-Kramer HSD test; the level of significance was set at $P < 0.05$.

RESULTS

Survival and Embryo Growth Are Reduced by SCNT

After flushing Day 17 pregnant recipient heifers and removing uterine mucous and cellular debris, AI, IVF and SCNT embryos were selected and measured; three in vivo-produced (AI), six IVF, and eight SCNT embryos were used in this experiment. Recovery rates for IVF and SCNT were 50% and 53%, respectively, and only intact embryos were counted during the recovery. No significant differences in sizes were found between in vivo-produced and IVF embryos at Day 17 (13.0 ± 2.22 cm and 8.98 ± 1.46 cm, respectively; Fig. 1A). However, SCNT embryos were significantly smaller than the in vivo-produced and in vitro-produced Day 17 embryos (4.23 ± 1.26 cm). Similar results were also reported in previous studies in bovine species [6]. Postimplantation recovery rates were 40.5% (17 pregnancies in 42 transfers) and 18.6% (26 pregnancies in 140 transfers) for IVF and SCNT, respectively, indicating a significant decrease in implantation rate in SCNT pregnancies. Also, fetus size was significantly reduced in SCNT compared with AI and IVF fetuses (Fig. 1B).

IGF2 *Is Aberrantly Transcribed in SCNT Day 17 Embryos but Not at Days 30–50*

To verify whether gene expression accounted for the different sizes and pregnancy rates of SCNT embryos, we proceeded with relative transcript abundance analysis of *H19* and *IGF2* using primers designed for quantitative real-time PCR (Table 1). Transcript abundances were normalized by the average values of three housekeeping genes (i.e. *GAPDH*, *ACTB*, and *H2AFZ*). Results for Day 17 embryos are shown in Figure 2. There was a great variability in abundance of *H19* transcript in the AI compared with IVF and SCNT groups (Fig. 2A), and similar variability was also observed for *IGF2* transcript levels (Fig. 2B). However, *IGF2* transcript abundance was significantly higher in the AI group compared with IVF and SCNT. A tendency toward a correlation between *IGF2* transcript abundance and embryo size was observed ($P = 0.1$). At the postimplantation stage (between Days 30 and 50), *H19* and *IGF2* transcript abundance was analyzed in extraembryonic (placenta) and embryonic (muscle) tissues; AI and SCNT had similar levels of *H19* transcript in both muscle and placenta tissues (Fig. 3A), whereas in the IVF group, *H19* transcript abundance was lower in the placenta than in the muscle. In the placenta, *IGF2* transcript levels in the SCNT group were higher than in the IVF group and tended to be higher than the AI group ($P < 0.07$; Fig. 3B). Contrary to what was observed in preimplantation embryos, *IGF2* expression in muscle of the SCNT group was not different from AI and tended to be lower than the IVF group ($P < 0.16$). Although SCNT embryo sizes were also reduced during the postimplantation stage compared with AI and IVF, this difference cannot be directly attributed to *IGF2* transcript abundance at this stage.

H19 *Gene Is Biallelically Expressed in SCNT Day 17 Embryos but Not at Days 30–50*

Aberrant imprinting patterns observed in clones generally results in deregulation of parental gene expression. Results on the transcript abundance data encouraged us to assess the allelic expression of the paternally imprinted *H19* gene in bovine F1 animals. Sequencing of genomic DNA and cDNA samples obtained from animals of the *B. taurus* and *B. indicus* subspecies enabled the identification of an SNP for use in allele-specific expression profiles from F1 samples. Genome walking sequences stretching from exons 1 to 4 then made it possible to detect a guanine to adenine (G/A; *B. taurus*/*B. indicus*) SNP at position +30 downstream of exon 3 of the *H19* gene (Fig. 4A). The SNP was confirmed by sequencing of PCR fragments obtained with *H19* primers using genomic DNA samples obtained from *B. taurus* (maternal control) and *B. indicus* (paternal control). Real-time FRET analysis results validated the imprinted status of *H19* in preimplantation embryos produced by AI (in vivo), where only one of three embryos showed some minor paternal transcript expression (Fig. 5). These results are in accordance with previously reported data in sheep [26] where monoallelic expression was observed after the blastocyst stage. Most IVF embryos maintained imprinted status of *H19*; only one of six showed biallelic expression (Fig. 5). These results suggest that the in vitro culture system did not affect the imprinted status of the *H19* gene in the majority of embryos analyzed. However, in

