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Dynamic methylation pattern of H19DMR and KvDMR1 in bovine oocytes and preimplantation embryos

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Abstract

Purpose This study aimed to evaluate the epigenetic reprogramming of ICR1 (KvDMR1) and ICR2 (H19DMR) and expression of genes controlled by them as well as those involved in methylation, demethylation, and pluripotency.

Methods We collected germinal vesicle (GV) and metaphase II (MII) oocytes, and preimplantation embryos at five stages [zygote, 4–8 cells, 8–16 cells, morula, and expanded blastocysts (ExB)]. DNA methylation was assessed by BiSeq, and the gene expression was evaluated using qPCR.

Results H19DMR showed an increased DNA methylation from GV to MII oocytes (68.04% and 98.05%, respectively), decreasing in zygotes (85.83%) until morula (61.65%), and ExB (63.63%). *H19* and *IGF2* showed increased expression in zygotes, which decreased in further stages. KvDMR1 was hypermethylated in both GV (71.82%) and MII (69.43%) and in zygotes (73.70%) up to morula (77.84%), with a loss of methylation at the ExB (36.64%). The zygote had higher expression of most genes, except for *CDKN1C* and *PHLDA2*, which were highly expressed in MII and GV oocytes, respectively. *DNMTs* showed increased expression in oocytes, followed by a reduction in the earliest stages of embryo development. *TET1* was downregulated until 4–8-cell and upregulated in 8–16-cell embryos. *TET2* and *TET3* showed higher expression in oocytes, and a downregulation in MII oocytes and 4–8-cell embryo.

Conclusion We highlighted the heterogeneity in the DNA methylation of H19DMR and KvDMR1 and a dynamic expression pattern of genes controlled by them. The expression of *DNMTs* and *TETs* genes was also dynamic owing to epigenetic reprogramming.

Keywords Epigenetic reprogramming · ICR1 and ICR2 · DNA methylation · Gene expression · In vitro produced embryos

Introduction

Assisted reproductive technologies (ARTs) are an often used strategy by infertile couples to achieve pregnancy [1]. In contrast to humans, when used in domestic animals, such as bovines, ARTs are helpful in improving embryo production rate per year, genetic gain, and genetic and phenotypic selection [2, 3]. The in vitro production (IVP) of bovine embryos is the primary biotechnological tool used in both commercial and research laboratories. More than 350 thousand embryos were produced in vitro in 2020 for commercial purpose [4]. Additionally, IVP of bovine embryos is a useful strategy for better understanding of gametogenesis and early embryonic development, including those studies related to animal models of human relevance [5–7]. During IVP of embryos, oocytes undergo in vitro maturation until metaphase II (MII) to achieve nuclear and cytoplasmic competence for

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fertilization and support the initial cleavages during early embryo development prior to embryonic genome activation [8, 9]. Oocyte growth and maturation are accompanied by epigenetic reprogramming, although genome-wide remethylation and imprinting stablishment affect oocyte competence and fertilization, and embryo development [10, 11].

As the embryo develops, the undifferentiated zygote undergoes differentiation to become a blastocyst, with two cell lineage, an inner cell mass (ICM) and a trophectoderm (TE) [12]. In this moment, genes as *NANOG*, *OCT4*, and *SOX2*, are essential to the proper cell differentiation and embryo pluripotency maintenance [13–15]. During the embryo in vitro culture, an epigenetic reprogramming occurs, in which, the genome loses its epigenetic marks brought by gametes due to a DNA demethylation wave [16, 17]. The DNA demethylation is mainly active in the maternal and mainly passive in the paternal genome [18, 19]. The ten-eleven translocation (TET) enzymes are primarily responsible for the active wave of DNA demethylation [20, 21], whereas the exclusion of nuclear DNA methyltransferase 1 (*DNMT1*) is the main mechanism associated with passive DNA demethylation [22, 23]. After genome DNA demethylation, de novo DNA methylation and remethylation are carried out by DNMT enzymes family [18, 19]. At this developmental stage, epigenetic reprogramming occurs throughout the genome except for the genes controlled by genomic imprinting [24].

Genomic imprinting is an important epigenetic mechanism that regulates gene expression, and consequently, embryo development [25]. In general, imprinted genes present an expression pattern based on their parental origin; maternal or paternal imprinted genes are found in clusters and may be regulated by imprinting control regions (ICRs) [17, 24, 26]. Additionally, at least one differentially methylated region (DMR) is found surrounding the ICRs [27]. Modification of epigenetic mechanisms, such as DNA methylation, can imbalance the expression of genes located at these ICRs and lead to the development of syndromes [25, 28].

On the telomeric region of bovine chromosome 29, two ICRs, namely ICR1 and ICR2, seem to be related to developmental disorders such as the large offspring syndrome (LOS) [29–31]. H19DMR is found in the ICR1, and it harbors and regulates the expression of *H19* and *IGF2* genes. The maternal chromosome transcribes *H19* but does not express *IGF2*, and the opposite occurs in the paternal chromosome. This is possible due to a complex imprinting model involving CTCF-binding protein and enhancer competition model [32–34]. In ICR2, KvDMR1 is located in intron 10 of *KCNQ1* and in the promoter region of its antisense *KCNQ1OT1* [35, 36]. Besides *KCNQ1* and *KCNQ1OT1*, several other genes, including *CDNQ1C*, *PHLDA2*, and *SLC18A22*, are influenced by the KvDMR1 [37–39].

Previous studies have shown a relationship between ARTs and aberrant epigenetic reprogramming of gene expression during embryo development in cattle [30, 40, 41] as well as humans [42–44]. Loss of imprinting due to alterations in DNA methylation and misregulation of imprinted genes have been cited as frequent causes underlying LOS in cattle and Beckwith–Wiedemann syndrome (BWS) in humans [43]. The present study aimed to evaluate the DNA methylation patterns of H19DMR and KvDMR1 as well as the expression of imprinted genes from ICR1 and ICR2 during bovine oocyte maturation and early embryo development; we also evaluated the expression patterns of important genes associated with epigenetic reprogramming (*DNMTs* and *TETs*) and pluripotency. In this study, we show the DNA methylation of H19DMR and KvDMR1 and a dynamic expression pattern of the genes controlled by them, besides a global epigenetic reprogramming using *DNMTs*, *TETs*, and pluripotency genes in bovine oocytes and embryos.

