Theriogenology 186 (2022) 95–107

Contents lists available at ScienceDirect

Theriogenology

journal homepage: www.theriojournal.com

Multi-locus DNA methylation analysis of imprinted genes in cattle from somatic cell nuclear transfer

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ARTICLE INFO

Article history:
Received 24 November 2021
Received in revised form
6 April 2022
Accepted 8 April 2022
Available online 12 April 2022

Keywords:
Multi-locus methylation defects
Multi-locus imprinting disturbances
Beckwith-wiedemann
Abnormal offspring syndrome
Large offspring syndrome

ABSTRACT

Multi-locus methylation defects (MLMDs) in imprinted loci have been reported in Beckwith-Wiedemann Syndrome (BWS). Large offspring syndrome (LOS), a phenotypic subgroup of abnormal offspring syndrome (AOS), is considered a molecular and phenotypic model for BWS. Both LOS and BWS have presented epigenetic defects in some common imprinted loci. In this study, methylation-specific restriction digestion assay - quantitative PCR was used to analyze the DNA methylation pattern in differentially methylated regions (DMRs) of the *H19* (H19-DMR), *KCNQ1OT1* (KvDMR1) and *PEG1/MEST* (PEG1-DMR) genes in bovine clone tissues from calves that did not survive after birth. Individual and tissue-specific changes in DNA methylation levels in the bovine KvDMR1, H19-DMR, and PEG1-DMR were observed. In contrast to what has been reported in the literature on BWS and AOS/LOS, the KvDMR1 showed gain (GOM) and loss (LOM) of DNA methylation. LOM and GOM events were found in the DMRs studied in animals produced by the same nucleus donor cell line. This is the first report of epimutations in the PEG1-DMR and GOM at the KvDMR1 found in bovine clones. The findings showed that epigenetic modification in imprinted loci in cloned cattle occurred in a multi-locus pattern similar to that seen in human imprinting disorders. Other multi-locus analyzes must be done to elucidate the MLMD pattern in AOS in bovine clones.

1. Introduction

Genomic imprinting is an epigenetic mechanism that results in monoallelic parental tissue-specific expression of a set of genes in placental and marsupial mammals [1,2]. This monoallelic parental-specific expression is regulated by imprinting control regions (ICRs), whose function is regulated by differential allele-specific patterns of DNA methylation and histone modifications [1,3–5]. Functionally, imprinted genes are involved in prenatal and postnatal processes such as fetal growth and placental development, glucose and fat metabolism, and the establishment of mother-offspring interactions and adult behaviors [6].

Due to their regulatory roles, loss of ICR parental specific DNA methylation and histone modification marks (loss of imprinting - LOI) prevents monoallelic transcription, affecting important biological processes regulated by imprinted genes [7,8]. The gain or loss of DNA methylation (GOM or LOM, respectively) has been widely reported in individuals born by assisted reproductive technology (ART) in human and animal models using DNA methylation as a marker to analyze the occurrence of LOI events [9–16].

In ruminants, LOI events are commonly associated with somatic cell nuclear transfer (SCNT) derived animals [8,9,12,13,15], in turn associated with developmentally abnormal phenotypes involved in the etiology of abnormal offspring syndrome (AOS) [17]. Because of the occurrence of overgrowth phenotypes observed initially in the reports of this syndrome, it was originally described as large

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https://doi.org/10.1016/j.theriogenology.2022.04.006
0093-691X/

offspring syndrome (LOS) [17–20]. However, because not all the animals born through IVF (*in vitro* fertilization) or SCNT have increased body weight phenotypes, especially when comparing this phenomenon with human counterparts, the expression abnormal offspring syndrome is more accurate to describe of this phenomenon [17].

LOS has been linked to neonatal death; aberrant growth of several organs such as the heart, brain, skeletal muscle and liver; extended gestation; developmental failure; and higher occurrence of hydrallantois, in addition to the increased weight phenotype [21–23]. In this context, considering cattle as an example, it is important to note that LOS affects this subgroup of animals affected by AOS with specific overgrowth phenotypes. AOS can be categorized into four types (I-IV) based on aberrant phenotypes, which depend on the varied aspects of the syndrome. Type I through III animals have defective fetal development and die before delivery, although type III animals sometimes survive with severe defects that cause perinatal death. Only type IV causes moderate abnormal development, in which the calf is born alive, although with abnormal phenotypes [17,24].

In humans, LOI events are linked to abnormal phenotypic development, including hypergrowth, macroglossia, umbilical hernia, visceromegaly, and neonatal hypoglycemia [25]. Beckwith-Wiedemann Syndrome (BWS) is the most common overgrowth syndrome in humans, with a nine fold increase in cases among children born from *in vitro* fertilization (IVF) [26,27]. Interestingly, BWS and LOS involve LOI events in common homolog imprinted loci. Therefore, LOS-affected animals have been considered as models for BWS [25].

IGF2, *H19*, and *KCNQ1OT1* are some of the most commonly affected genes in BWS and LOS [8,12,28]. Several studies have reported that the *H19/IGF2* and *KCNQ1OT1/CDKN1C* clusters exhibit specific alterations in BWS. In around 10% of BWS patients, the *H19/IGF2* genes controlled by ICR1 show a GOM pattern. In contrast, the LOM in the *KCNQ1OT1* gene, which is controlled by ICR2, is responsible for 50–60% of BWS cases [29–32]. In addition to epigenetic failures, BWS can also occur by uniparental disomy (Upd(11p15)pat), which affects about 20% of BWS patients [31–33]. However, the occurrence of epimutation reported in patients affected by BWS includes other imprinted genes (e.g., *IGF2R*, *SNRPN*, *GRB10*, *PEG10* and *PEG1/MEST*), with epigenetic disruption detected in a multi-locus pattern [8,15,34–37].

Imprinting disorders (IDs) with alterations at several loci have recently been documented in the literature as a result of multi-locus methylation defect (MLMD) events [33,34,38–41]. These epigenetic disorders are called multi-locus imprinting disturbances (MLIDs) [42,43]. MLIDs may arise from different genomic and epigenomic alterations, such as uniparental disomy, mutations, epimutations, GOM and LOM, and point mutations in imprinted genes [33,44]. As a result, the imprinting disturbances lead to variations in growth, behavior, development, and metabolism [45]. According to previous studies, almost half of BWS patients with hypomethylation in ICR2 have multi-locus imprinting defects [41,46,47].

In cattle, most MLMD studies focus on animals from IVF. In contrast, the SCNT protocol has a higher failure rate compared to other reproductive biotechnologies [48], so MLMD studies in bovine clones are necessary to understand the failures that occurred during embryonic development in these animals. In addition, bovine clones show epimutations similar to those seen in children from ART.

These multi-locus epimutation patterns have been associated with clinical differences among patients affected by BWS [26]. Patients with ICR2 hypomethylation have a 5% greater chance of developing some type of tumor, such as hepatoblastoma. Similarly, hemihyperplasia and omphalocele phenotypes are associated with this molecular etiology of LOM [26,49,50]. On the other hand, patients with ICR1 hypermethylation are 25% more likely to develop additional malignancies such Wilms' tumor and hepatoblastoma [26,27]. Likewise, BWS patients coming from uniparental disomy present the same features, as well as hemihyperplasia in various body parts [26,27,50]. In LOS affected animals, many human homolog imprinted genes included in MLID have yet to be studied [8,25,51]. As a result, the clinical significance of the relationship with multi-locus occurrences has yet to be determined.

