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DNA methylation of the endogenous retrovirus Fematrin-1 in fetal placenta is associated with survival rate of cloned calves

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ABSTRACT

Introduction: The expression of retroviral envelope proteins in the placenta facilitates generation of the multinuclear syncytiotrophoblast as an outer cellular layer of the placenta by fusion of the trophoblastic cells. This process is essential for placenta development in eutherians and for successful pregnancy.
Methods: We tested the hypothesis that alterations in DNA methylation and gene expression profiles of the endogenous retroviruses (ERVs) and genes related to epigenetic reprogramming in placenta of cloned calves result in abnormal offspring phenotypes. The fetal cotyledons in 13 somatic cell nuclear transfer (SCNT) pregnancies were collected. DNA methylation level of *Fematrin-1* was analyzed using bisulfite PCR and mRNA levels of *Fematrin-1, Syncytin-Rum1, DNMT1, DNMT3A, DNMT3B, TET1, TET2* and *TET3* measured by RT-qPCR.
Results: Methylation of Fematrin-1 in placenta of control animals produced by artificial insemination (AI) was similar to live SCNT-produced calves, but hypermethylated than dead SCNT-produced calves. The levels of mRNA differed between SCNT-produced calves and AI animals for all genes, except *TET3*. However, no differences were observed between the live and dead cloned calves for all genes. Moreover, no differences were found between mRNA levels of *Fematrin-1* and *Syncytin-Rum1*.
Discussion: Our results suggest that this altered DNA methylation, deregulation in the expression of ERVs and in the genes of epigenetic machinery in fetal cotyledons of cloned calves may be associated with abnormal placentogenesis found in SCNT-produced animals. Further studies characterizing other mechanisms involved in the regulation of ERVs are important to support the development of new strategies to improve the efficiency of cloning.

1. Introduction

The somatic cell nuclear transfer (SCNT) technique allows a highly differentiated genome from a somatic cell to be reprogrammed into a totipotent state, thereby facilitating the generation of organisms from a single donor cell [1]. SCNT has tremendous potential applications such as in reproductive cloning to accelerate animal genetic improvement, production of transgenic and genome-edited farm animals for various purposes which include animal welfare, disease resistance, performance, and biomedical applications [2,3], for the generation of human pluripotent cells from cloned blastocysts for use in regenerative medicine [4], and as human disease models [5].

In 1962, using frogs, Dr. John Gurdon first demonstrated that animals could be cloned by the transfer of a somatic cell nuclei into an

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unfertilized egg [6]. Later, in 1997, Dolly the sheep, the first cloned mammal created using a somatic cell from an adult animal was born [7]. Since then, successful cloning of more than 20 mammalian species has been reported [8], and recently the birth of two cynomolgus monkeys (*Macaca fascicularis*), the first non-human primates cloned by SCNT, has been reported [9]. Despite its success, technical barriers have limited the practical use of SCNT. Developmental anomalies are frequent in cloned animals, and losses throughout early preimplantation, postimplantation, and pre- and postnatal development are suggested to have epigenetic errors as origin [10]. Reprogramming of the epigenetic patterns of the somatic cell represents a barrier in SCNT because the enucleated oocyte needs to reprogram the somatic cell nucleus into a totipotent stage, capable of developing into a viable offspring [11]. Abnormal epigenetic reprogramming occurs mainly in the trophoblast cells, with these cells frequently revealing aberrant hypermethylation patterns in SCNT pregnancies [12]. Since the placenta originates from the outer trophectoderm layer of blastocysts, this tissue is considered as the origin of most pathologies in cloning by nuclear transfer [13]. Besides that, the parturition difficulties and other abnormalities including defects in the cardiovascular, musculoskeletal, neurological and respiratory systems affect the post-natal viability of the cloned animals [14,15].

Placental tissue comprises fetal trophoblast cells and maternal uterine cells, derived from the chorion and endometrium, respectively [16]. However, in eutherians, the placental morphology differs among the species. In cattle, placenta is classified as synepitheliochorial and cotyledonary [17]. The main feature of this placenta is the adhesion of the trophectoderm cells to the uterine caruncles, constituting the placentomes [16]. The synepitheliochorial placentation is characterized by a heterologous cell-fusion process between the fetal and maternal cells [18]; the long terminal repeat (LTR) retrotransposons play a crucial role in this process [19]. Retrotransposons are transposable elements (TEs), classified as RNA transposon class I, which multiply themselves in the host genome through a copy and paste mechanism mediated by a reverse transcriptase enzyme [20]. Approximately 40% of the mammalian genome comprises retrotransposons [21], including some of the most abundant repetitive sequences, of which about one-fourth are endogenous retroviruses (ERVs) [22]. LTR retrotransposons, related to ERVs, are inherited genetic elements closely resembling the proviruses formed following exogenous retrovirus infection [21].

For several years, TEs were considered as junk DNA or purely selfish, with the absence of favorable effects for the organisms [23]. However, recent advances in genome sequencing have recognized the participation of TEs as important components of transcriptional regulatory networks [24], playing essential roles in the evolution and biology of most organisms [25]. For example, in the bovine placenta, the expression of the envelope proteins of ERVs, such as *Fematrin-1* and *Syncytin-Rum1*, has been co-opted to serve as a fetomaternal cell-to-cell fusion [26], playing an essential role in placental morphogenesis (Fig. 1) [19]. The transcriptional control of retrotransposons is regulated by epigenetic mechanisms, mainly during early embryogenesis [27,28]. DNA methylation is the main epigenetic modification of the mammalian genome [29] and is considered extremely significant to retrotransposons, since it is the best established mechanism of retroelement silencing [30]. The enzymes responsible for its generation and maintenance are the family of DNA methyltransferases (DNMTs) [31]. Furthermore, ten-eleven translocation (TET) proteins are directly responsible for the DNA demethylation process in cell reprogramming and this process may lead to the activation of retrotransposon promoters in the embryonic stem cell (ESC) [32,33].