Material and methods

Ethics statement

The project was approved by the Ribeirao Preto Medical School Animal Ethics Committee (CEUA-FMRP no. 004/2019–1).

In vitro production of bovine embryos

In vitro maturation of oocytes

A total of 747 bovine ovaries were collected from a slaughterhouse located near Ribeirao Preto-SP (Brazil). The follicles ranging from 2 to 8 mm were aspirated using an 18-gauge needle connected to a 10-mL syringe [9]. A total of 3960 grade I cumulus-oocyte complexes (COCs) were selected as previously described [45]. The GV oocytes destined for DNA methylation and gene expression analysis were denuded using hyaluronidase solution (4 µg/mL). The denuded oocytes were pooled ($n = 40$) in 10 µL of phosphate-buffered saline solution (PBS), immersed in liquid nitrogen, and stored at − 80 °C for subsequent DNA methylation and gene expression analysis (Figure S1).

Oocytes were in vitro matured in microdrops (20 COCs/microdrop) of in vitro maturation (IVM) medium [TCM199 with Earle's salts, glutamine, $NaHCO_3$ pyruvate (22 µg/mL), 10% FBS, FSH (0.5 mg/mL), LH (50 µg/mL), amikacin (83 µg/mL), and estradiol (1.0 µg/mL)], for 22–24 h in maximum humidity, at 38.8 °C and 5% CO_2. After IVM, MII oocytes were used for in vitro fertilization or molecular analysis. Those destined for DNA methylation and gene expression analysis were denuded using hyaluronidase

solution (4 µg/mL). The denuded oocytes were pooled ($n = 40$) in 10 µL of PBS, immersed in liquid nitrogen, and stored at − 80 °C for subsequent DNA methylation and gene expression analysis (Figure S1).

In vitro fertilization

Semen samples from a single bull and from the same batch were used for each experiment. Semen was centrifuged ($342 \times g$) using a Percoll (Sigma-Aldrich, USA) gradient (45:90) for 30 min. After centrifugation, the pellet was analyzed for sperm concentration and motility. Matured COCs were placed with spermatozoids (20 COCs/microdrop) in in vitro fertilization (IVF) medium [TALP supplemented with heparin (10 mg/mL), pyruvate (22 µg/mL), BSA FAF (Fatty acid free) (6 µg/mL), PHE solution (2 µM of penicillamine, 1 µM of hypotaurine, and 0.25 µM of epinephrine), and amikacin (83 µg/mL)]. Each drop contained approximately two million sperm per milliliter (2×10^6 sperms/mL). Oocytes and sperms were left together in the IVF medium for 18 h in maximum humidity at 38.8 °C and 5% CO_2.

In vitro culture of embryos

At the end of the fertilization period, presumptive zygotes (single-cell embryos; D0) were transferred to CR2 culture medium [46] (10 embryos/microdrop) with modifications [47]. The embryos were maintained in maximum humidity at 38.8 °C and 5% CO_2 for 7 days for preimplantation embryonic development. The cleavage rate was measured 48 h after IVF. On day 4 (D4), the embryos were evaluated and those considered degenerated were discarded; a part of the in vitro culture medium was replaced with fresh medium. On day 7 of development (D7), embryos were classified according to the International Embryo Transfer Society (IETS) as initial blastocysts, blastocysts, expanded blastocysts (ExB), and hatched blastocysts according to blastocoel size, inner cell mass (ICM) position, and trophectoderm (TE) formation. Embryo production was recorded based on viable embryos on D7 in relation to the zygotes cultured on D0. The collection period was based on the method described by Hafez and Hafez [48], and pools were collected as follows: zygotes ($n = 40$), 4–8 cells ($n = 30$), 8–16 cells ($n = 30$), morula ($n = 20$), and ExB ($n = 10$) (Figure S1). Samples were pooled in 10 µL of PBS, immersed in liquid nitrogen and stored at − 80 °C for subsequent DNA methylation and gene expression analysis.

Analysis of gene expression using real-time quantitative PCR (qPCR)

RNA was extracted using the PureLink™ RNA Mini Kit (ThermoFisher Scientific, Waltham, MA, USA) following the manufacturer's protocol. Total RNA was quantified using a NanoDrop 2000 spectrophotometer (ThermoFisher Scientific, Waltham, MA, USA). The complementary DNA (cDNA) synthesis was performed using the SuperScript® IV First-strand Synthesis System Kit (ThermoFisher Scientific, Waltham, MA, USA) using oligo dT and random hexamer primers (70/30 proportion), following the manufacturer's instructions.

Gene expression analysis was performed using a StepOne Real-Time PCR System (Applied Biosystems, USA) containing SYBR® Green PCR Master Mix, 2 pmol of each primer, and 2 µL of 1:2 diluted cDNA in a final volume of 10 µL. The amplification conditions were as follows: 95 °C for 10 min, followed by 45 cycles of 95 °C for 15 s and 65 °C for 1 min. The reactions were performed and analyzed in triplicates, and only those with a standard deviation > 0.3 were included. Negative controls were used to detect any contamination. The primer sequences for *IGF2*, *KCNQ1OT1*, and the reference gene *GAPDH* were as described by Verruma et al. [49]. The primer sequences for *H19*, *PHLDA2*, *CDKN1C*, *KCNQ1*, *DNMT1*, *DNMT3a*, *DNMT3b*, *TET1*, *TET2*, *TET3*, *OCT4*, and *NANOG* genes were designed using the Primer 3Plus software based on bovine genome reference (assembly ARS-UCD1.3) obtained using NCBI database (ncbi.nlm.nih.gov). *ACTB* reference gene primer sequence was previously described by Rios et al. [50]. The primer sequences are listed in supplementary table S1. Primer efficiencies were obtained using linear regression (5 points, 1:2 dilution range), and those with efficiencies between 90 and 105% were considered suitable for gene expression analysis, following the manufacturer's recommendations. Relative gene expression was evaluated using the method described by Pfaffl [51].