Because BWS and LOS have similar clinical and molecular characteristics, bovine clones could be used as an animal model to research molecular pathways linked with BWS etiology, including embryos and fetal tissues. Although the present study presents preliminary data of multi-locus alterations in bovine clones in only two tissues (liver and heart), it also presents information from phenotypic data associated with the altered DNA methylation pattern observed in imprinted genes. In addition, investigation of the epigenetic changes can contribute to the identification of factors that influence the molecular stability of imprinted genes. Based on this, we examined the DNA methylation pattern in the H19-DMR, KvDMR1 and PEG1-DMR in somatic tissues of SCNT-generated bovine clones that did not survive after delivery to see if the animals died due to MLMD events.

2. Material and methods

2.1. Samples

The tissue samples used in the present study were obtained from the bovine SCNT tissue bank of the company GENEAL - Genetics and Animal Biotechnology (Uberaba, MG, Brazil). GENEAL is authorized by the Ministry of Agriculture, Animal Husbandry and Supply (MAPA) and uses the technology patented by VIAGEN LC by Brazil's National Industrial Property Institute (INPI). In addition, GENEAL has a team of specialized veterinarians that accompany all recipients during pregnancy, delivery and calf development up to three months of age.

According to information obtained from GENEAL, the SCNT protocol was performed with skin fibroblast cells and oocytes. The oocytes were enucleated in metaphase II, in which the *in vitro* maturation process showed 70% rate. The originated embryos had a 45% blastocyst rate. The synchronization protocol was not revealed by GENEAL. The cloned animals in the present study were Nellore females, but there was no identification of animal age used as cell nucleus donor. All tissues from bovine clones were collected during autopsies, kept in microtubes with RNAlater®, and stored at −80 °C in a freezer until DNA extraction.

The DNA methylation study in three differentially imprinted methylated regions (DMR) was performed using liver and heart tissues (KvDMR1, H19-DMR, and PEG1-DMR). The tissue samples derived from 12 female bovine clones were divided into groups based on the donor nucleus cell source: A (A1, A2, A3, and A4), B (B1, B2, B3, and B4), C (C1 and C2), D1, and E1.

All control tissue samples (liver and heart) were obtained from 12 naturally reproduced animals in a slaughterhouse in the city of Campos dos Goytacazes, Rio de Janeiro state, Brazil. The control animal tissues were obtained shortly after death. After collection, all tissues were stored in RNAlater® (Ambion, Austin, TX, USA), and frozen at −70 °C until DNA extraction.

In addition to tissues earmarked for molecular analysis, the phenotypic data of the cloned animals were reported, including data of birth weight, gestation time of the recipient, clone survival time, and biochemical and organ abnormalities (Table 1). The clones were classified as per the AOS I-IV parameters [17]. The classification of LOS was performed based on the difference of birth

Table 1
Phenotypic parameters of bovine clones from SCNT, and control animals (conceived naturally). Clinical data collected from clones and indicative classification of the development of AOS in the clones according to the parameters postulated by Farin et al. (2006) [17]. ***Data not collected. AOS: abnormal offspring syndrome.

Clones	Birth weight (Kg)	Pregnancy (days)/ Survival time after birth	Phenotypic features	AOS I-IV parameters (Farin et al., 2006) [17]
A1	28	266/miscarriage	***	Type II
A2	48	288/17 h	Clot in thoracic cavity	Type III
A3	53	289/7 days	Enlarged heart Areas of ischemia in the heart Pulmonary edema Thick navel	Type III
A4	23	283/2 days	Anemia; Thin navel	Type III
B1	49	289/9 days	Alopecia in several parts Consolidated lung in the cranial parts Bleeding of the umbilical arteries Thick navel	Type III
B2	35	294/40 h	Severe hemorrhagic enteritis Liver with yellowish appearance	Type III
B3	30	301/51 days	Sudden calf death Splenomegaly Liver congestion	Type III
B4	50	308/12 h	Large cattle with folded neck	Type III
C1	30	286/36 h	***	Type III
C2	50	280/2 days	Liver yellowish color and increased size Hematomas in the pericardium sac, spleen and intestine Lung with dark red color (congestion) Foam inside the bronchi, bronchioles and trachea (pulmonary edema) and also yellowish viscous fluid	Type III
D1	62	291/fetal death	***	Type III
E1	45	295/2 h	Thick navel Hemorrhage in one of the umbilical arteries	Type III
Naturally conceived animals (controls)				
N1–N12	***			

weight of each clone relative to normal values for the *Bos indicus* species. The normal birth weight of a Nellore calf was assumed to be in the range 30–35 kg, with abnormal birth weights being either below 30 kg, or above 35 kg [9,52]. Tissue samples derived from the same nucleus cell donor were included in the same sample group (A-B-C). On the other hand, the phenotypic data of the control animals were not collected, which did not allow phenotypic analysis.

2.2. Somatic cell nuclear transfer protocol

For the SCNT protocol, oocytes from cyclized cows were used, which were collected through ultrasound guided follicular aspiration. After collection, the oocytes were selected and matured in a controlled incubator at 38.8 °C and 5% CO_2 and 20% O_2 saturation. The oocytes were denuded, and chosen by the presence of the first polar corpuscle (maturation) and labeled with Hoechst - Bisbenzimide 33342 (Sigma, St. Louis MO, USA).

The oocyte nuclei were removed by micromanipulation and reconstructed with the cell nucleus of bovine skin fibroblast from the GENEAL cell line bank. The cells were isolated using DMEM (Gibco BRL, Burlington, ON, Canada) plus 10% fetal bovine serum (Gibco BRL) and amikacin (Teuto, Anápolis, GO, Brazil), placed in cell culture bottles and frozen in liquid nitrogen when reaching 80% confluence. For the SCNT protocol, the cells were thawed and cultured in the same culture medium until reaching confluence again. The cycle of the cells used for the SCNT protocol was not identified by GENEAL.

Thus, enucleated oocytes and core donor cells were fused by electrofusion with BTX® equipment followed by activation with ionomycin (Sigma, St. Louis MO, USA) and 6-DMAP (Sigma, St. Louis MO, USA). After this procedure, the reconstructed structures were placed in synthetic oviduct culture supplemented with essential and non-essential amino acids (Sigma, St. Louis MO, USA), glucose (Sigma, St. Louis MO, USA), antibiotics (SOFaaci) (Gibco BRL, Burlington, ON, Canada), and mineral oil (Fujifilm Irvine Scientific, Santa Ana, CA, USA) in a 38.8 °C incubator with 5% CO_2 and 20% O_2.

The reconstructed oocytes remained in culture medium until the seventh day, when the formed embryos were selected, classified and shipped in straws to be transferred to recipients. With the formed embryos, recipient cows were chosen and submitted to transcervical transfer of the embryos. After 30–35 days of gestation, the pregnancy rate was assessed by transrectal ultrasonography, in which the average pregnancy rate was 53.56%. All cows were monitored monthly for evaluation of placental and fetal development with the help of the team of veterinarians of GENEAL.