The hypomethylation of 5′ LTR promoter of the ERVs can result in gene activation, encoding the retroviral envelope proteins, and in turn promoting cell-to-cell fusion during the placentation process [34]. Thus, the altered epigenetic regulation of ERVs may lead to aberrant placentation and development anomalies [35]. Therefore, in this study, we hypothesized that DNA methylation and gene transcription deregulation of syncytin-like genes are associated with abnormal placentation and neonatal mortality in cloned cattle. To test this hypothesis, we evaluated the DNA methylation profile of *Fematrin-1* and the transcript levels of *Fematrin-1, Syncytin-Rum1, DNMT1, DNMT3A, DNMT3B, TET1, TET2*, and *TET3* in the placenta of cloned calves presenting different placental phenotypes.

2. Materials and methods

Ethics approval

The Ethics Committee on Animal Use (CEUA protocol no. 078/16) of the Federal University of Uberlândia, Brazil, approved all the performed procedures.

2.1. Experimental design

Skin biopsies were surgically collected from two Nellore bulls (*Bos taurus indicus*). Fibroblasts were cultured *in vitro* and subsequently used in SCNT procedures (GENEAL Genetics and Animal Biotechnology, Uberaba, Minas Gerais, Brazil). SCNT-produced embryos were transferred to the recipient cows, and placental samples (fetal cotyledon) of 13 male cloned calves were collected at birth. Cloned calves were classified according to their viability during the first 3 months of life. Calves that died during this period were considered as dead offspring, whereas those that survived beyond this period were considered as live offspring (Fig. 2 a). Phenotypes observed in the placenta while calving of each cloned calf are represented in Fig. 2 and the following characteristics of each phenotype were analyzed as previously described by Silveira et al. [36]: enlarged placentomes, placental edema, meconium-stained amniotic fluid, enlarged umbilical cord, and large

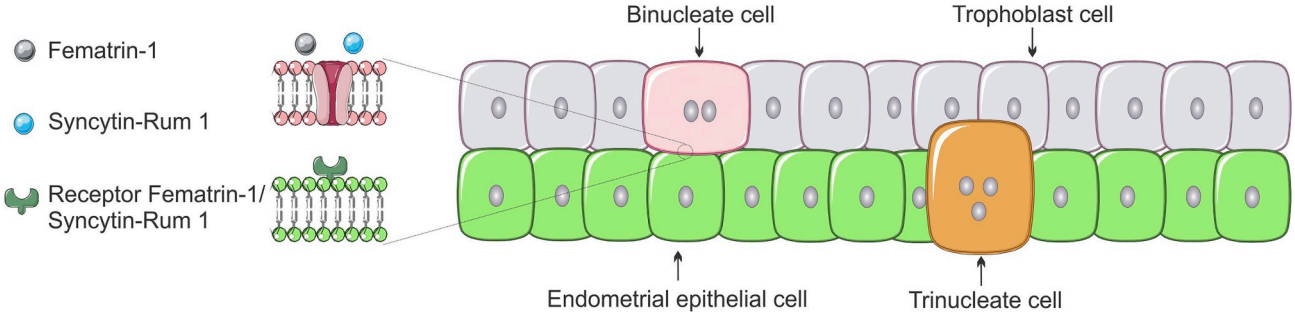


Fig. 1. Role of endogenous retroviruses in multinucleated cell formation in the bovine placenta. In bovines, trophectoderm forms binucleate cells through acytokinetic mitosis, which tend to migrate through the surrounding fetal trophoblast until they come in contact with maternal uterine epithelial cells with which they fuse resulting in the formation of the trinucleate cells that localize in the uterine stroma. This fusion process is coordinated by retroviral envelope proteins of two endogenous retroviruses, *Fematrin-1* and *Syncytin-Rum 1*, which are expressed in the binucleated trophoblast cells and, presumably, interact with the endometrial cell receptors to initiate cell fusion. Adapted from Imakawa et al. [50].