Genomic DNA isolation and bisulfite modification

Genomic DNA was extracted using lysis protocol with 2 µL Proteinase K (20 ng/µL) (Invitrogen, USA) and 18 µL Tris HCl (10 mM) for 1 h at 55 °C followed by 10 min at 95 °C for enzyme inactivation. DNA concentration and integrity were evaluated using a Nanodrop 2000 spectrophotometer (ThermoFisher Scientific, Waltham, MA, USA). Bisulfite conversion of DNA was carried out using the EpiTect Bisulfite Kit (Qiagen, Hilden, Germany) following the manufacturer's protocol. The DNA was stored at − 20 °C until further use.

The CpG islands at H19DMR and KvDMR1 as well as the primer sequences were predicted using the MethPrimer software [52]; sequences are listed in Supplementary Table S1. The amplification was carried out using a Bio-Rad T100 Thermal Cycler (Bio-Rad Laboratories, USA); each reaction tube contained 10 × PCR buffer II, $MgCl_2$ (25 mM), 5 mM of dNTPs (10 mM/µL), 5 pmol of each primer (10 pmol/µL), 0.04 IU of AmpliTaq Gold® DNA Polymerase (5 U/µL; ThermoFisher Scientific, Waltham,

MA, USA), 3 µL of bissulfite converted DNA, and ultrapure water in a final volume of 25 µL. Amplification conditions were as follows: 5 min at 95 °C, followed by 50 cycles of 45 s at 94 °C, 45 s at 59 °C, and 45 s at 72 °C. The final extension step was performed for 10 min at 72 °C. The PCR products were stored at − 20 °C until next generation sequencing.

DNA methylation analysis using BisSeq

Targeted bisulfite sequencing was performed using the MiSeq (Illumina, USA) platform, covering 10 CpG sites in H19DMR (chr29: 51,168,375–51,168,468) and 18 CpG sites in KvDMR1 (chr29:50,580,053–50.580,210), as shown on Fig. 1. PCR products containing the adapters were barcoded using the Illumina Nextera XT library preparation kit, and sequencing was performed using the 600 bp V3 reagents kit according to the manufacturer's instructions. The FASTQ files for individual samples were generated using the Illumina pipeline (bcl2fastq2-v2-20). Trimmomatic v0.38.124 [53] was used to remove the adapters and indices from the sequences. The paired read sequences were merged using the default settings of FLASH v1.2.11.425 [54] and aligned to the bisulfite-converted genome using the Bismark v0.18.2 [55] with setting–ambig_bam26, which was also used to count the reads with different DNA methylation patterns. Methylated CpGs were visualized using the UCSC Genome Browser.

Statistical analysis

The statistical analysis was performed using the RStudio software (v. 1.4.1717). The data distribution was estimated using the Shapiro–Wilk test. The expression profiles of each