2.3. DNA isolation and MSRED-qPCR assay (methylation-sensitive restriction enzyme digestion – quantitative polymerase chain reaction)

The DNA was extracted from the liver and heart tissues of clones and naturally conceived animals (controls) [53]. The tissue samples were digested overnight at 55 °C with proteinase K (Invitrogen, Carlsbad, CA, USA). Subsequently, 5 M NaCl was added to the solution for DNA isolation and centrifuged at 20,000×*g* for 10 min. The DNA was precipitated by adding 70% ethanol and centrifuged for 10 min at 20,000×*g*. After that, a NanoDrop ND-2000 spectrophotometer was used to quantify the extracted DNA. The 260/280 nm ratio was used to determine the purity of the DNA. Samples with the optical density ratio (260/280) between 1.8 and 2.0 were considered to have good quality for use. Approximately 200 ng of each DNA sample was submitted to methylation-sensitive digestion with 5U of *Hpa*II enzyme (New England Biolabs, Ipswich, MA, USA) following the manufacturer's instructions. The enzyme digestion assay was performed at 37 °C for 4 h in a final volume of 20 μL. Each digested sample was diluted ¼ (v/v) with ddH2O to adjust the optimum DNA concentration for subsequent qPCR assays.

In order to analyze the DNA methylation pattern of the DMR in the *H19*, *KCNQ1OT1*, and *PEG1/MEST* genes, real-time PCR was performed using the StepOnePlus™-Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) through the modified MSRED-qPCR protocol with SYBR Green Master Mix (Applied Biosystems, Foster City, CA, USA) [14].

The primers used for MSRE-qPCR were: H19-DMR fwd-5′-GGCTGACCAAATAGACCC-3′, rev-5′-ACCTTGCTGGTCTGCACAG-3′, KvDMR1 fwd-5′-GGTGGATCAGCATAGCGT-3′, rev-5′-GGCA-CACCGCTTTCCACAC-3′, PEG1-DMR fwd-5′-GGGTGGGCTCTAAAAGTCG-3′, and rev-5′-CCTCCTCTGCGGCAACCG-3' (Fig. 1). All primers used for MSRED-qPCR were designed using sequences from the bovine reference genome bosTau7, obtained from the UCSC Genome Browser. Since we analyzed Nellore animals, the amplicon sequences including primer sequences and *Hpa*II restriction sites were aligned with *Bos indicus* genome sequences (NCBI Reference Sequence: NC_032678.1 and NC_032653.1) using Mutalin program [54]. No nucleotide differences were identified that overlapped sequences from primer pairs of *Hpa*II restriction sites (Supplementary Fig. 1). The GeneRunner version 3.05 software (https://gene-runner.software.informer.com/) was used to design the primers. The methylation percentage of the DNA of each sample was calculated according to the formula 5 mC % = 100 × $1/2^{(\Delta Ct)}$ (ΔCt = Ct of the digested sample - mean Ct of the undigested sample) [14]. Each reaction was performed in triplicate.

2.4. Statistical analysis

For the statistical analysis of the 5 mC% data, one-way ANOVA and Tukey's multiple comparisons test were used to obtain the results of the general average of the clones versus control for the KvDMR1, H19-DMR and PEG1-DMR regions, and also for the data obtained in the groups of clones from the same donor nucleus (groups A and B). The data from group C were statistically analyzed by Welch's *t*-test due to the small number of clones in the group. In all statistical analyses, the confidence interval was 95% and differences were considered significant at $p < 0.05$. All analyses were performed using the GraphPad Prism 8.0 software.

3. Results

3.1. Phenotypic features

The bovine clones analyzed were classified as being included in type II - AOS and III - AOS, since the clone A1 died between gestational day 42 and 280 (type II AOS). For the others, death occurred around the time of parturition, or during the neonatal period. Exceptionally, clone B3 died 51 days after birth. However, since it did not survive, this animal was included as type III - AOS.

Since the normal weight of a Nellore calf at birth ranges from 30 to 35 kg, about 66% of the clones presented abnormal low (23–28 kg) and high (45–62 kg) birth weights [9,52,55]. Among the 12 clones analyzed, seven presented birth overgrowth, indicated by birth weight higher than 35 kg. These cloned calves were classified as LOS animals. Besides the weight, the survival time also varied, but with no significant values among the individual clones, from a few hours to a maximum of nine days (Table 1). Both classification parameters of AOS and LOS varied even among clones and among animals generated by SCNT from the same nucleus donor cell source (Table 1).

In addition, other phenotypic characteristics in cloned calves were similar to those found in LOS, with predominant morphological alterations in different organs. Abnormalities ranged from anemia and changes in navel size to morphological changes in various organs (including lung, heart, and liver), bleeding, and anemia [9,55–57].

3.2. DNA methylation levels in KvDMR1, H19-DMR and PEG1-DMR

The results of DNA methylation quantification in each DMR demonstrated specific levels of 5 mC%. For the DMR region of the *H19* gene, a similar pattern of DNA methylation between the clones and the control was observed in both tissues. The liver tissue presented mean 5 mC% values of 54.09% (SD ± 12.81) and 54.85% (SD ± 11.25) in the clones and controls, respectively. Although there was no statistical difference ($p > 0.05$), in the heart tissue we observed a difference of 12% in the methylation of DNA pattern in

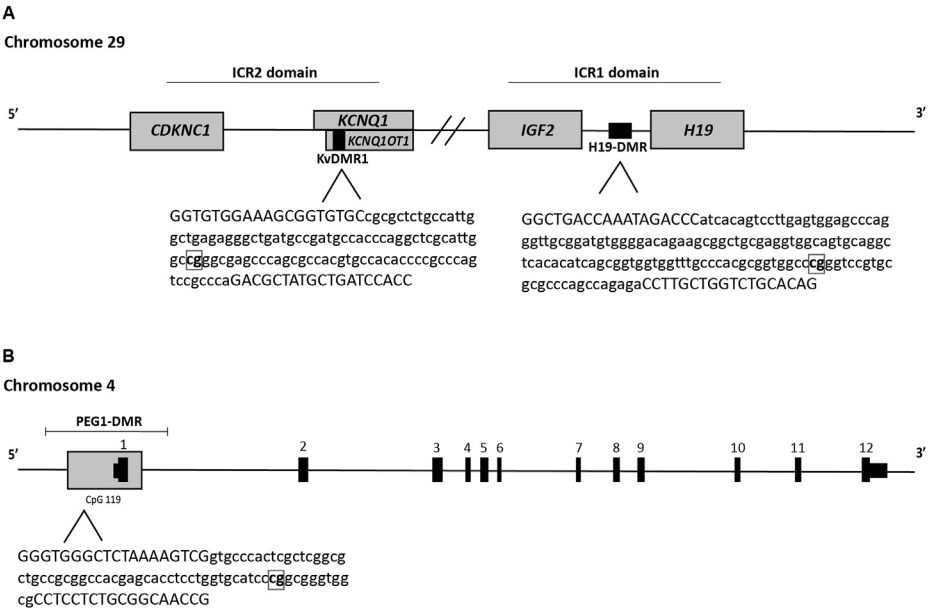

Fig. 1. DMR regions analyzed in imprinted genes. (A) Position of DMRs in *KCNQ1OT1* and *H19* genes. Amplicon analyzed with primers in uppercase, CG highlighted in dark black and gray squares. (B) Position of PEG1-DMR. Region analyzed located in CpG 119 in *PEG1* gene promoter, with primers in uppercase, CG highlighted in dark black and gray squares.

clones compared to control animals (Fig. 2A).