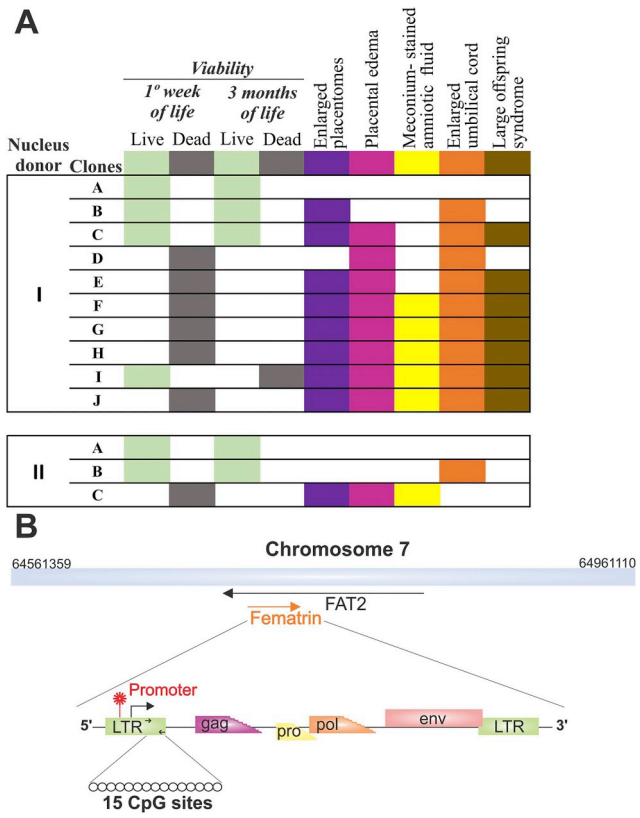


Fig. 2. Different phenotypes of Nellore (*Bos taurus indicus*) cloned calves at birth and achematic representation of the *Frematrin-1* locus in the bovine genome. (A) Each phenotypic trait is represented by a different color. Each line represents a calf. I and II represent different cell lines (nucleus donor animals). Letters represent different cloned animals. Controls are calves produced by artificial insemination (AI). (B) *Frematrin-1* was integrated in a reverse orientation into the intron 18 of the bovine FAT tumor suppressor homolog 2 (FAT2) gene. The conserved *Frematrin-1* structure corresponds to 5′LTR, env (cell fusion) and 3′LTR. Gag (polyprotein Gag), pro (viral protease), and pol (reverse transcriptase, RNase H and integrase) genes are degenerated. The 5′LTR contains a promoter, recognized by the host RNA polymerase II that transcribes the mRNA of the env (the start-site of transcription is indicated by the right-angled arrow). The region selected for methylation analysis is within the 5′LTR; black arrows represent the PCR primer position and white circles represent the CpGs dinucleotides evaluated in methylation analysis. Part B adapted from Nakaya et al. [53].

offspring syndrome. Three Nellore male calves produced by artificial insemination (AI) were used as controls. Placental samples (fetal cotyledon) were collected during cesarean sections, immediately snap frozen on dry ice and stored at −80 °C until genomic DNA/RNA isolation. Two molecular analyses were performed as follows: DNA methylation analysis of *Frematrin*-1 by bisulfite PCR and the mRNA relative abundance quantification of *Frematrin-1, Syncytin-Rum1, DNMT1, DNMT3A, DNMT3B, TET1, TET2,* and *TET3* genes by RT-qPCR.

2.2. DNA isolation

Genomic DNA was isolated from the placental biopsies as reported by Biase et al. [37]. DNA quality, concentration, and purity were evaluated as described in a study by Silveira et al. [36]. DNA samples were stored at −20 °C until further use.

2.3. Sodium bisulfite treatment, PCR amplification, cloning, and bisulfite sequencing

Primers were designed using the Bisulfite Primer Seeker (http://www.zymoresearch.com/tools/bisulfite-primer-seeker) to flank and amplify a CpG island in ERV sequence from the *Bos taurus* bovine endogenous retrovirus (BERV-K1) 5′ LTR, fetomaternal trinucleate cell inducer 1 (*Frematrin- 1*). Primer sequences, GenBank accession number, CpG numbers, and amplicon size are listed in Table 1. The 5' LTR region in the ERV structure was selected for methylation analysis. The LTRs are crucial as they comprise promoter sequences responsible for regulating the ERV expression (Fig. 2 b) [35].

DNA samples were treated with sodium bisulfite using the EZ DNA Methylation-Lightning™ Kit (Zymo Research, Orange, CA, USA) according to the manufacturer's instructions. After sodium bisulfite treatment, the DNA samples were stored at −80 °C until PCR amplification. Sodium bisulfite-treated DNA samples were subjected to PCR amplification in duplicate for each sample. PCR was performed in a total volume of 20 μL comprising 1 × Taq buffer, 1.5 mM $MgCl_2$, 0.4 mM dNTPs, 1 U Platinum™ Taq polymerase (Invitrogen, CA, USA), 0.5 μM of each primer (forward and reverse), and 2 μL of bisulfite-treated DNA. The reaction commenced with an initial denaturation step at 94 °C for 3 min, followed by 41 cycles at 94 °C for 40 s, 50 °C for 1 min, and 72 °C for 1 min, with a final extension at 72 °C for 15 min. The process of DNA cloning, sequencing, and sequence analysis criteria were based on a previous study conducted by Silveira et al. [36]. Briefly, regarding the sequence analysis criteria, only sequences that arisen from clones with ≥95% of identity and bisulphite efficiency were used. The conversion rate of non-CpG cytosines was used to calculate the efficiency of the bisulphite treatment, and the methylation profile was used to identify different alleles. Thus, to avoid clonal duplicates, the exclusion criteria were percentage of methylation, bisulphite conversion rate and methylation profile (which cytosines were converted). If two or more DNA clones were the same in all criteria, only one sequence was used for analysis. The raw values from the bisulphite-treated DNA sequence data for CpG methylation analysis are listed in Supplemental Table 1.

2.4. RNA isolation and cDNA synthesis

Total RNA from the placental samples was isolated using the TRIzol™ Plus RNA Purification Kit (Invitrogen, Carlsbad, CA, USA), according to the manufacturer's instructions. DNAse treatment (10 U) was performed during RNA isolation. Total RNA samples were stored at −80 °C for further cDNA synthesis. Immediately before cDNA synthesis, total RNA samples (1 μg) were treated again with DNase using 1 U of RQ1 RNase-Free DNase® (Promega, Madison, WI, USA) according to the manufacturer's instructions. cDNA was synthesized from 1 μg of total RNA by using the SuperScript™ III First-Strand Synthesis SuperMix, with oligo$(dT)_{20}$ (2.5 μM) and random hexamer (2.5 ng/μL) primers (Invitrogen, Carlsbad, CA, USA) in a final volume of 20 μL, according to the manufacturer's instructions. cDNA samples were stored at −20 °C until further use.