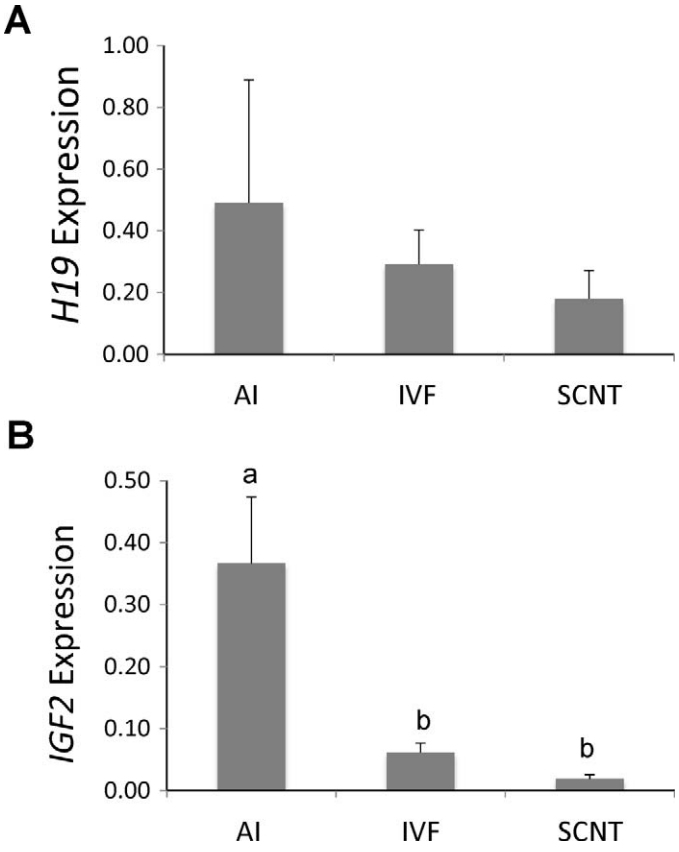

FIG. 2. Expression of *H19* and *IGF2* genes in Day 17 embryos produced by SCNT, IVF, and AI. An average of quantitative real-time PCR analysis of *H19* and *IGF2* gene transcripts. Values were normalized by *GAPDH*, *ACTB*, and *H2AFZ* housekeeping gene expression using GeNorm VBA version 3.5 software. Data are mean ± SEM; different letters represent significant difference ($P < 0.05$).

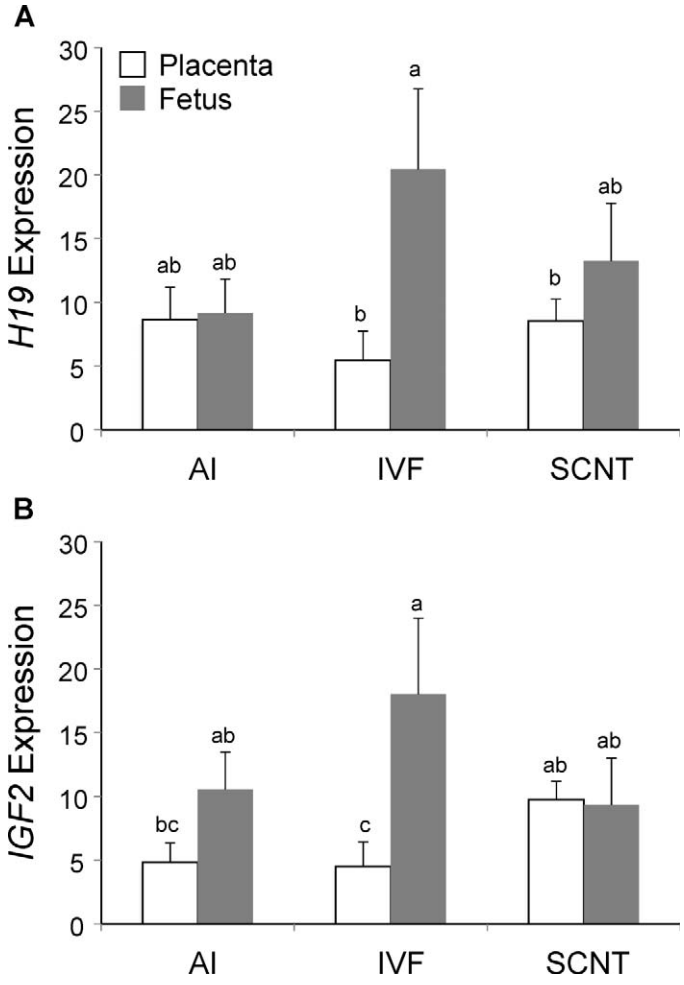

FIG. 3. Expression of *H19* and *IGF2* genes in Day 35–45 fetuses and placenta produced by SCNT, IVF, and AI. An average of quantitative real-time PCR analysis of *H19* and *IGF2* gene transcripts is shown. Values were normalized by *GAPDH*, *ACTB*, and *H2AFZ* housekeeping gene expression using GeNorm VBA version 3.5 software. Data are mean ± SEM; different letters represent significant difference ($P < 0.05$).