The KvDMR1 showed a significant decrease of 5 mC% in the heart tissue of the clones (28.04% SD ± 13.54) compared to the control (56.45% SD ± 17.38) ($p < 0.001$). In contrast, the DNA methylation pattern in the liver tissue of the clones' animals showed an increase of almost 10% in comparison with the controls ($p > 0.05$) (Fig. 2B).

In the DMR region of the *PEG1* gene, the DNA methylation pattern was similar in both tissues analyzed, as was the difference between the clones and controls. The 5 mC% mean values were 55.37% (SD ± 14.65) and 34.64% (SD ± 15.76) ($p < 0.05$) between the clones and controls, respectively, for liver tissue. For heart tissue, the clones had 55.42% (SD ± 23.47) while controls had 38.96% (SD ± 14.64) ($p > 0.05$) (Fig. 2C).

In the multi-locus analysis of the 5 mC pattern, almost all regions showed a difference of 5 mC% greater than 10% in heart tissue between clones and the control group. Separately, the levels of 5 mC% found in the heart tissue showed a decrease in the H19-DMR ($p > 0.05$) and KvDMR1 ($p < 0.001$) in the clones compared to the control group. In contrast, liver tissue in the KvDMR1 ($p > 0.05$) and PEG1-DMR ($p < 0.05$) regions showed GOM in clones compared to controls.

Because the clones in this study (A1 to A4, B1 to B4, and C1 to C2) all came from the same cell nucleus donor origin, there was a significant variance in 5 mC percent levels between clones within the same group. When compared to each other, these clones

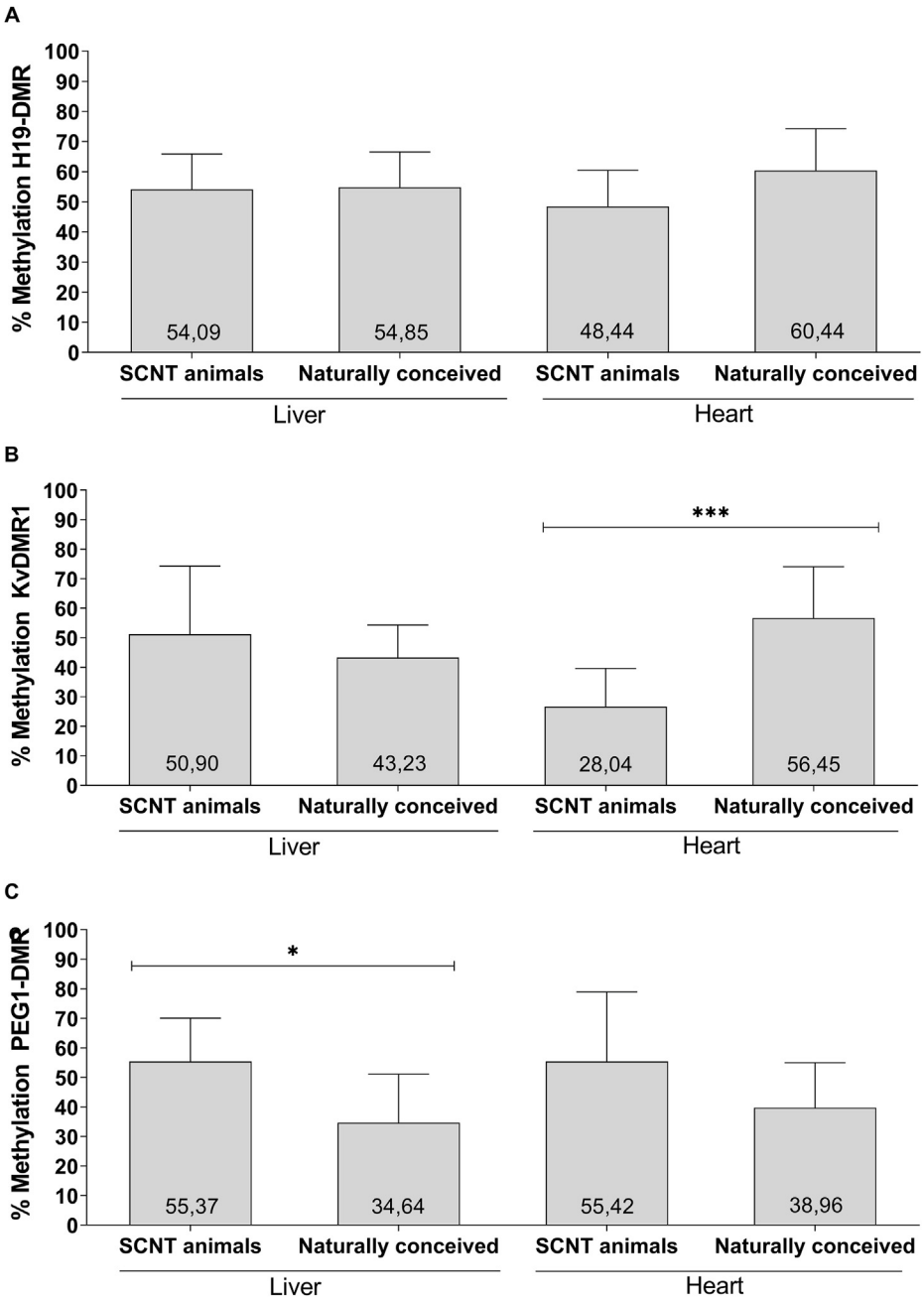

Fig. 2. DNA methylation levels (5 mC%) in KvDMR1, H19-DMR and PEG1-DMR. Data of 5 mC% in liver and heart samples from clones and naturally conceived animals (control animals) in H19-DMR (A), KvDMR1 (B) and PEG1-DMR (C). Values inside bars indicate the 5 mC% average in each sample. *$p < 0.05$/***$p < 0.001$.

showed a difference in the 5 mC% value in both tissues studied.

The group of clones from the nucleus donor group A, in the H19-DMR, reached a difference of 20% (A1 - A2) in the liver tissue ($p > 0.05$). For the heart, the difference reached 29% in clones A1 - A4 ($p < 0.001$) (Fig. 3A). Interestingly, group A clones showed no significant difference ($p > 0.05$) regarding KvDMR1 5 mC% in heart tissue, although the methylation pattern in this tissue presented a decrease of DNA methylation compared to the liver tissue. In contrast, a difference of 38% was observed between clones A1 and A3 ($p < 0.01$) in the liver (Fig. 3B). For the PEG1-DMR region, a very significant difference in the levels of 5 mC% between the clones of group A was observed, reaching almost 42% ($p < 0.001$) in the liver tissue, in which clone A1 showed levels of loss of methylation (28.52%) compared to the other clones in this group. Interestingly, for the heart tissue, the PEG1-DMR region also showed a significant difference of 5 mC% between the clones of groups A ($p < 0.05$) (Fig. 3C).

In both tissues, the clones from nucleus donor source B showed loss and gain of DNA methylation in all DMRs analyzed. As shown in H19-DMR, liver tissue had a change of up to 40% in 5 mC% ($p < 0.001$), with a gain of methylation of clone B3 (80.14%). Although the heart tissue showed a significant difference, the change in the 5 mC% levels achieved only 13% difference between clones B1 and B3 ($p < 0.01$) (Fig. 4A).