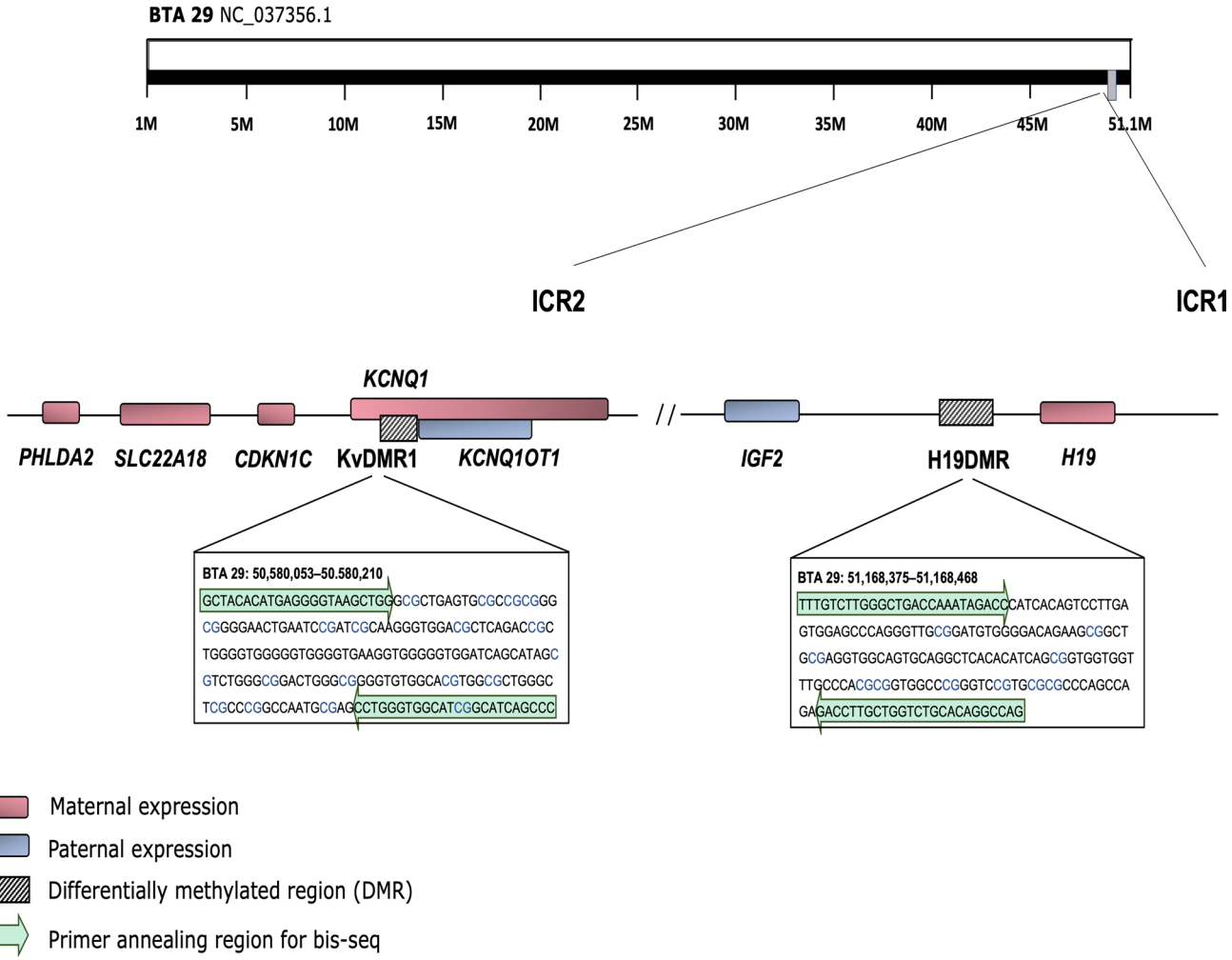

Fig. 1 Schematic representation of telomeric region of BTA29, highlighting the H19DMR (in the ICR1) and KvDMR1 (in the ICR2) sequenced regions and the genes controlled by theses ICRs. Genes with paternal expression (maternal imprinting) are represented in blue (*IGF2,* in the ICR1, and *KCNQ1OT1,* in the ICR2) and maternal expression (paternal imprinting) are represented in pink (*H19,* in the ICR1, and *CDKN1C*, *KCNQ1*, and *PHLDA2,* in the ICR2)

gene during the seven stages were compared using ANOVA followed by Tukey's post hoc test, with a level of significance set at 5% ($p < 0.05$).

Results

Thirteen replicates were performed to collect all samples during oocyte maturation and embryo preimplantation development. A total of 2478 zygotes were initially cultured in vitro. We observed a cleavage rate of 86.50% ± 5.0% and embryo production at D7 of 36.26% ± 10.7%. Preimplantation developmental stages were selected according to the method described by Hafez and Hafez [42] during oocyte maturation (GV and MII) and embryo preimplantation development before (zygotes and 4-8 cells embryos), during (8-16 cells embryos), and after (morula and ExB) bovine genome activation, as well as during the embryo differentiation into two cell lineages, the ICM and TE.

H19DMR and KvDMR1 methylation pattern and gene expression

In ICR1, the H19DMR controls the *H19* and *IGF2* genes. Ten CpGs were analyzed in H19DMR. H19DMR methylation increased during oocyte maturation (from 68.04% in GV to 98.05% in MII oocytes) and remained higher in zygote (85.83%) and 4–8 cells embryo (83.23%). Subsequently, DNA methylation at H19DMR decreased in 8–16 cells (69.15%) and morula (61.65%), while maintaining its DNA methylation level in ExB (63.63%) as observed in Fig. 2A.

There are no changes in *H19* and *IGF2* expression during oocyte maturation. (Fig. 2B and C). After oocyte maturation, both *H19* ($F_{(6)} = 41.89$; $p < 0.01$) and *IGF2* ($F_{(6)} = 44.2$; $p < 0.01$) showed an upregulation in the zygote stage, the highest expression. After upregulation in the zygotic stage, both genes were downregulated at 4–8 cells stages, thereafter, maintaining low levels with no significant difference in the expression until the blastocyst stage. Notably, the expression of *H19* was detected at all evaluated stages; however, the *IGF2* expression was not detected in the ExB stage (Fig. 2B and C).

In ICR2, the KvDMR1 controls a higher number of genes when compared to the H19DMR. A total of 18 CpGs were analyzed in the KvDMR1 (Fig. 3A). The KvDMR1 methylation remained stable between GV (71.82%) and MII oocytes (69.43%). During embryo development, the hypermethylation status was maintained between zygote and morula stage (~ 74.44%). Between morula and ExB, it was observed a reduction in the KvDMR1 methylation and ExB was hypomethylated (36.64%) (Fig. 3A).

Several genes are influenced by the KvDMR1 methylation pattern, including *CDKN1C*, *KCNQ1*, *KCNQ1OT1*, and *PHLDA2* (Fig. 3B, C, D, and E, respectively). Similar to *IGF2* and *H19*, zygote was the stage with higher expression in the *KCNQ1* ($F_{(6)} = 5.96$; $p < 0.01$) and *KCNQ1OT1* ($F_{(6)} = 20.04$; $p < 0.01$) (Fig. 3C and D). *CDKN1C* showed a major expression in MII oocytes ($F_{(6)} = 14.95$; $p < 0.01$), whereas GV was the higher expression in *PHLDA2* ($F_{(6)} = 44.2$; $p < 0.01$), as shown in the Fig. 3B and E. All genes, except *CDKN1C*, were downregulated in the 4–8 cells. *CDKN1C*, *KCNQ1OT1*, and *PHLDA2* were detected at all the evaluated stages. *KCNQ1* showed the lowest expression among all genes of ICR2; its expression was not detected during GV oocyte, morula, and ExB stages.

Expression of DNA methylation, demethylation, and pluripotency-related genes

The expression of *DNMTs* (*DNMT1*, *DNMT3a*, and *DNMT3b*) and *TETs* (*TET1*, *TET2*, and *TET3*) was detected at all evaluated stages. In the *DNMT* family, the MII oocyte showed the highest expression, followed by the GV stage (Fig. 4A, B, and C). Downregulation was observed between oocyte maturation and zygote for *DNMT1* ($F_{(6)} = 84.43$; $p < 0.01$) and *DNMT3b* ($F_{(6)} = 153.2$; $p < 0.01$), and between MII and 4–8 cells for *DNMT3a* ($F_{(6)} = 63.22$; $p < 0.01$). Although in different proportions, the expression of *DNMT3a* and *DNMT3b* presented a similar pattern during oocyte maturation and preimplantation embryo development (Fig. 4B and C).

In the *TET* family, *TET1* ($F_{(6)} = 128.8$; $p < 0.01$) showed the highest expression in the morula stage (Fig. 4D), whereas *TET2* ($F_{(6)} = 22.53$; $p < 0.01$) and *TET3* ($F_{(6)} = 73.99$; $p < 0.01$) showed the highest expression in the GV stage among all the studied stages (Fig. 4E and F). *TET1* gene maintained a low expression until the 4–8 cells stage, and then, it was upregulated until the morula stage, followed by a downregulation until the ExB stage. *TET2* and *TET3* presented similar expression patterns during oocyte maturation and early embryonic development, with a downregulation between GV oocyte and 4–8 cells, and then remaining relatively low until the ExB stage (Fig. 4E and F).