SCNT embryos, a severe loss of imprinting could be observed: approximately 90% of SCNT Day 17 embryos showed biallelic expression of the *H19* gene, the degree of biallelism varying from low (10%) to complete biallelic expression (50% paternal *H19* expression). Furthermore, a negative correlation ($P < 0.02$) between vesicle size and *IGF2* gene transcript abundance was observed in Day 17 embryos.

Although embryo loss is mainly observed in the preimplantation period, we also assessed the *H19* gene imprinting status in Day 30–50 postimplantation embryos. Surprisingly, monoallelic maternal expression was present in all tissues (placenta and muscle) of all treatment groups (AI, IVF, and SCNT), indicating normal *H19* imprinting of embryos that were able to survive beyond implantation.

Loss of Methylation Is Observed in Day 17 Embryos but Not in Day 30–50 SCNT

Previous studies in mice and humans have indicated that *H19* imprinting is controlled by epigenetic alteration of a DMD located upstream of its promoter [21, 27, 28]. A bovine genomic library was created to amplify and obtain the 5′ *H19* sequences to determine whether or not the paternal *H19* gene expression observed in our samples was due to abnormal methylation patterns at the bovine *H19* DMD. In this way, confirmation of the existence of an *H19* in the bovine DMD was possible. Using primers designed in the putative *H19* DMD, a bisulfite analysis was performed in DNA samples obtained from bovine spermatozoa and oocytes. Genomic DNA was bisulfite treated, and sequence analysis showed a hypermethylated and hypomethylated DNA status in sperm and oocytes samples, respectively (data not shown), indicating that the putative bovine *H19* DMD was indeed differentially methylated in the male and female gametes.

In other species *H19-IGF2* imprinting is controlled by differential methylation at the CTCF-binding sites [18, 21, 25, 29]. To determine if this was the case in the bovine, a search for CTCF-binding sites within the DMD was conducted. Using mice, human, and rat CTCF and CTCF consensus sequence from other studies [30], we found a CTCF-binding site sequence within a region comprising 14 CpG islands (Fig. 4A). Homology across different species confirmed the CTCF-binding motif (Fig. 4B) and that the ''putative'' DMD was actually a DMD in the bovine. An SNP (A/G *B. taurus*/*B. indicus*) found upstream of the CTCF-binding site enabled us to distinguish paternal from maternal alleles (Fig. 4A) and confirm that the CTCF-binding site, along with the *H19* DMD, is hypomethylated and hypermethylated in maternal and paternal alleles, respectively, in adult somatic tissues.