2.5. RT-qPCR

The mRNA relative abundance of the ERV genes, implicated in bovine placentation, BERV-K1 *env* (*Frematrin-1*) and *Syncytin-Rum1*, and genes related to DNA methylation reprogramming, *DNMT1, DNMT3A, DNMT3B, TET1, TET2* and *TET3* were determined by RT-qPCR. Primers were designed using the PrimerQuest Tool (http://www.idtdna.com/PrimerQuest) and are listed in Table 2. RT-qPCR amplification mixtures comprised 1 μL of cDNA, 0.2 μM of each primer (forward and reverse), 12.5 μL of Fast SYBR Green Master Mix (Applied Biosystems, Foster City, California, USA) and 10.5 μL of nuclease-free H_2O. Reactions were performed on a 7500 *Fast Real Time PCR System* (Applied Biosystem, Foster City, California, USA). The cycling conditions included 40 cycles at 95 °C for 3 s (denaturation) and 60 °C for 30 s (annealing/extension), with a dissociation (melting curve) at 60 °C–95 °C. Each sample was analyzed in triplicate and the specificity of PCR product was determined by the melting curve analysis and amplicon size in the agarose gel.

Table 1
Primers for methylation analysis of *Fematrin- 1* gene.

Genomic region	Primer Sequence (5′-3′)	GenBank accession number	CpG sites	Amplicon length (bp)
Fematrin- 1	F: TAAAGTATTTGTATATGATAAGTTTGTAGAAAAG R: ATCCAAAAATATCCTCTAAAAAAC	AB751366.1	15	481

F (forward); R (reverse); bp (base pair).

Table 2
Primers for RT-qPCR analysis.

Gene	Primer Sequence (5′-3′)	GenBank accession number	Amplicon length (bp)	Primers efficiency (%)
Fematrin- 1	F: CCCTATCTTATGGTGCCTGTAAC R: CTAACCGTCGTGATCGCATTA	NM_001245951.2	103	92.011
Syncytin-Rum1	F: CCGGTTCCGACTGGAAATATAG R: CTACCCAGCCAACTGGTAAC	NM_001305454.1	108	80.981
DNMT1	F: TTGGCTTTAGCACCTCATTTGCCG R: TCCTGCATCACGCTGAATAGTGGT	NM_182651.2	82	91.244
DNMT3A	F: TTTCCAATGTGCCATGACAGCGAC R: GGGCCCACTCGATCATTTGTTTGT	NM_001206502.1	82	114.726
DNMT3B	F: CAACAAGCAACCAGAGAATAAG R: CAACATCCGAAGCCATTTG	NM_181813.2	161	112.048
TET1	F: GTATGCTCCAGCTGCTTATC R: CCACTGTGCTCCCATTATTC	XM_015469834.1	167	108.166
TET2	F: GTAGGGACATTTCCTCCTTATTC R: CAGCTGCACTGTAGTTATGG	XM_010828077.2	157	105.302
TET3	F: GTAACCCAGGTGATTCTGATAC R: CAGCAGCCTATCTGCTAATC	XM_015465317.1	200	101.853
ACTB	F: GGCACCCAGCACAATGAAGATCAA R: ATCGTACTCCTGCTTGCTGATCCA	NM_173979.3	134	109.929
GAPDH	F: GGCGTGAACCACGAGAAGTATAA R: CCCTCCACGATGCCAAAGT	NM_001034034.2	119	101.988

F (forward); R (reverse); bp (base pair).

Threshold was set at 0.2 ΔRN (variation report signal) and baseline in default of the qPCR program.

The transcript levels were normalized relative to the amount of the housekeeping genes encoding *Bos taurus* actin beta (ACTB) and *Bos taurus* glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The geometric average of the Ct (cycle threshold) as well as the geometric average of efficiencies of the housekeeping genes were used for data normalization [38]. The mean values of the control group (AI-produced calves) were used as the reference sample. The relative abundance of each gene was calculated using ΔΔCt method with efficiency correction by the Pfaffl method [39].

2.6. Statistical analysis

Data were analyzed using the GraphPad Prism software (https://www.graphpad.com/scientific-software/prism/). Methylation data were compared among the experimental groups using the Kruskal–Wallis test followed by the Dunn's multiple comparison test or the Mann–Whitney test. Gene expression data were compared among the experimental groups using one-way analysis of variance (ANOVA), followed by the Tukey–Kramer multiple comparison test or the independent samples *t*-test. The results are presented as mean ± standard error of the mean (SEM) or with error bars representing the standard deviation (SD). *P* value ≤ 0.05 denotes a statistically significant difference.