The expression of *NANOG* gene was not detected in oocytes (GV and MII) and zygote. Its expression was detected only from the 4–8 cells onwards. From 4-8 cells until morula stage, its expression was upregulated ($F_{(6)} = 11.8$; $p < 0.01$), followed by a significant downregulation in the ExB stage (Fig. 4G). Different of *NANOG*, the expression of *OCT4* was detected in oocytes and embryos. The *OCT4* showed two moments of upregulation ($F_{(6)} = 115.9$; $p < 0.01$), first during oocyte maturation and the second during the morula stage. After upregulation in the morula stage, a downregulation was observed in the ExB stage (Fig. 4H).

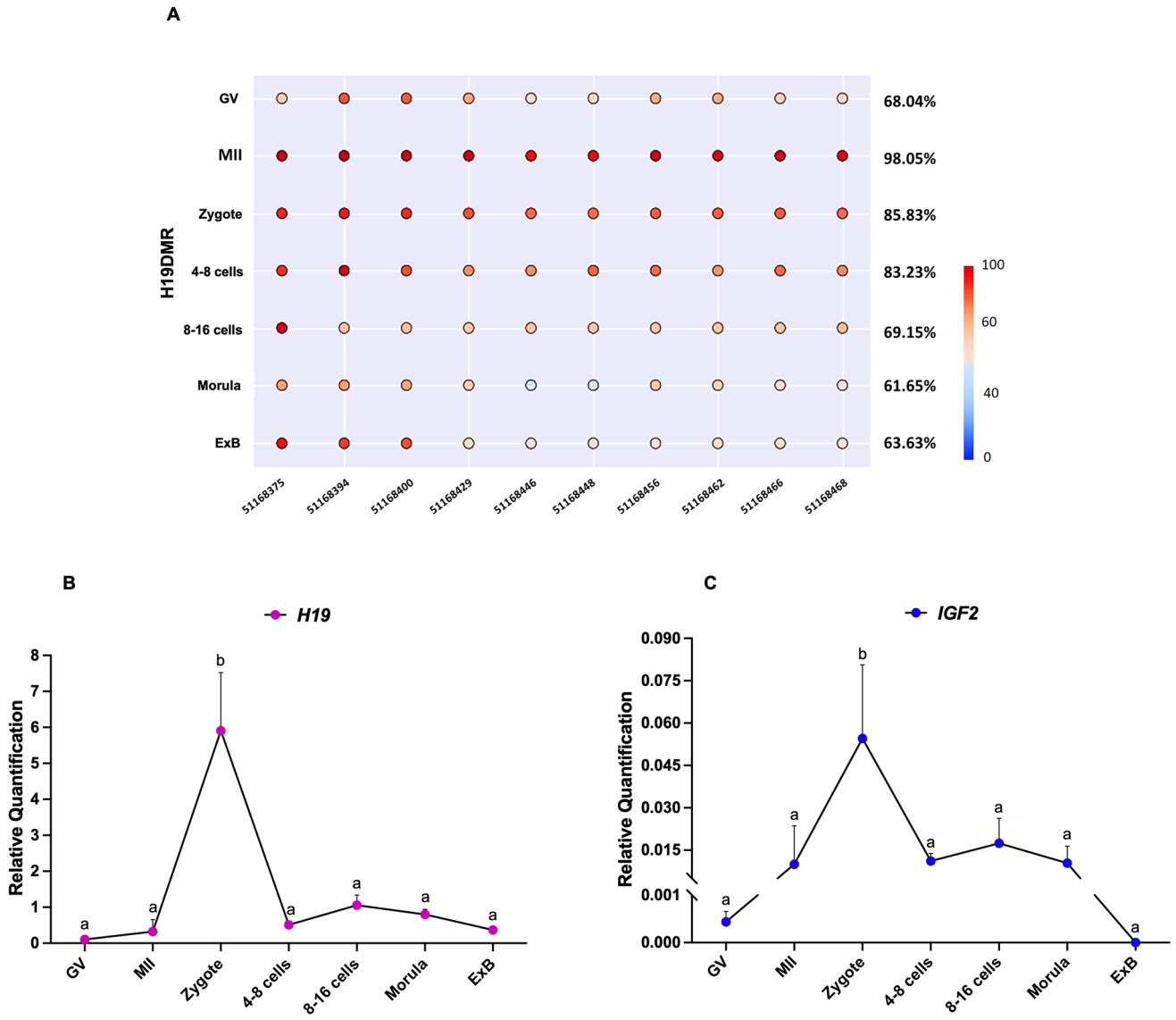

Fig. 2 ICR1 analysis during oocyte maturation and embryo preimplantation development. **A** H19DMR methylation pattern, measure in percentage that alters between hypomethylation (blue) to hypermethylation (red). **B** and **C** *H19* and *IGF2* gene expression, respectively, where pink marks represent gene that present maternal monoallelic expression (paternal imprinting) and blue marks represent gene that present paternal monoallelic expression (maternal imprinting). Different letters in different developmental stages indicate statistical difference ($p < 0.05$)

Discussion

The early embryo development is crucial for pregnancy success. The IVP of bovine embryos is a useful tool for obtaining biological material for research related to gametogenesis and early embryo development. Imprinted genes are essential during this period, and in our study, their expression pattern changed during oocyte maturation and early embryo development. In addition, genes related to DNA methylation and demethylation also showed a dynamic expression. Epigenetic processes, including genomic imprinting, are fundamental to fetal and placental development, and some imprinted genes may be embryo, tissue, and/or specie specific, making their analysis difficult [5, 56, 57].