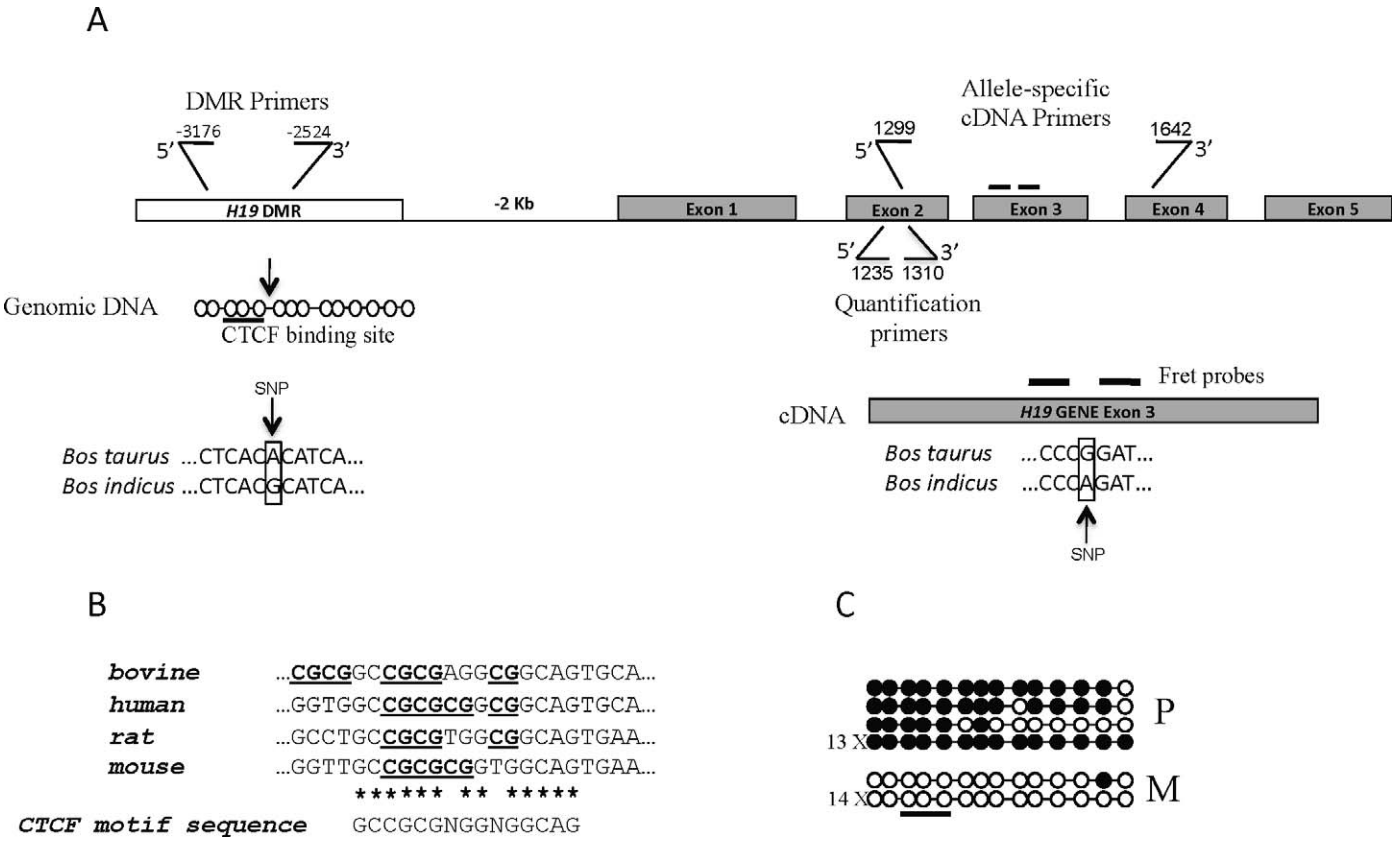

FIG. 4. Identification of SNPs in the *H19* gene and the CTCF-binding site. **A**) Schematic representation of the *H19* gene and the SNP identified between maternal allele (G; *Bos taurus*) and paternal allele (A; *Bos indicus*) present in exon 3, and the SNP present at the maternal allele (A) and paternal allele (G) *H19* DMD CTCF-binding site. Positions of primers, as well as their locations, are described in intronic and exonic regions. **B**) Representative figure showing homology of the sequence for the CTCF-binding site in bovine, human, rat, and mouse, creating a CTCF consensus sequence. **C**) Representative samples of bisulfite analysis of a fetal donor cell line used in SCNT. Each line represents an individual clone that was sequenced. Black filled circles represent methylated CpG islands, and open circles represent unmethylated Cpg islands. M, maternal alleles; P, paternal alleles.

Results from bisulfite analysis from Day 17 samples demonstrated that, in accordance with the monoallelic maternal expression observed in AI and IVF embryos, a demethylated maternal allele and methylated paternal allele were present (Fig. 6; Supplemental Data S1, all Supplemental Data are available online at www.biolreprod.org). On the other hand, embryos in the SCNT group showed severe loss of methylation on the paternal allele, whereas all three AI embryos showed a uniform methylated DMD covering the CTCF-binding site and most of the 14 CpG islands, with approximately 100% of the paternal allele being methylated. In the IVF group, however, only one embryo showed a low paternal methylation level