Similarly, a pattern of difference in the 5 mC% levels was observed in the liver tissue for the KvDMR1 region in the clones from nucleus donor source B, which reached 52% ($p < 0.01$) between clones B3 and B1, which exhibited values classified as hypermethylation and hypomethylation, respectively. The same was observed in the heart tissue, with a very significant difference of 40% (B3 − B4) ($p < 0.001$) in DNA methylation (Fig. 4B).

There was also a 5 mC change in the PEG1-DMR region between the group B clones. When comparing the values of the clones to each other in liver tissue, there was a very significant difference of up to 30% in the DNA methylation level ($p < 0.001$). In the heart tissue, however, the difference in 5 mC% values reached 72% ($p < 0.001$), with GOM and LOM values of clones B3 and B4, respectively (Fig. 4C).

Unlike the others, the group C had just two clones, and it was possible to observe DNA methylation changes in all DMRs from the tissues analyzed. The H19-DMR exhibited 5 mC% differences of 15% in the liver tissue ($p > 0.05$) and 12% ($p < 0.01$) in the heart tissue (Fig. 5A). The KvDMR1 showed only a significant result in liver tissue ($p < 0.05$), which reached a difference of 23% in 5 mC% between clones C1 and C2 (Fig. 5B). Finally, PEG1-DMR had a significant result in both tissues, with 19% ($p < 0.01$) and 55% ($p < 0.001$) in the liver and heart, respectively (Fig. 5C).

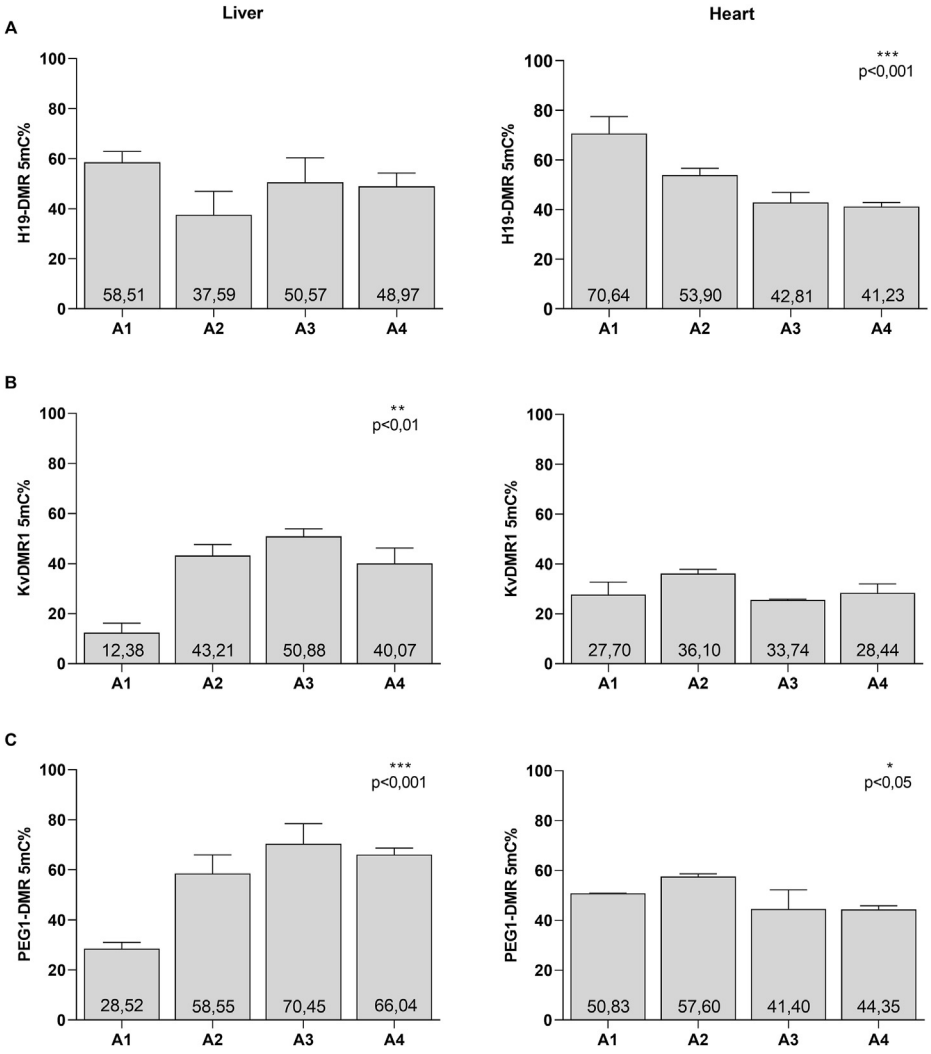

Fig. 3. DNA methylation levels (5 mC%) in group A clones. Data of 5 mC% in liver and heart samples from clones in H19-DMR (A), KvDMR1 (B) and PEG1-DMR (C). Values inside bars indicate the 5 mC% average in each sample. *$p < 0.05$/**$p < 0.01$/***$p < 0.001$.