In ICR1 (H19DMR), our study showed hypermethylation during oocyte maturation. The hypermethylation in MII oocytes corroborates with previous data obtained by our research group (unpublished data), as well as in recent data published by Vargas et al. [58]. However, the literature describes an hypomethylation status in mammal MII oocytes [59–61]. Contradictory data are frequently found when DNA methylation and gene expression patterns are compared between in vitro and in vivo oocytes and embryos [62, 63]. The hypermethylation at H19DMR found in our results may

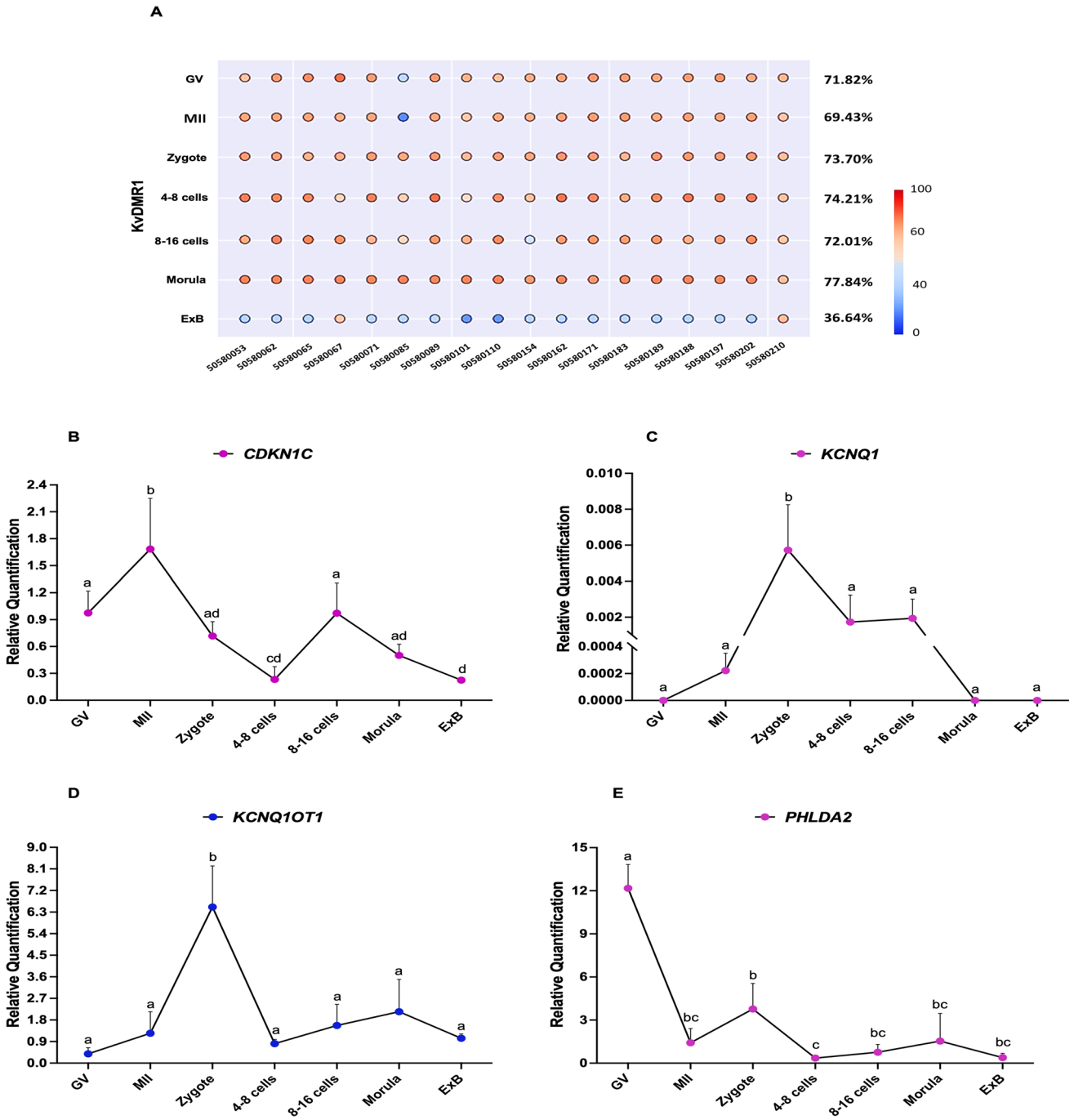

Fig. 3 ICR2 analysis during oocyte maturation and embryo preimplantation development. **A** KvDMR1 methylation pattern, measure in percentage that alters between hypomethylation (blue) to hypermethylation (red). **B**, **C**, **D**, and **E** *CDKN1C*, *KCNQ1*, *KCNQ1OT1*, and *PHLDA2* gene expression, respectively, where pink marks represent genes that present maternal monoallelic expression (paternal imprinting) and blue marks represent gene that present paternal monoallelic expression (maternal imprinting). Different letters in different developmental stages indicate statistical difference ($p < 0.05$)

be related to some factors, such as the region selected for sequencing. Al-Khtib et al. [64] observed that human MII oocytes were generally unmethylated in the H19DMR after IVM; however, some CpG islands in this region showed a higher methylation status. In addition, the in vitro maturation environment should also be considered. It is known that the in vitro manipulation could influence the DNA methylation pattern [65, 66].

A reduction in the H19DMR methylation status was observed between 4–8 and 8–16 cells, maintained stable