3. Results

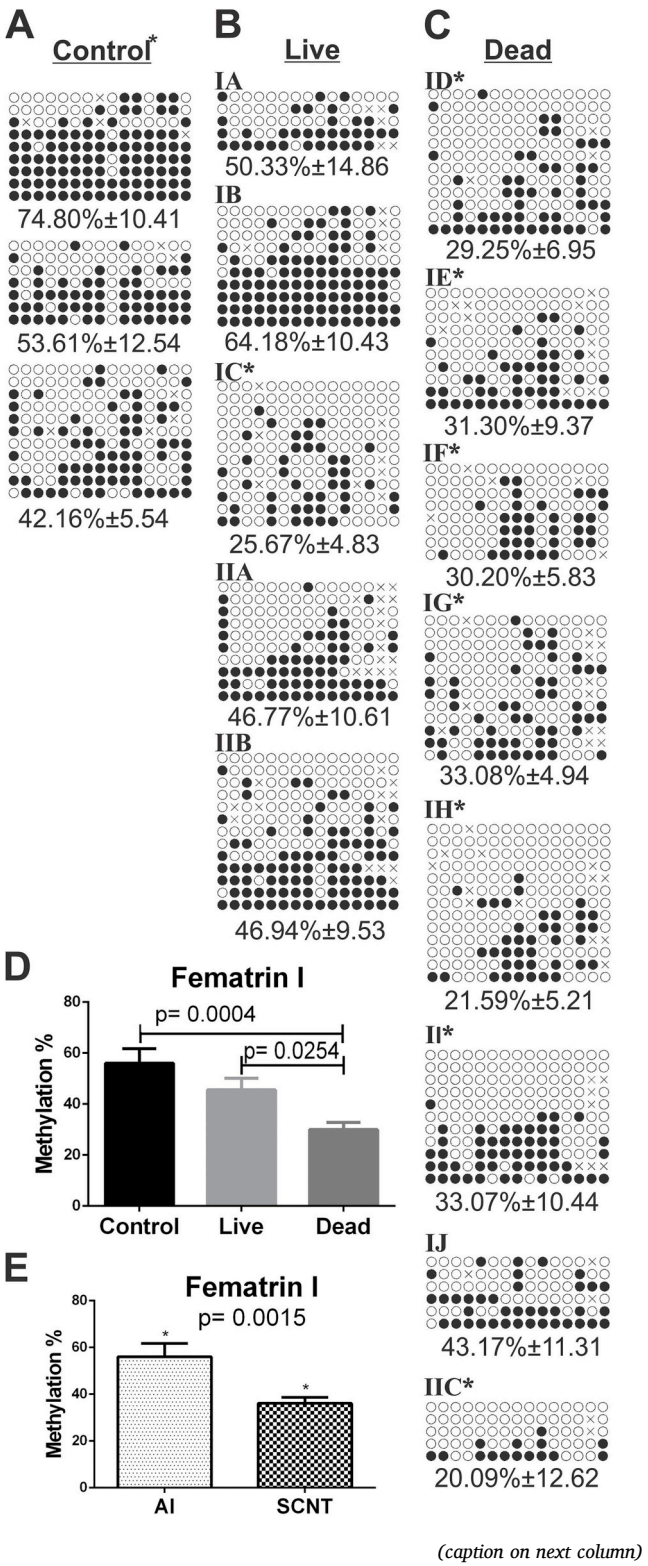
3.1. DNA methylation profile of the endogenous retrovirus Fematrin-1

We evaluated the methylation pattern in the endogenous retrovirus *Fematrin-1* in the placenta of cloned calves with different viability at 3 months of life. The bisulfite sequencing results are presented in Fig. 3, which displays the methylation mean values of *Fematrin-1* in the placenta sample of each cloned calve. *Fematrin-1* was less methylated in the placenta of dead SCNT-produced calves (ID: 29.25%; IE: 31.30%; IF: 30.20%; IG: 33.08%; IH: 21.59%; II: 33.07%; and IIC: 20.09%) compared to that of AI controls (74.80%; 53.61%; 42.16%; $p < 0.05$; Fig. 3 a, c), except in the animal IJ (43.17%), which revealed similar DNA methylation pattern as the controls (Fig. 3 c). Nevertheless, placenta of live cloned calves revealed methylation patterns (IA: 50.33%; IB: 64.18%; IIA: 46.77%; and IIB: 46.94%) similar to those of the AI controls (74.80%; 53.61%; 42.16%; Fig. 3 a, b), except in the animal IC (25.67%), which presented lower DNA methylation levels than the controls ($p < 0.05$; Fig. 3 b).

Bisulfite sequencing analysis demonstrated that the placenta from the control animals had higher DNA methylation in the endogenous retrovirus *Fematrin-1* (56.01% ± 5.74) than the placenta from dead cloned animals (ID; IE; IF; IG; IH; II; IJ; IIC) (29.96% ± 2.78; $p < 0.05$; Fig. 3 d). In addition, the placenta of live cloned animals (IA; IB; IC; IIA; IIB) also had higher DNA methylation levels (45.59% ± 4.52) than those from dead animals (29.96% ± 2.78) for *Fematrin-1* ($p < 0.05$) and no differences were observed between placenta of the control and live animals (Fig. 3 d). Placenta from SCNT-produced calves (IA; IB; IC; ID; IE; IF; IG; IH; II; IJ; IIA; IIB; and IIC) presented lower levels of DNA methylation in *Fematrin-1* (36.11% ± 2.53) compared to the AI animals (56.01% ± 5.74; $p < 0.05$; Fig. 3 e).

3.2. Gene expression analyses

mRNA levels of *BERV-K1 env Fematrin-1, Syncytin-Rum1, DNMT1, DNMT3A, DNMT3B, TET1, TET2*, and *TET3* genes were analyzed in the bovine fetal placenta during calving, and the results are shown in Fig. 4. Placenta from the cloned calves revealed higher levels of transcripts for *Fematrin-1*, *Syncytin*, *DNMT3B*, and *TET1* than those from the controls ($p < 0.05$), whereas placenta from AI controls presented higher levels of transcripts for *DNMT1, DNMT3A*, and *TET2* genes than those from



(caption on next column)