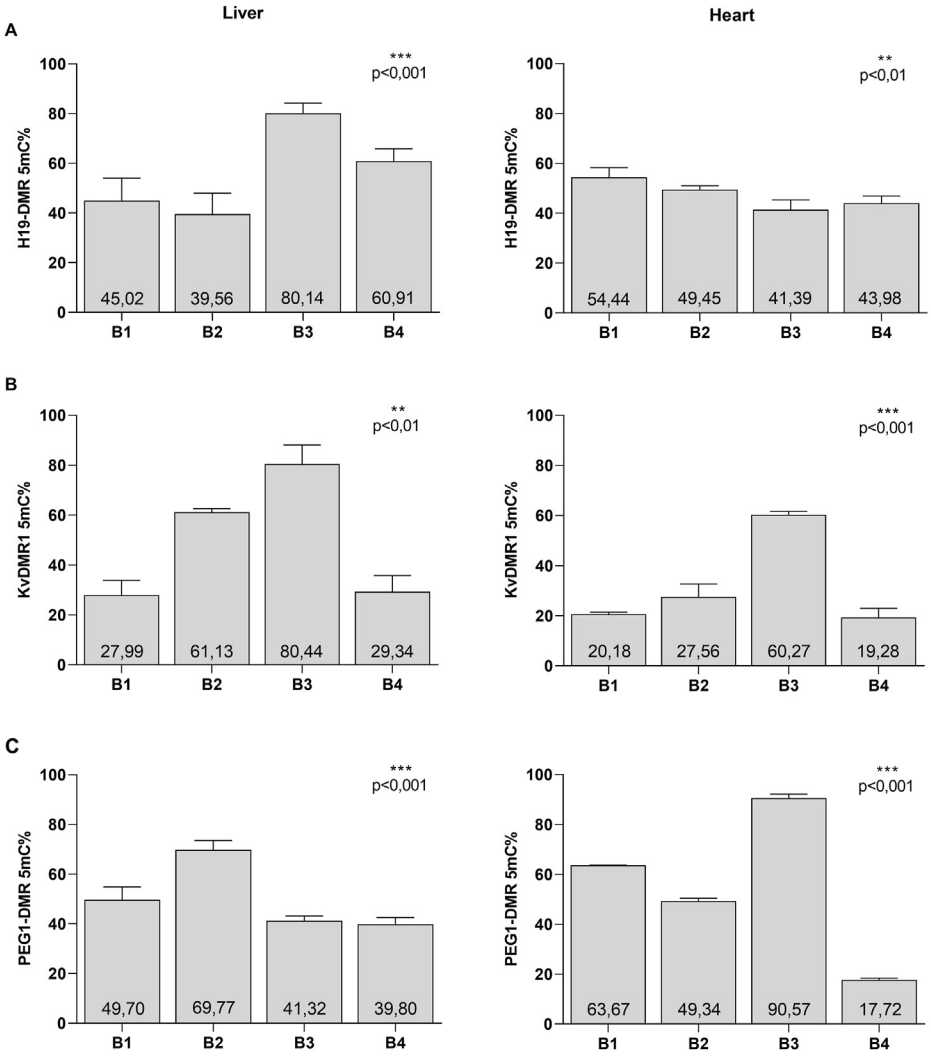

Fig. 4. DNA methylation levels (5 mC%) in group B clones. Data of 5 mC% in liver and heart samples from clones in H19-DMR (A), KvDMR1 (B) and PEG1-DMR (C). Values inside bars indicate the 5 mC% average in each sample. *p < 0.05/**p < 0.01/***p < 0.001.

4. Discussion

The clinical features of the AOS/LOS include alterations in the morphology of different organs, increased birth weight, physiological alterations, and dystocia [17,58]. In this study, we observed the occurrence of morphological alterations in the heart, liver and navel of the clones analyzed, similar to findings of other researchers [56,59]. Moreover, a previous study also reported survival rates starting at a few hours, with only one clone surviving up to six months, similar to the survival period found in our study [59].

According to previous studies, SCNT calves had a considerably high postnatal mortality rate of 16% and a stillbirth rate of 14% [52]. Furthermore, when compared to normal reproduction, animals who survive this breeding technology have a high incidence of congenital abnormalities [23]. Another relevant aspect of the current study was comparison of the 5 mC% data of bovine clones with naturally born animals, instead of comparison with IVF or insemination animals, as has occurred in many studies [8–10,60]. The DNA methylation pattern of ART-born animals may be different from those born naturally, due to possible failures in epigenetic reprogramming at the beginning of embryonic development [61–63]. Therefore, we used naturally born animals with a "normal" DNA methylation pattern for comparison with the 5 mC% data observed in SCNT-born animals.

Recently, a new characterization of hypergrowth syndrome (OGS) was described in the literature. According to this description, OGS can be classified into generalized and localized/partial, both of which have specific parameters of weight, height, head circumference, and number of affected organs [21].

Seven of the clones categorized as type-II and type-III AOS had higher birth weights than expected for the species (>35 kg). These clones were sub-classified as AOS/LOS or just LOS. Indeed, the birth weight data found in our sample demonstrated that these clones had different phenotypic outcomes. Furthermore, not all the clones were born with increased weight, which corroborates the AOS classification [17,24].

Cattle with OGS usually have a birth weight range of 59 kg–98 kg [21]. However, only one clone in the study had weight greater than 59 kg (Clone D1). Some clones did not present increased birth weight, and two animals had lower birth weight than expected for Nellore calves (clones A1 and A4). Interestingly, this low weight report may represent an additional phenotypic spectrum occurring in AOS.

It is important to remember that SRS is affected by MLID through GOM and LOM events [51]. This syndrome is characterized by intrauterine restriction and lower birth weight values [64]. The lower birth weight of the clones and the similar imprinted DMR defects may indicate that AOS is a phenotypic and molecular mirror

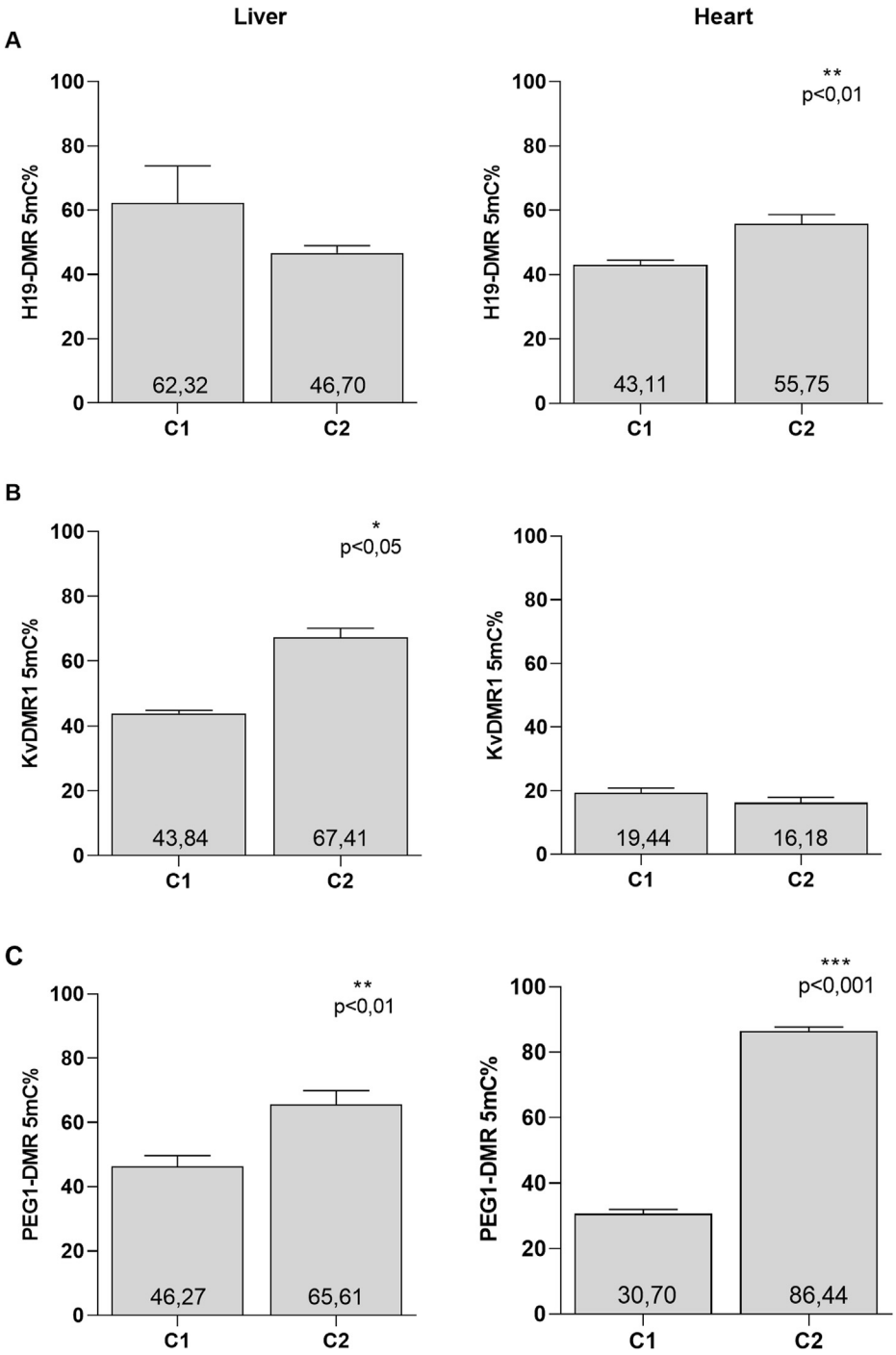

Fig. 5. DNA methylation levels (5 mC%) in group C clones. Data of 5 mC% in liver and heart samples from clones in H19-DMR (A), KvDMR1 (B) and PEG1-DMR (C). Values inside bars indicate the 5 mC% average in each sample. *p < 0.05/**p < 0.01/***p < 0.001.

of other imprinting related syndromes [65].