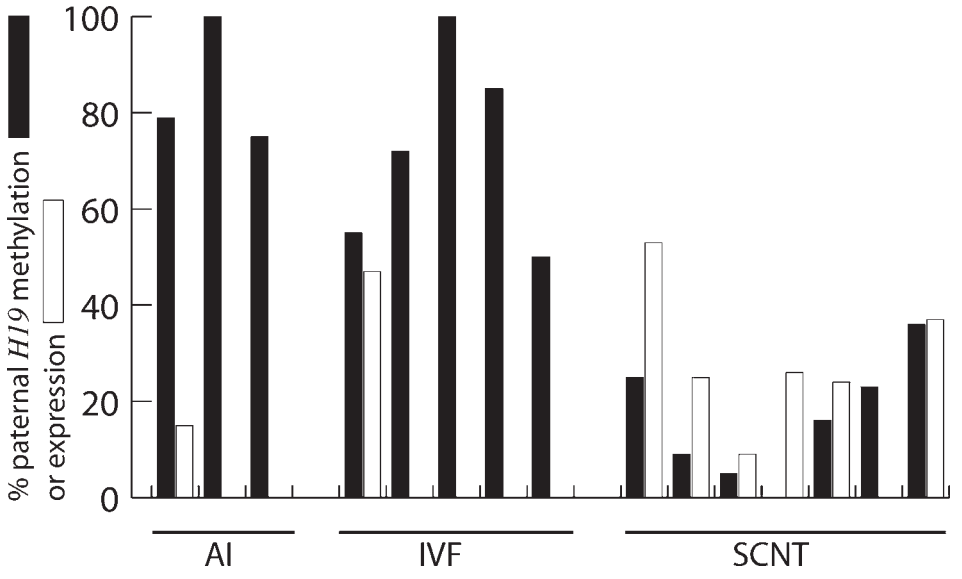

FIG. 5. Paternal DMD methylation level and expression of *H19* gene in Day 17 embryos produced by AI, IVF, and SCNT. Real-time FRET PCR analysis of paternal (*B. indicus* "A" allele) *H19* gene expression in individual Day 17 embryos (white bars), and comparative methylation levels at the paternal (*B. indicus* "G" allele) *H19* DMD (gray bars). Bar values were based on the total number of methylated CpG sites over the total number of CpG sites of the paternal alleles.