Fig. 3. DNA methylation profile of *Fematrin-1* in the fetal placenta (cotyledon) of SCNT-produced calves. (A) Represent AI-produced calves. (B) Represent live SCNT-produced calves: IA, IB, IC, IIA, and IIB. (C) Represent dead SCNT-produced calves: ID, IE, IF, IG, IH, II, IJ, and IIC. I and II are different cell lines (nucleus donor animals). (D) Percentage of methylation in the *Fematrin-1* according to the offspring viability. (E) Percentage of methylation in the *Fematrin-1* according to the assisted reproductive technique. Each line represents an individual DNA clone and each circle represents a CpG dinucleotide (15 CpGs). White circles represent unmethylated cytosines, filled black circles represent methylated cytosines, and X represents a cytosine that could not be analyzed. The numbers at the bottom of each group represent the DNA methylation means ± standard error of the mean. (*) represent significantly different mean methylation levels between each SCNT-produced calf compared to the AI controls using the Mann–Whitney test ($p \leq 0.05$). Percentage of offspring viabilities and assisted reproductive techniques are represented by means ± standard deviation. *P-value* represents the significantly different means between offspring viabilities or assisted reproductive techniques using the Kruskal–Wallis and Mann-Whitney tests, respectively ($p \leq 0.05$). SCNT (somatic cell nuclear transfer); AI (artificial insemination).

cloned calves ($p < 0.05$; Fig. 4 a).

Gene expression analysis also revealed that placenta of the control animals exhibited lower mRNA levels of *Fematrin-1* and *Syncytin* than the live and dead cloned calves ($p < 0.05$; Fig. 4 b); the control animals showed lower levels of *DNMT3B* and *TET1* only when compared with dead cloned calves ($p < 0.05$; Fig. 4 b). In contrast, the placenta from control animals exhibited higher levels of transcripts of *DNMT1, DNMT3A*, and *TET2* compared to that from the dead cloned calves ($p < 0.05$; Fig. 4 b). In addition, no differences were found between placenta of the live and dead cloned calves for all genes (Fig. 4 b) and for *TET3* (Fig. 4 a, b).

A descriptive analysis for each cloned calf was individually performed (Fig. 4 d). It was observed that the patterns of upregulation and downregulation for all genes in placenta were similar for most animals, except in the animals II and IIA, whose behavioral gene pattern differed from the others, with a downregulated and upregulated pattern for II and IIA, respectively (Fig. 4 d).

4. Discussion

In SCNT, approximately only 5–10% of the transferred embryos produce a viable offspring [40]. Such high losses are considered to be related to an incorrect epigenetic reprogramming of the somatic epigenome [41], specifically in trophoblast cells [12], which results in placental pathologies and the onset of several developmental abnormalities [42]. The incorrect epigenetic reprogramming in some types of repetitive DNA has been reported during cloning by nuclear transfer [36,43,44]. However, in cattle, the relationship between endogenous retroviruses (ERVs) involved in placentogenesis, epigenetic reprogramming, and placental problems remains unclear. Therefore, in this study, we aimed to test the hypothesis that DNA methylation patterns and gene expression in the syncytin-like genes are associated with abnormal placentation and neonatal mortality in cloned cattle. To test this hypothesis, we evaluated the DNA methylation and expression profiles of the envelope protein coding sequences of bovine endogenous retroviruses *Fematrin-1* and *Syncytin-Rum1*. Additionally, we evaluated the expression profile of genes related to the epigenetic machinery such *DNMT1, DNMT3A, DNMT3B, TET1, TET2,* and *TET3* in the placenta of cloned calves exhibiting different placental phenotypes.

In ruminants, the expression of retroviral envelope proteins in binucleated trophoblastic cells throughout gestation induces fusion with bovine endometrial cells forming hybrid cells termed as trinucleate cells (TNCs), and this unique fusogenic process is essential for placenta formation and consequently for successful pregnancy [19]. The methylation analysis of the bovine endogenous retroviral envelope gene, *Fematrin-1*, revealed that placenta from SCNT-produced calves was hypomethylated in this region compared to animals produced by AI