The normal gestational age of cattle ranges from 280 to 290 days, in which a longer gestation time may be associated with increased birth weight [66]. Except for one animal with a gestation period of 308 days, all of the clones studied had gestation periods that were equivalent to those of naturally conceived animals [65]. Animals that present gestational age over 300 days, as observed in our study, can be characterized as post-term cattle [21].

Despite the fact that ART has progressed significantly in recent decades, its negative impact on gene regulation during the embryonic and fetal stages, as well as shortly after birth and in adulthood, is still controversial. Epigenetic multi-locus defects have been reported in humans, especially LOI events [67]. These ART-induced epimutations do not occur in the same way in mice and humans, highlighting the need for better comparative models with other species [26,68].

These epigenetic abnormalities and accompanying symptoms appear to arise in ruminants in a similar way as in humans, who are impacted by different imprinted loci [60,69–71]. As a result, various researchers have suggested employing bovines produced

through somatic nuclear transfer as a model to study epigenetic abnormalities in humans resulting from various ART techniques [13,65]. Furthermore, our entire sample group (clones and naturally born animals) were females to avoid any potential sex-specific effects on DNA methylation [72–75].

In this study, the multi-locus pattern of GOM observed in two regions (PEG1-DMR and KvDMR1) in liver tissue, and LOM pattern in H19-DMR and KvDMR1 regions in heart tissue in the clones generated by SCNT (Fig. 2), can be compared to those found in MLID patients, i.e., with multi-locus alterations in two or more imprinted genes [42]. When comparing the current results with MLID in humans, two points should be highlighted. First, the DMRs studied here are the same ones that have been linked to human BWS. Second, for medical and ethical reasons, analogous epigenetic research in humans has concentrated on live-born children's peripheral tissues (blood and buccal epithelial cells). This scarcity of tissue makes it difficult to gain a better knowledge of MLID in humans [76,77]. Comparison of the MLID of humans with our findings in cloned calves indicate that this type of epigenetic disruption can affect a higher number of tissues than previously reported in humans [55]. Similar to humans, the occurrence of LOM or GOM in calves from ART also is tissue-specific, since some studies have revealed both alterations in the 5 mC% pattern in liver, kidney, placenta, muscle, brain and tongue, in different genes [8,25,26,78]. These alterations may influence the clinical outcomes derived from the effects of these epimutations [26]. The difference between the cell types studied in BWS and LOS demonstrates the need for further studies of different tissues to determine the pattern of epimutations occurring due to ART.

We focused on liver and heart tissues because bovine clones (LOS/AOS) and humans (BWS) have abnormalities reported in other studies. In humans, the methylation alterations in imprinted loci in liver-derived cells (hepatocellular carcinoma) and cardiac tissues are associated with congenital heart disease [79,80]. We hypothesized that these imprinting defects may also occur in LOS/AOS, so the analysis of liver and heart tissues may shed light on the multi-locus epimutations and tissue-specific nature of imprinting disorders.

Particularly for the KvDMR1, H19-DMR, and PEG1-DMR regions, changes in the DNA methylation pattern can affect liver and heart tissues. For KvDMR1, the LOM pattern may be associated with the development of neoplasms in different human tissues, such as the liver and breast [81,82]. Also, the loss of methylation has already been reported in BWS patients with liver carcinomas [83]. In cattle, KvDMR1 is differentially methylated in the heart, liver, and spleen. Changes in the methylation pattern of this region in liver tissue were observed in cattle [84].

The gain of DNA methylation in H19-DMR, and consequently the increase in *IGF2* gene expression, allows the development of hepatoblastoma [81,85]. Similar to these findings, another study reported that the differentially methylated region of *H19* was related to increased *IGF2* expression and BWS characteristics [86].

The extensive phenotypic variety of these overgrowth syndromes (BWS and LOS) in humans and cattle may be due not just to the variability of the affected tissues, but also the multi-locus heterogeneity of these imprinting errors [26,51,55,60]. In this study, tissues derived from different germ layers were analyzed, and the findings may indicate that these epigenetic alterations occur in a different developmental window, during or after gastrulation. LOS and BWS cause structural and physiological changes in a variety of organs. Epimutations in the tissues studied indicated that epigenetic abnormalities may play a role in the clinical heterogeneity of these disorders, according to other reports [8,25,64,87].

In humans, the frequency of imprinting defects varies and is dependent on the affected loci [8,35]. Multi-locus patterns of GOM and LOM with varied occurrence rates were discovered among the DMRs investigated in the current study. Similar to humans, the LOM pattern at the KvDMR1 region observed in the heart tissue in clones compared to control animals reinforces the molecular similarities of this animal model with humans [59,88]. Although comparison with identical human tissues of bovine clone samples was not performed in this study, the DNA methylation changes were similar to those reported in humans for BWS and SRS.

Interestingly, GOM in the KvDMR1 in liver tissues was also detected (Fig. 2B). Although the difference in the GOM pattern observed in the KvDMR1 region in the clones compared to the control animals was not significant ($p > 0.05$), we believe the GOM in KvDMR1 found in cloned calves in this work is the first evidence of a novel epigenetic change in this DMR. Prior to the present report, GOM in KvDMR1 has not been detected in humans or ruminants. The only evidence found regarding GOM in KvDMR1 comes from a study of mice offspring from Nlrp2 defective females, which presented GOM in KvDMR1 [89]. Because tissues that are not commonly investigated in humans were analyzed, we can hypothesize that the findings of the present study reflect tissue-specific epigenetic alterations. On the other hand, the lack of reports about GOM in KvDMR1 in mammals can indicate this epigenetic disruption is lethal for some species. The real effects of this new epimutation pattern need to be investigated, not only regarding the phenotypic outcomes, but also to identify regulatory effects of imprinted genes and which regulation is associated with KvDMR1 in mammals.

The H19-DMR did not show a change of methylation pattern among clones and control animals. However, the heart tissue of clones had a 12% decrease of DNA methylation compared to the control animals ($p > 0.05$). In general, the findings of this study are consistent with those found in the literature. A recent study found a hypomethylation pattern in the *H19* gene in all bovine clones compared to control animals [59]. However, these data contradict the findings of some studies of OGS, such as BWS, in which 5% of the patients presented hypermethylation of ICR1, inducing biallelic expression of the *IGF2* gene [26,67].

The co-occurrence of LOM in clone E1 of KvDMR1 (8.56%) and H19-DMR (23.33%) in heart tissues was observed. This same animal also showed hypermethylation in KvDMR1 in the liver sample (92.11%). Less common methylation alterations were also found to occur in cattle, similar to those observed in human MLID. These less common patterns reflect the heterogeneity of methylation defect alterations in a multi-locus context [51,90].