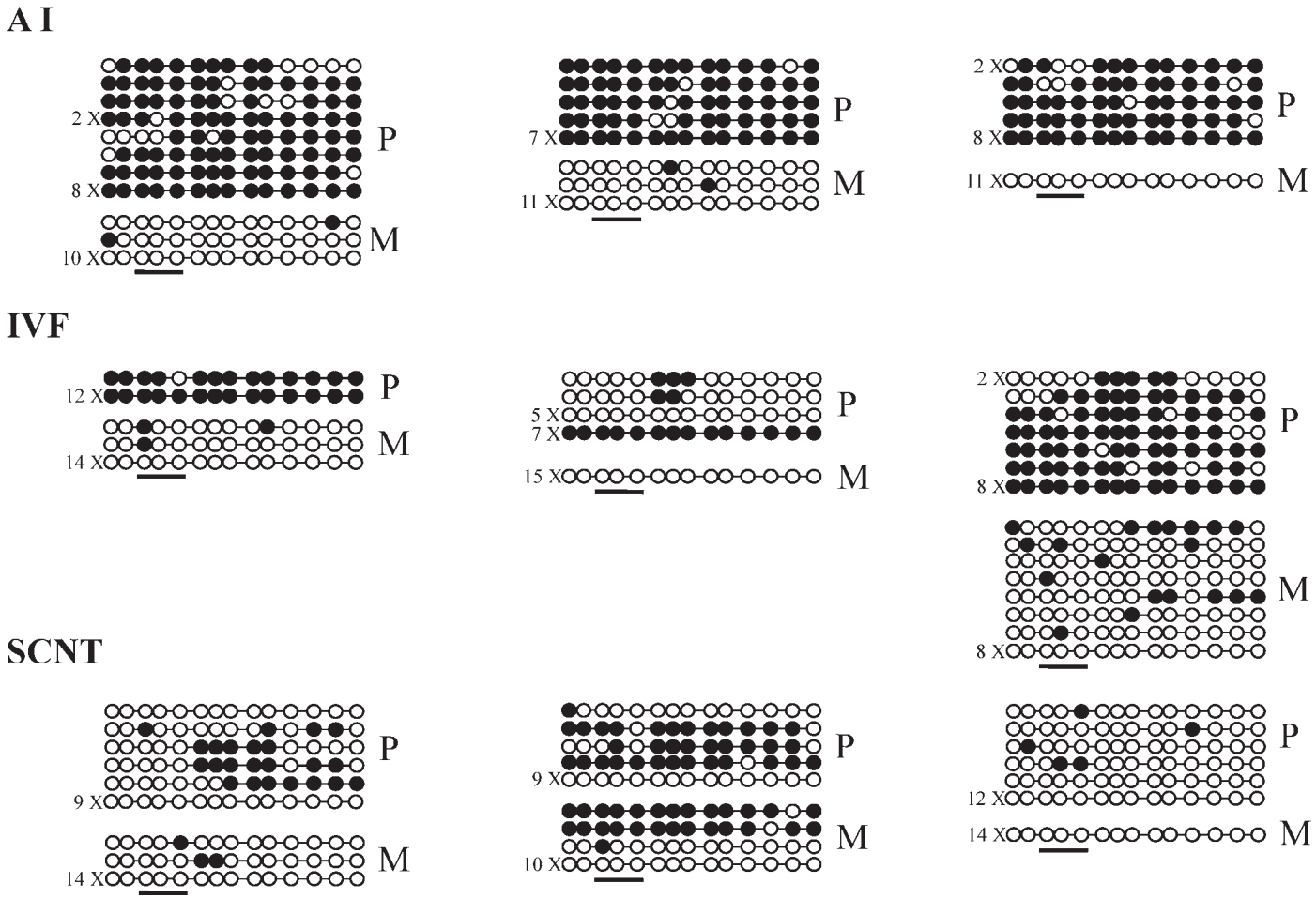

FIG. 6. *H19* DMD methylation levels in Day 17 embryos produced by SCNT, IVF, and AI. Representative samples of bisulfite analysis of Day 17 embryos produced by SCNT, IVF, and AI. Each line represents an individual clone that was sequenced. Black filled circles represent methylated CpG islands, and open circles represent unmethylated Cpg islands. M, maternal alleles; P, paternal alleles.

(approximately 55%); interestingly, the same embryo was also the only one showing paternal expression of the *H19* gene. In general, Day 17 SCNT embryos showed paternal methylation levels not greater than 40% and simultaneous paternal expression of the *H19* gene (Fig. 5). Analysis of the paternal CTCF methylation levels showed that embryos in the SCNT group are hypomethylated compared with AI and IVF embryos (Fig. 7). When assessed for gene expression and DNA methylation levels, donor fibroblasts used for SCNT showed normal monoallelic expression of *H19* and hypermethylated paternal allele (Fig. 4C), indicating that *H19* imprinting status was disrupted after oocyte reconstruction by SCNT.

The results of DNA methylation and *H19* gene expression in Day 17 embryos encouraged us to verify a possible correlation between the paternal CTCF methylation level and paternal expression of *H19* gene. Figure 8 illustrates a significant negative correlation between CTCF methylation and gene expression, suggesting that, similarly to the mouse, bovine *H19* gene expression is controlled by paternal CTCF methylation at the preimplantation stage.

In postimplantation embryos, paternal CTCF methylation was assessed in samples of muscle and placenta. In agreement with previous studies in mice [31], placenta methylation level was generally lower than that in muscle. As expected with monoallelic expression of *H19* gene, no differences in methylation levels at the paternal CTCF-binding site were found between AI, IVF, and SCNT in Day 30–50 embryos (Fig. 9; Supplemental Data S2). In muscle samples there was no difference among the three groups, which is in accordance with monoallelic *H19* transcript expression present at post-implantation.

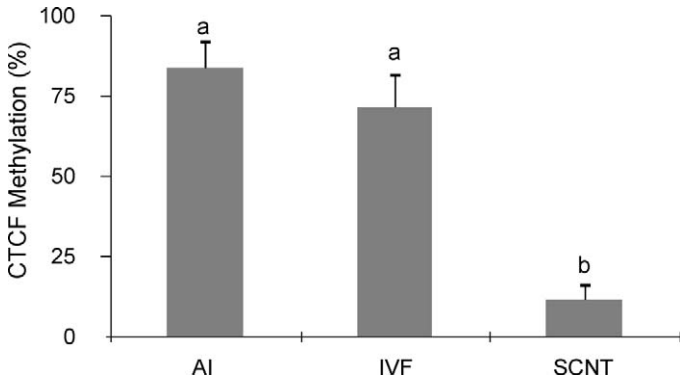

FIG. 7. Paternal methylation of *H19* DMD of Day 17 embryos produced by SCNT, IVF, and AI. Bisulfite methylation data (mean ± SEM) obtained by computing the number of methylated CpG sites over the total number of CpG sites present in the paternal alleles. Different letters represent significant differences within groups ($P < 0.05$).