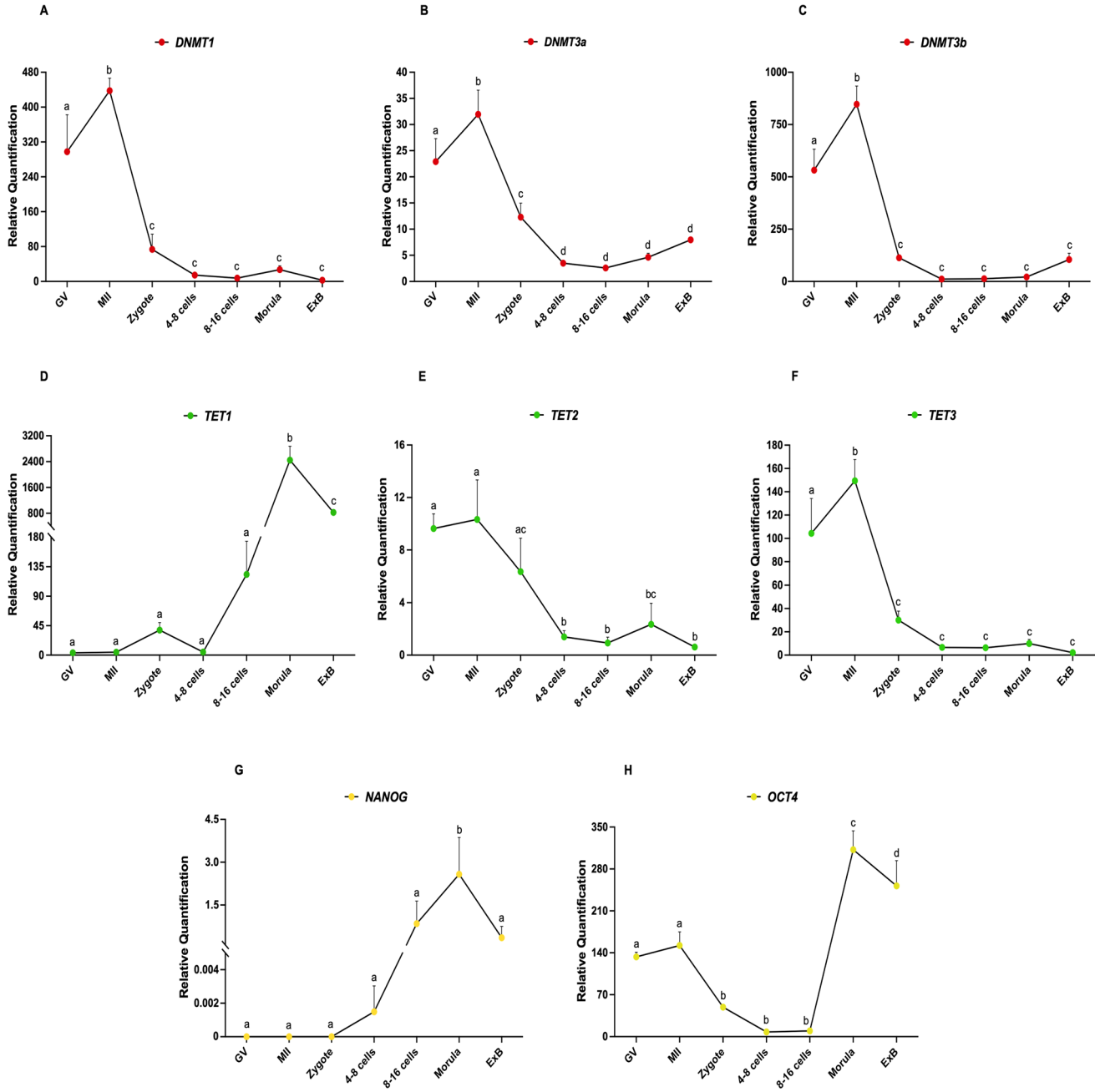

Fig. 4 Expression pattern of *DNMTs*, *TETs*, and pluripotency genes during oocyte maturation and embryo development. Expression pattern of **A** *DNMT1*, **B** *DNTM3a*, **C** *DNMT3b*, **D** *TET1*, **E** *TET2*, **F** *TET3*, **G** *NANOG*, and **H** *OCT4*. Different letters in different developmental stages indicate statistical difference ($p < 0.05$)

until ExB (~65%). After fertilization, the embryo undergoes to an epigenetic reprogramming that erases the epigenetic marks brought by the parental genomes [24]. However, ICRs and imprinted genes maintained its marks and, due to the parental of origin, the ICRs are usually 50% (± 10%) methylated [67, 68]. In bovine, around 8–16 cells occur the genome activation and new epigenetic marks are stablished in the embryo [19, 69].

IGF2 and *H19*, both imprinted genes, are found in the neighboring region of H19DMR. *IGF2* exhibits maternal imprinting and low monoallelic expression during early bovine development [70–73]. In the current study, the *IGF2* expression was detected at all stages, with the exception of ExB; the zygote has shown the highest expression [71–73]. In addition to its importance during early embryo development, a recent study has demonstrated its relevance in the

later stages of pregnancy, wherein fetal *IGF2* seems to be involved in placental vascularization [74]. The *H19*, which is found near *IGF2*, produces a conserved long noncoding RNA (lncRNA), the first functional lncRNA described in the literature [75, 76]. *H19* plays an oppositive role of *IGF2*, repressing embryo weight, growth, and modifications in the *H19* expression during early embryo development may lead to phenotypic alterations [73, 77–79]. Our results showed that at all evaluated stages, the expression of *H19* was higher than *IGF2*; however, both genes presented similar patterns, with higher expression in the zygote, followed by a downregulation ($p < 0.05$) and stable expression during the remainder of the preimplantation development.

In ICR2, the KvDMR1 influences a higher number of genes that modulate imprinting, including *CDKN1C*, *KCNQ1*, *KCNQ1OT1*, *PHLDA2*, and *SLC22A18* [31], and its DNA methylation pattern regulates the expression of these genes [29, 35, 80, 81]. In our study, the KvDMR1 was found to be hypermethylated during oocyte maturation. In accordance to our results in MII oocytes, the KvDMR1 is hypermethylated in oocytes [82, 83]. Contrary to the hypermethylation found in the oocytes, spermatozoa is hypomethylated in the KvDMR1 [84]. The hypermethylation status was maintained until the morula stage, with ExB being the only hypomethylated stage (36.64%). A hypomethylation status in KvDMR1 was also observed by Khoueiry et al. [85] in human embryos considered suitable for transfer.

The *KCNQ1* belongs to a large family involved in potassium channel formation, which is fundamental to biological processes, including ion exchange for cell volume maintenance [86]. In our study, this gene presented a higher expression level in the zygote. After that, a downregulation was observed between the 4–8 and 8–16-cells stages. Its expression was undetectable in morula and ExB stages. Antisense of *KCNQ1*, the *KCNQ1OT1* is paternally expressed and influences the activity of several surrounding genes. In our study, its expression was stable during oocyte maturation and embryo development, presenting an increase only in zygotes, followed by a downregulation in the next stage. *KCNQ1OT1* transcribe an lncRNA that regulates chromatin and the nucleus, influencing the expression of several genes [38, 87].

