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Expression pattern of *JMJD1C* in oocytes and its impact on early embryonic development

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ABSTRACT. Cell reprogramming mediated by histone methylation and demethylation is crucial for the activation of the embryonic genome in early embryonic development. In this study, we employed quantitative real-time polymerase chain reaction (qRT-PCR) to detect mRNA levels and expression patterns of all known histone demethylases in early germinal vesicle stage and *in vitro*-matured metaphase II (MII) oocytes (which are commonly used as donor cells for nuclear transfer). On screening, the Jumonji domain containing 1C *(JMJD1C)* gene had the highest level of expression and hence was used for subsequent experiments. We also found that *JMJD1C* was primarily expressed in the nucleus and showed relatively high levels of expression at the 2-cell, 4-cell, 8-cell, 16-cell, morula, and blastocyst stages of embryos developed from MII oocytes fertilized *in vitro*. Further, we knocked down the *JMJD1C* gene in MII oocytes using siRNA

and monitored the cleavage of zygotes and development of early embryos after *in vitro* fertilization. The results showed that the zygote cleavage and blastocyst rates of the transfection group were reduced by 57.1 ± 0.07 and 50 ± 0.01% respectively, which were significantly lower than those of the negative control group ($P < 0.05$). These data suggest that *JMJD1C* plays a key role in the normal development of early bovine embryos. Our results also provide a theoretical basis for the investigation of the role and molecular mechanism of histone demethylation in the early development of bovine embryos.

Key words: JMJD1C; Demethylase; Oocyte; Early embryo; Expression pattern

INTRODUCTION

In germ cells and early embryos, the entire genome is dynamic due to genome-wide reprogramming. The discovery of histone demethylase (HDM) demonstrated that methylation is a reversible process and opened a new research area for detailed investigation of histone modifications. Recent research shows that the level of H3K9 methylation is crucial for pluripotent stem cells to stay in an intermediate state as evidenced by its role in full reprogramming of somatic cells to induced pluripotent stem cells (Chen et al., 2013). HDM proteins can be classified further into subfamilies on the basis of sequence homology and members of a subfamily can only perform the demethylation on the same lysine residues.

JMJD1C gene is one of the three members in the *JMJD1* family. JMJD1C can specifically prevent H3K9me1 and H3K9me2 methylation, and has no catalytic effect on H3K9me3 (Okada et al., 2007; Kim et al., 2010; Kim et al., 2012). Studies have shown that the promoter region of *JMJD1C* gene has POU5F1/OCT-4 binding sites. Since POU5F1/OCT-4 is the key transcription factor for stem cells to maintain pluripotency, POU5F1/OCT-4-mediated *JMJD1C* transcription might play an important role in the regulation of gene expression in undifferentiated stem cells (Chen et al., 2013). However, the function of *JMJD1C* in early bovine embryonic development remains unclear.

To further elucidate the role of HDM proteins during early embryonic development in bovines and to screen for marker HDM proteins that play important roles in this process, we have 1) analyzed mRNA levels and expression patterns of HDM family of genes at the GV stage and in MII bovine oocytes, 2) identified *JMJD1C* as the gene with highest relative expression in bovine oocytes, 3) detected the expression pattern of *JMJD1C* during early bovine embryonic development after IVF, 4) knocked down *JMJD1C* in oocytes using siRNA microinjection, and 5) measured zygote cleavage and early embryonic development after IVF. We have also presented the expression patterns of bovine HDM family members in oocytes and discussed the functions of *JMJD1C* in early embryonic development. Our analysis provides a theoretical basis for improving the procedures that are involved in somatic cell nuclear transfer.

MATERIAL AND METHODS

Materials

An oocyte maturation medium was prepared by mixing M199 medium (Gibco, Life technologies, Grand Island, NY, USA) with 10% (v/v) FBS (Hyclone, Logan, UT, USA), 1% Pyruvate

(Sigma-Aldrich, Shanghai, China), 0.5% FSH (Sigma-Aldrich, Shanghai, China), 0.1% β-estradiol (Sigma-Aldrich, Shanghai, China) and 1% penicillin/streptomycin (PAA, Cambridge, England). The medium was divided into 100 µL droplets, overlaid with mineral oil and incubated at 39°C and 5% CO_2 to reach equilibrium.