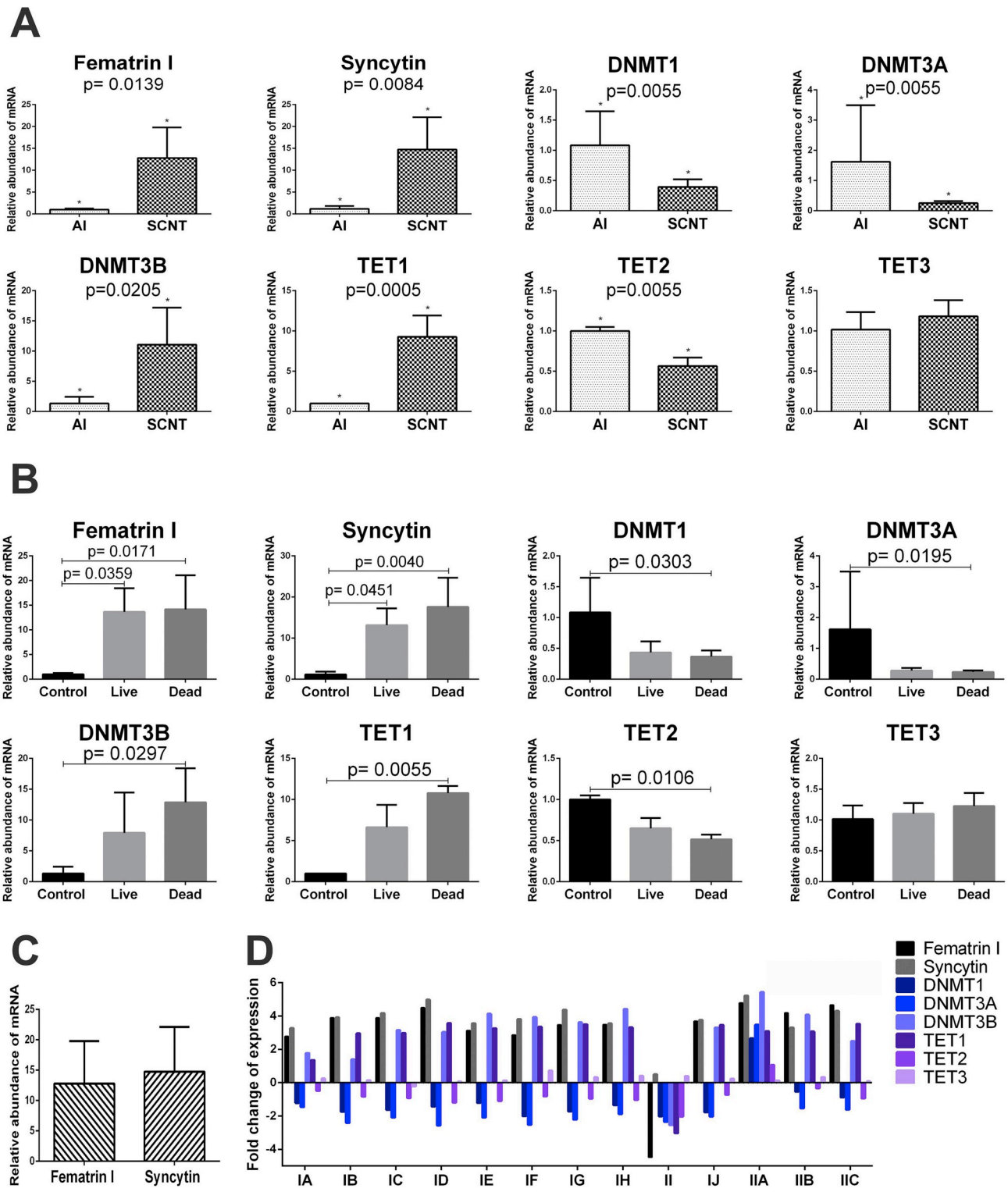


Fig. 4. Relative abundance of mRNA of BERV-K1 env (*Fematrin I*), Syncytin-Rum1 (*Syncytin*), *DNMT1, DNMT3A, DNMT3B, TET1, TET2* and *TET3* genes in fetal placenta (cotyledon) of SCNT-produced calves and AI-produced calves. (A) Relative abundance of mRNA according to assisted reproductive technique. (B) Relative abundance of mRNA according to the offspring viability. (*) represent significantly different mean using the Mann-Whitney test ($p \leq 0.05$). (C) Comparison of relative abundance of mRNA between *Fematrin-1* and *Syncytin-Rum1* in all clones. *P-value* represents the significantly different using the Kruskal–Wallis test ($p \leq 0.05$). (D) Fold change values for all genes for each cloned calf individually compared to the control group (value 0). I and II represent different cell lines (nucleus donor animals) and letters indicate various cloned animals. SCNT (somatic cell nuclear transfer); AI (artificial insemination).

(Fig. 3 e). In addition, the placenta from cloned calves that did not survive were hypomethylated in *Fematrin-1* compared to those from the live cloned calves (Fig. 3 a–d). The methylation patterns between SCNT-produced calves and animals produced by IA seems to extend to mRNA levels, demonstrating inverse correlation between methylation and gene expression in *Fematrin-1* (Fig. 4 a, b). Although other epigenetic mechanisms than DNA methylation may be also involved in the regulation of the retrotransposon expression, Fematrin I showed less methylated and more expressed in the SCNT-produced animals (Fig. 3 e and 4 a). Therefore, this profile suggests the relevance of DNA methylation for Fematrin I regulation, despite we found some hypomethylated alleles in the AI group, suggesting that the DNA methylation may not be the primary epigenetic mark suppressing Fematrin I transcription. These data suggest an epigenetic misregulation in the locus of the *Fematrin*-1 that may be associated to the placental abnormalities indicated by the cloned animals that were evaluated in this study (Fig. 2 a), which may be related to the low efficiency of SCNT procedures and high offspring mortality rates reported in several studies [45–48]. Furthermore, a loss of methylation in ERVs can lead to a deregulation of other genes [35]. ERVs have the common provirus structure of coding open reading frames (ORFs) flanked by LTRs (Fig. 2 b) [20]. LTRs contain promoter sequences and enhancer elements [35], which can regulate not only ERV expression but others genes, specifically when LTRs are located in the intergenic regions [49,50]. The 5′ LTR hypomethylation can increase promoter activity, that could disrupt the normal gene expression or transcript processing [35], which can contribute to aberrant phenotypes culminating in early mortality. HERV-W (human endogenous retroviruses group W), which encodes syncytin-1, has also been associated with neurological disorders, autoimmune disease [35], and cancers [34,51] in situations of de-repression of the LTRs.