The highest variation in gene expression was observed for *CDKN1C*. Between oocyte maturation and embryo development, we observed two upregulations in this gene, first in GV oocytes and second in 8–16-cells embryos, followed by a downregulation. *CDKN1C* is a cyclin-dependent kinase complex (CDKs) controlled by lncRNAs. It regulates the cell cycle and encodes a protein (p57Kip2) whose function is largely associated with correct embryo development and pregnancy evolution [38, 88]. In bovines, Driver et al. [37] have demonstrated that silencing *CDKN1C* leads to a reduction in bovine embryo production rate. The p57Kip2 may be related to the nutrients provided to the fetus by the placenta and, when associated with other genes, such as *PHLDA2*, it plays crucial roles during early embryo development [37, 89, 90]. Contrary to the other KvDMR1 genes, the *PHLDA2* was the only gene that showed a downregulation during oocyte maturation. Similar to the results described by Jiang et al. [56], in the present study, *PHLDA2* was found to be upregulated after embryo genome activation until the morula stage, followed by a reduction of its expression in the ExB, with a relative expression close to zero.

Von Meyenn and Reik [91] have shown that epigenetic reprogramming is fundamental to mammalian development and appears to be conserved among several species. In our study, we observed alterations in the expression of *DNMT1*, *DNMT3a*, and *DNMT3b* before (GV) and after (MII) oocyte maturation and in the five evaluated periods during embryo development. These findings corroborate with previously published data [92, 93]. Oocyte growth and maturation are accompanied by increased expression of *DNMT3a* and *DNMT3b* [11]. During oocyte maturation and early embryo development, *DNMT* genes exhibit a dynamic pattern, with upregulation and downregulation depending on the developmental stage [93]. Although in different proportions, in our study, the *DNMTs* demonstrated similar behavior between GV oocytes and 4–8-cells embryos. An upregulation was observed in the *DNMTs* expression during oocyte maturation, followed by downregulation in the zygote (higher expression rate) and 4–8 cells. In bovines, the epigenetic reprogramming that occurs during early embryo development has been shown to be due to the action of *TET* genes in addition to the lower expression of *DNMT1* in a passive demethylation process [16, 94]. Reduced *DNMT1* expression was also observed in our study, with lower expression levels in 4–8 and 8–16 cells embryos, prior to genome activation. Using murine model, Uysal et al. [95] demonstrated the importance of *Dnmt1* and *Dnmt3a* during preimplantation development. Their knockdown upregulated pluripotency genes, such as *NANOG*, and increased apoptosis and reactive oxygen species levels.

Analysis of the results showed that *TET1* had higher expression rates in the later stages of embryo preimplantation development (morula and ExB), whereas for *TET2* and *TET3*, higher expression was observed during oocyte maturation (GV and MII). Bovine *TET3* is believed to be involved in maternal DNA demethylation and, in addition to the oxidation process, is capable of controlling DNA methylation levels, preventing the addition of new methyl groups during de novo DNA methylation [18, 96]. In accordance with our data, Wossidlo et al. [97] showed in bovine that *TET3* transcript is elevated in the oocytes and rapidly decreases in the early embryo development.

In the murine model, the *Tet1* seems to be related to ICM specification, besides the pluripotency maintenance,

influencing *NANOG* gene regulation, for example [98]. Our data showed that the expression of *NANOG* was detected only after 4–8 cells. In bovine embryos, this gene is not expressed during early stages and is only detected in 8-cells embryos.[99, 100]. In addition, the expression patterns observed for both *OCT4* and *NANOG* during oocyte maturation and embryo development corroborate with the literature [99, 101]. *NANOG* seems to be active by the activity of other pluripotency genes, such as the *OCT4* gene [13, 101].

Graphical analysis showed that *OCT4* was upregulated twice, first during the oocyte maturation and then during the morula (higher expression) and ExB stages and, similar pattern was described by Khan et al. [99]. Simmet et al. [13] demonstrated that embryos produced by somatic cell nuclear transfer, whose donor cell was *OCT4* knockout, presented maternal *OCT4* mRNA in the morula, demonstrating the importance of its high expression level in the oocytes. The second stage of increased expression could be related to its main function, as this gene is responsible for cell differentiation. Between the stages of morula and blastocyst occurs the blastocoel cavitation and cell differentiation into the first two lineages, ICM and TE [102, 103].

A limitation of this study was the lack of H19DMR and KvDMR1 methylation analysis in semen. However, the use of only one bull was a fixed variable to evaluate possible variation in oocytes and embryos. The main strength of our study is that we evaluated H19DMR and KvDMR1 DNA methylation, expression of genes controlled by them, and global epigenetic reprogramming using *DNMTs*, *TETs*, and pluripotency genes at multiple stages during preimplantation development of bovine embryos. Evaluating five different stages allows us to track gene expression as the embryo develops. These data could be helpful and assist to clarify some gaps in embryonic development and the influence of ARTs on these crucial stages. Furthermore, it would be interesting to compare our results with the in vivo production of bovine embryos in future experiments.

ICR1 and ICR2 are highly conserved in different species, including humans, dogs, and cattle [67], and the *DNMTs* and *TETs* are crucial for DNA methylation maintenance and epigenetic reprogramming during embryo preimplantation development. In the current study, H19DMR and KvDMR1 were hypermethylated during almost all evaluated stages, and their neighboring imprinted genes presented a dynamic expression pattern during the main embryo preimplantation development. Zygotes presented the highest expression rate compared to other embryonic stages; this could be due to maternal storage during the final oocyte maturation, which is consumed during the first cleavages [104, 105]. In addition, the expression pattern of pluripotency-related genes, owing to their increase at the beginning of cell differentiation, indicated that the embryo development was appropriate. Our findings should assist future studies on epigenetic reprogramming and the influence of ARTs on bovine embryos.

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Data Availability All data is available from the corresponding author and can be made available on request.

Declarations

Conflict of interest The authors declare no competing interests.

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