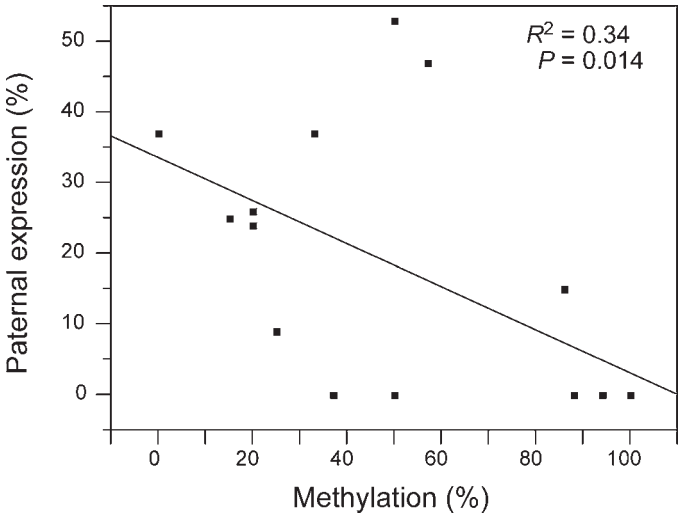

FIG. 8. Paternal methylation of *H19* DMD of Day 35–45 fetuses and placenta produced by SCNT, IVF, and AI. Average percentage methylation levels of Day 35–45 embryos and placenta tissues derived from AI, IVF, and SCNT. Bisulfite methylation data were obtained by computing the number of methylated CpG sites over the total number of CpG sites present in the paternal alleles.

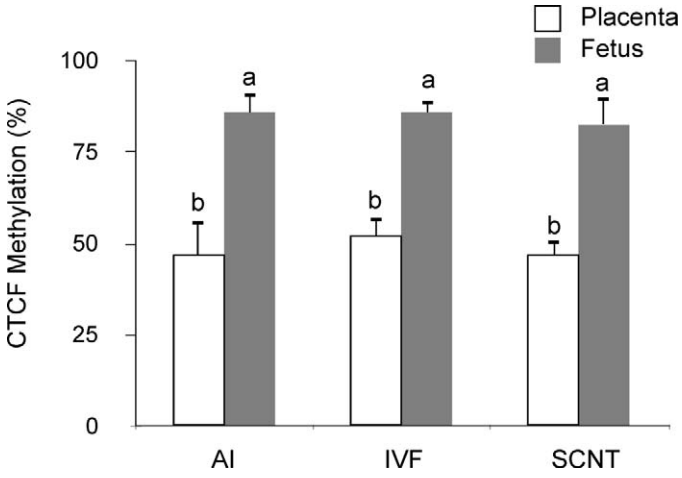

FIG. 9. Bivariate analysis of paternal *H19* DMD methylation and paternal expression of *H19* transcripts in Day 17 embryos. Data are mean ± SEM; different letters represent significant differences within groups ($P < 0.05$).