In addition to the evaluation of DNA methylation and gene expression in *Fematrin-1*, we evaluated the relative abundance of transcripts encoding *Syncytin-Rum1*, and compared the mRNA levels between *Fematrin-1* and *Syncytin-Rum1* (Fig. 4). Analysis of the expression levels of *Syncytin-Rum1*, is consistent with the results obtained for *Fematrin-1*, demonstrating that the placenta obtained from cloned animals indicated higher levels of mRNA for these genes compared to the placenta of control animals (Fig. 4 a). Moreover, the placenta from cloned calves that did not survive exhibited higher levels of mRNA than the control calves (Fig. 4 b). Due to the fusogenic activity of syncytin-like genes during placentation [18], the higher expression of these genes in dead cloned calves may be related to placental abnormalities present in these animals (Fig. 2 a). Furthermore, we compared the mRNA levels between *Fematrin-1* and *Syncytin-Rum1*, and no significant differences were found (Fig. 4 c). The presence of the transcripts of both genes in placenta may be supported by the baton-pass hypothesis. Baton-pass hypothesis, which was proposed by Nakamura and Imakawa [52] comprises multiple successive ERV variants which gradually replace the cell-fusion role of a pre-existing gene during endogenization, thereby resulting in increased trophoblast cell fusion, morphological variations in placental structures, and enhanced reproductive success in placental mammals [19]. This suggests that *Syncytin-Rum1* contributed, in an evolutionary sense, to the appearance of synepitheliochorial placenta and has both fusogenic [18] and immunosuppressive activity to subdue maternal immunity for avoiding fetal rejection [53]. Nevertheless, the fusogenic activity of *Syncytin-Rum1* might have been attenuated during evolution after the acquisition of *Fematrin-1* [54], and according to the evidence demonstrated in the previous study conducted by Nakaya et al. [55], *Fematrin*-1 might play a pivotal role in the formation of fetomaternal hybrid cells in present bovine species.

Additionally, the fold change analysis revealed that syncytin-like genes were upregulated in the placenta of cloned animals during calving (Fig. 4 d). However, at the time of calving, these genes should typically be downregulated, since in physiological conditions, a gradual reduction occurs in the expression of the retroviral envelope proteins throughout gestation [18,55]. Therefore, these data suggest that there may be a deregulation in the expressions of *Fematrin-1* and *Syncytin--Rum1* during nuclear transfer cloning; however, there was an exception, the animal II presented a different behavior compared to the other animals (Fig. 4 d). Interestingly, this cloned calf survived the first week, but it died before the first three months of life (Fig. 2 a), more precisely in the second week due to cardiac abnormalities that may have been a result of the cloning procedure [56].

Placenta-specific expression of the endogenous retroviral envelope genes such as *Syncytin-Rum1* and *Fematrin-1*, are subject to a dynamic modulation by the epigenetic machinery, such as DNMTs and TETs, during the progression of gestation [57]. *DNMT1* and *DNMT3A* exhibited lower levels, while *DNMT3B* demonstrated higher levels of mRNA in the placenta of SCNT-produced calves compared to the placenta of control animals (Fig. 4 a). This difference in the expression of *de novo DNMT3A/B* may be related to specificity in the recruitment of these enzymes. Certain studies have reported that *3A* and *3B* isoforms are required for the proper establishment of genomic imprinting patterns [58] and methylation of satellite regions [59], respectively. The refined control mechanism of this machinery in ERVs has still not been elucidated. Therefore, despite the higher levels of mRNA of *DNMT3B* in SCNT-produced calves, it is possible to propose an association between the lower levels of *DNMT1/3A* transcripts, the hypomethylated pattern in the *Fematrin-1* promoter (Fig. 3 e) and higher levels of expression of *Fematrin-1* observed in cloned animals (Fig. 4 a). However, considering that *DNMT3B* is associated with methylation of repetitive DNA [59], the higher levels of *DNMT3B* mRNA found in the SCNT-produced calves should be investigated in further studies. Additionally, TET enzymes also participate in the process of DNA methylation reprogramming, acting on the demethylation mechanism, thus being essential for the regulation of gene expression during development [60]. In the present study, we found higher mRNA levels of *TET1* and lower mRNA levels of *TET2* in placenta from cloned calves compared to the AI controls (Fig. 4 a). Extrapolating to other species, these findings may be related to the dynamic role that these enzymes play in the regulation of transcriptional activity in the placenta, such as in mice and humans [61]. Based on our results, the higher levels of mRNA of *TET1* collectively with the lower levels of mRNA of *DNMT1* and *DNMT3A* (Fig. 4 a) may be related to the hypomethylation pattern found for *Fematrin-1* in the placenta of cloned calves (Fig. 3 e). However, further studies may be important to determine if the methylation patterns observed are due to the methylation, hydroxymethylation or a combination of both.

Thus, as demonstrated by the present study, cloned pregnancies serve as important animal models for studying the epigenetic reprogramming of placental development embryology, and consequently provide a better understanding about the assisted reproduction techniques in both animals and humans. In the present study, a healthy cloned calf originated from each donor animal, which in turn was used as a control for the other cloned calf with abnormal placental phenotype (Fig. 2 a), thus removing any genetic effect that could intervene in the DNA methylation and expression analyses.

5. Conclusion

In summary, our results suggest that this altered DNA methylation, deregulation in the expression patterns of ERVs, and in the epigenetic machinery involved in the fetal cotyledons of cloned calves may be associated with the abnormal placentogenesis found in some SCNT-produced animals. Further studies characterizing other mechanisms involved in the regulation of ERVs, as the retrotransposon transcriptional repression complex, are also important to improve our understanding about the role of ERVs in cloning. A better understanding of the function of these ERVs in placentogenesis can support new strategies to improve the efficiency of present SCNT procedures.

Authors' contributions

Henrique Xavier Salgado Bayão, Rodolfo Rumpf, Márcia Marques Silveira, and Naiara Araújo Borges: sample collection. Maurício Machaim Franco, Márcia Marques Silveira, Luna Nascimento Vargas, and Alexandre Rodrigues Caetano: performed genomic analyses. Márcia Marques Silveira, Luna Nascimento Vargas, and Maurício Machaim Franco: designed the experiment, interpreted the results, and wrote the manuscript. All authors read and approved the final manuscript.

Conflicts of interest

None.

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Appendix A. Supplementary data

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