From the 2000s to the present time, some studies have shown that the imprinted genes altered in LOS and BWS are orthologs. Other genes, including *PEG1/MEST*, *GRB10*, *GNAS*, *SNRPN*, *PLAGL1*, *BEGAIN*, *DIRAS3* and *CDKN1C*, have recently been linked to LOI in these syndromes [8,51]. Therefore in cattle LOI may occur at different loci than in the *IGF2/H19* and *KCQN1OT1* genes [8].

PEG1/MEST is involved with the adipose tissue in which it is upregulated. Studies in mice show a link between changes in the expression of this gene and obesity, implying that epigenetic control is involved [91,92]. Therefore, alterations in the DNA methylation pattern in *PEG1/MEST* may be associated with the increased body weight found in clones with AOS/LOS characteristics.

Compared with other MLIDs, the epigenetic changes in PEG1-DMR in bovine clones confirm the multi-locus changes in animals born by SCNT analyzed. The DNA methylation pattern in PEG1-DMR was the most homogeneous among the three analyzed DMRs. GOM was observed in heart and liver tissues, with a 5 mC% increase of approximately 20% compared to controls. GOM was detected in SRS patients conceived through *in vitro* fertilization [64]. In contrast, hypomethylation was found in PEG1-DMR in patients with BWS from ART [16].

In SCNT-derived animals, the occurrence of DNA methylation changes of the bovine *PEG1/MEST* homolog has not been reported in the literature. As a result, this might be the first study of PEG1-DMR to show a significant increase in DNA methylation in AOS clones. Therefore, molecular characterization of epimutation patterns in bovine homolog imprinted genes associated with the etiology of BWS will permit a better understanding of the molecular mechanisms involved in the onset of both pathologies.

We observed a higher prevalence in the increase of methylation levels in PEG1-DMR in the tissues analyzed, in which both tissues presented GOM status compared to the control animals. Differences in the detection of GOM and LOM were reported to be tissue-specific in both BWS and SRS, with disagreement only in peripheral blood and buccal epithelial cells [76,77].

The SCNT protocol involves cellular and epigenetic reprogramming, so that after the fusion of the donor nucleus and cytoplasm of the receptor cell, the structure is able to reprogram itself, leading to embryo formation. It is coherent to say that SCNT animals are genetically identical, especially clones from the same nucleus donor [93].

However, the *in vitro* culture environment may influence the nucleus donor, compromising the total epigenetic reprogramming and the reestablishment of embryonic totipotency, leading to a lower rate of progeny formation [94]. For example, cell culture conditions can influence the embryo development by changing the expression pattern of imprinted genes [95,96]. One of the mechanisms involved in epigenetic reprogramming is DNA methylation. Therefore, analyzing the pattern of 5 mC% in embryos from ART sheds light on one of the causes of the low success rate of reproductive biotechnologies [94,97,98].

Although clones from the same donor nucleus have the same genetic background, the cellular and epigenetic reprogramming act in different ways. This explains the different levels of 5 mC% found in the clones in the present study coming from the same nucleus donor (Figs. 3–5). Corroborating this, the amount and functions of mitochondrial DNA present in recipient oocytes used in the SCNT protocol may affect the development and efficiency rate of the embryos produced [99,100], which might have influenced the different levels of 5 mC% that we observed in clones originated from the same core donor.

Another perspective to be considered is that there is no information on the cycle of the cells used in the SCNT protocol, which could influence the data variation of 5 mC% observed between clones from the same nucleus donor. Knowledge about the dynamics between DNA methylation and hydroxymethylation levels in cells used to produce SCNT embryos is important for understanding epigenetic reprogramming changes during embryonic development [101,102].

The different 5 mC% levels observed between clones from the same nucleus donor in the present study may reflect errors in epigenetic reprogramming, which led to the development of typical features of AOS and the consequent death of the animal. Although we observed a pattern of MLMD in liver and heart tissues, further studies in other tissues and in other imprinted genes are needed to demonstrate a tissue-specific pattern of change in SCNT-derived cattle.

The results here corroborate those described previously in the literature, according to which the specific occurrence or co-occurrence of epimutations for tissues derived from the different germ line indicates that this epimutation occurred at different stages of development.

5. Conclusions

Given the presence of hypergrowth characteristics in some clones in the present study, as well as organ abnormalities, it can be assumed that the death of these clones is related to the occurrence of AOS/LOS. The multi-locus pattern in cattle may be associated with the tissue-specific LOI due to DNA methylation alteration. The LOI pattern seen in KvDMR1 clones is comparable to that described in the literature for BWS syndrome. Surprisingly, GOM was discovered in KvDMR1 liver tissue in the current study. The tissue-specific methylation changes found in the H19-DMR, KvDMR1, and PEG1-DMR are an indication of a heterogeneous developmental feature of MLMD in the genes regulated by genomic imprinting. The GOM events found in bovine KvDMR1 indicate that variability of epigenetic defects may be higher than previously reported in mammals, or that some of these molecular abnormalities have species-specific patterns. Due to the mosaicism of the DNA methylation pattern found in genes *H19*, *KCNQ1OT1* and *PEG1/MEST*, other imprinting clusters should be analyzed regarding multi-locus events in clones generated by nuclear transfer. Studies of multi-locus DNA methylation modifications should be performed based on the phenotypic and molecular characteristics identified here, to elucidate the relationship of these events with the phenotypic traits observed in the clones.

CRediT authorship contribution statement

Paula Magnelli Mangiavacchi: Investigation, Formal analysis, Writing – original draft. **Maria Clara Caldas-Bussiere:** Supervision, Project administration, Writing – review & editing. **Mariana da Silva Mendonça:** Investigation, Writing – original draft. **Rodolfo Rumpf:** Resources, Production of bovine clones, Tissue autopsy. **Paulo Edson Soares Lemos Júnior:** Resources, Production of bovine clones, Tissue autopsy. **Carla Soares Alves:** Resources, Writing – review & editing, Production of bovine clones. **Warlei da Silva Carneiro:** Resources, Production of bovine clones, Tissue autopsy. **Angelo José Burla Dias:** Supervision, Writing – review & editing. **Álvaro Fabrício Lopes Rios:** Conceptualization, Supervision, Formal analysis, Project administration, Writing – review & editing.

Declaration of competing interest

None.

Acknowledgements

The authors have no competing interests to declare. This research was supported by FAPERJ (Fundação de Amparo à Pesquisa do Estado do Rio de Janeiro, Brazil - E-26/111.604/2010 and E-26/203.551/2015). Paula M. Mangiavacchi was initially a recipient of a fellowship from FAPERJ/UENF, and in the last six months of her PhD research, she received a scholarship from CAPES (Coordenação de Aperfeiçoamento de Pessoal de Nível Superior, Brazil). Each author contributed significantly to the development of the project and the manuscript. The authors Rodolfo Rumpf, Paulo Edson Soares Lemos Júnior, Carla Soares Alves, and Warlei da Silva Carneiro produced all the clones of the present project, as well as the collection of all the material analyzed. The current research was part of the doctoral project of Paula Magnelli Mangiavacchi, under the supervision of professors Álvaro Fabrício Lopes Rios and Maria Clara Caldas Bussiere. The author Angelo José Burla Dias participated in the project as a collaborating researcher. Mariana da Silva Mendonça participated in the execution of all the steps of the research together with Paula Magnelli Mangiavacchi.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.theriogenology.2022.04.006.

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