DISCUSSION

For the bovine species, a considerable number of studies show that epigenetic reprogramming is severely compromised in SCNT embryos: most of the abnormalities are based on the demethylated state of genomic DNA present at the early stages of development [12], the effect of methylation on imprinting in midgestation [13], and later gestational problems related to LOS [7, 22, 23]. However, much of what is known in the bovine is still based on speculation of putative DMDs and qualitative methods to determine parental expression. Although extensive studies have been performed to elucidate the *H19* DMD in bovine [22, 23], it is still defined as ''putative'' because there is no confirmation of the parental DNA methylation. Here, we have demonstrated for the first time in bovine embryos that the *H19* DMD CTCF-binding site is hypomethylated and hypermethylated in the maternal and paternal alleles, respectively. We also show that in vitro culture systems used to derive IVF embryos can occasionally cause the demethylation of the paternal allele in IVF. Furthermore, severe demethylation of the paternal allele could be observed in SCNT embryos (Fig. 6). Combining results from parental methylation with parental expression were important to establish a correlation between methylation and parental expression of the *H19* gene, which in many cases can be biased by the overall estimated percentage of methylated and nonmethylated sequences. As previously observed in mice [21], the CTCF-binding site sequence and paternal methylation are also conserved in bovine embryos. As demonstrated in Figure 5, the majority of SCNT embryos showed a significant loss of methylation at the CTCF site and, according to the results of parental-specific expression, SCNT embryos also show biallelical status during preimplantation. In general, there was a negative correlation between parental expression and methylation levels of the *H19* CTCF site (Fig. 8). However, for some Day 17 embryos (Fig. 5), although demethylation levels of the paternal DMD were practically complete, the paternal expression was rarely greater than 30%. There are two possible reasons for this: first, other mechanisms are known to share regulation of imprinting along with DNA methylation. Histone modification by acetyl, or even methyl, groups, for example, plays an important role in gene expression and gene regulation at ICRs, either by protecting sites from methylation [32] or by working together with DNA methylation [33]. Second, there are other CTCF-binding sites present at the bovine *H19* DMD [22] that could be interacting simultaneously on imprinting control. Nevertheless, it is worth emphasizing that none of the SCNT embryos at postimplantation stages presented abnormalities such as loss of methylation and biallelic expression of *H19* gene. It is unlikely that the preimplantation embryo would have acquired methylation on the *H19* DMD and repaired the abnormal expression pattern in time for implantation; thus, we believe that loss of methylation during early stages may have contributed to implantation failure and eventual embryo demise. Somatic cell nuclear transfer fetuses with apparently normal levels of DNA methylation present on the ICR and promoter regions of several imprinted genes have been reported in bovine [34], which supports the idea that a minimum of epigenetic remodeling is required for implantation of SCNT embryos. We do not exclude the possibility of a mosaic methylation pattern in the exact region we analyzed, which is commonly seen in bovine studies and also in mice [25]. However, these patterns were not seen in control AI and IVF embryos.

Interestingly, the abnormal biallelic expression of the *H19* gene did not account for any increase in transcript abundance in SCNT embryos. In fact, our results showed that *IGF2* transcripts were downregulated in IVF and SCNT Day 17 embryos (Fig. 2). Similar results were also reported in mice, where a large variability and generally low transcript levels were found in cloned embryos before implantation [35]. Moreover, other studies have shown that the in vitro culture of mouse embryos resulted in the downregulation of *IGF2* gene expression and smaller fetuses at Day 9.5 of gestation [36]. *IGF2* transcripts were downregulated during early pregnancy (Days 30–50) in muscle tissues of SCNT fetuses ($P < 0.16$), and those fetuses were smaller compared with AI fetuses, even though imprinting status of *H19* was normal. As previously reported [16], *IGF2* transcript abundance was upregulated in SCNT placental tissues. Generally, SCNT placental tissues have fewer placentomes, which are augmented in size and show other symptoms of arrested development [7]. It is likely

that the upregulation of *IGF2* transcripts in placental tissues is a compensatory effect to try to overcome growth retardation. It is probable that the paternal demethylation of the CTCF region of the *H19* gene has led to biallelic activation of the paternally expressed *IGF2* gene, causing the placental tissue to compete with the fetus for maternal nutrients. In fact, this theory would explain the reduced SCNT fetal sizes. Another explanation for the low development could be the direct effect of *H19* transcript on *IGF2*. Recently, it has been suggested that *H19* RNA is involved in the downregulation of *IGF2* mRNA expression by a *trans*-acting mechanism in mice [37]. In such a case, the biallelic expression of *H19* in SCNT Day 17 embryos would explain the reduced fetal size. However, in the present study, biallelic expression did not upregulate *H19* transcripts, suggesting that different imprinting mechanisms are present in ruminants and rodents.

In other cases, upregulation of the *IGF2* gene, which is often observed in fetuses late in pregnancy [38], may have developed as a similar compensatory mechanism to overcome retarded growth due to placenta malfunction. We do not exclude the possibility of more genes being aberrantly expressed in preimplantation SCNT embryos. In fact, the *H19* gene has been singled out as a fine-tuning mechanism that regulates the expression not only of *IGF2*, but also other genes present in the newly described imprinted gene network [37]. This supports the hypothesis that *H19* imprinting might be crucial for the establishment of viable pregnancies of SCNT bovine embryos.

In summary, we have demonstrated that paternal demethylation of the *H19* DMD caused by aberrant reprogramming of the donor cell led to biallelic expression of *H19* transcript, reduced embryo size, and low implantation rate in cattle. Although the involvement of *H19* gene in embryo growth and gestation, either by direct action of the gene itself or by controlling *IGF2* expression, remains yet to be solved, this creates new opportunities for future studies of imprinting mechanisms and reproductive technologies in the bovine.

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