Preparation of GV and MII bovine oocytes

Fresh bovine ovaries were acquired from slaughterhouses and washed with preheated saline at 39°C containing penicillin and streptomycin. A syringe with a 20-gauge needle was used to aspirate the follicular fluid from follicles that were 3-8 mm in diameter. The cumulus-oocyte complexes (COCs) were selected under a stereoscopic microscope. Some of the COCs were treated with 0.2% hyaluronidase in an incubator for 1 min, followed by vortexing to remove cumulus cells. These GV oocytes were collected and placed in liquid nitrogen.

The remaining COCs were transferred to droplets of the maturation medium and incubated at 39°C and 5% CO_2 for 22 h. The COCs that had matured *in vitro* were retrieved and the cumulus cells were removed using hyaluronidase. MII oocytes in which the first polar body had discharged were selected under a microscope, washed, collected and placed in liquid nitrogen.

RNA extraction and gene expression analysis

RNeasy Micro Kit (QIAGEN, Hilden, Germany) was used to extract total RNA. The number of GV and MII oocytes used and the RNA concentrations are presented in Table 1.

Table1. Total RNA yield from bovine oocytes.

Stage	Number of Oocytes	Quantity (ng/µl)	$A_{260/280}$
GV	180	60	1.88
MII	180	71	1.81

PrimeScript RT reagent kit (TaKaRa, Dalian, China) was used for reverse transcription to obtain cDNAs of GV and MII oocytes. SYBR Green Real-time PCR Master Mix (TaKaRa) dye and MasterCycler RealPlex (Eppendorf, Hamburg, Germany) were used to detect the relative levels of the expression of genes related to histone demethylation at GV and MII stages. The primer sequences and length of amplified fragments of individual genes are summarized in Table 2. The formula $2^{-\Delta\Delta Ct}$ was used to calculate the relative mRNA expression levels of *JMJD1C* and the HDM family genes in both oocytes and embryos treated with different siRNAs.

In vitro fertilization of bovine oocytes

Frozen semen was thawed in a 37°C water bath for 30 s and poured into 10 mL Dulbecco's phosphate-buffered saline (D-PBS) containing 10% penicillin/streptomycin at 37°C. After gentle mixing and centrifugation, cells were resuspended in 1 mL D-PBS with 10% penicillin/streptomycin. Following another centrifugation, the cells were resuspended in 1-2 mL *in vitro* fertilization (IVF) stock solution (Table 3) containing 60 µg/mL heparin. Sperm density was determined under a

microscope to be approximately 1 x 10^6/mL. Fertilization droplets were then produced.

Mature COCs were washed with D-PBS and IVF stock solution before being transferred to the IVF droplets. Oocytes were removed after 24-26 h, washed in D-PBS, shaken for 1.5 min to remove cumulus cells, and transferred to *in vitro* culture (IVC) droplets at a concentration of 10-15 cells per droplet. The composition of IVC medium is provided in Table 4.

Table 2. Sequence of qRT-PCR primers used in this study.

Gene	Primer sequence (5'-3')	PCR fagment length (bp)
GAPDH	F-GCCATCAATGACCCCTTCAT	70
	R-TGCCGTGGGTGGAATCA	
KDM1A	F-CGCAAAGGAAACTATGTAG	261
	R-ATTATTGAGGACGTTGAAGTC	
KDM2A	F-AGCCTTGACCTCAGTTGGACC	251
	R-CGGAGCTTGCTGCGATTG	
KDM2B	F-GAGTCAGAGGGCGTGGTC	202
	R-CACACTCACTCCTCCGCTTGG	
KDM3A	F-AACATGGTGTATTGCGGGTAG	232
	R-CCTTTGACGGCTCGCTTCC	
KDM4A	F-GGCCATGACTGTGCGAGAG	312
	R-AAGTCCATGTCTTCGGTGTGC	
KDM4B	F-AATGTGTGTACTGCCGGAAAC	284
	R-CTGCGTGGTGGTGCCGATGAC	
KDM5A	F-TTCGGGAGACCTATGGCTATG	282
	R-CTTTAGCTCCTGGCGACAAC	
KDM5B	F-TTCTCCTTGCCCGACGACTG	242
	R-CTCTTCTCACTGCCGGGTCTC	
KDM5C	F-ACTTCAACATGCCCGTACAC	300
	R-AGACCATGCCCACATAGAGCC	
KDM6A	F-AGGATGCCATTAAATGCTAC	336
	R-GGTCATCTACTTGGCTAGAAG	
KDM6B	F-CACGGCGTGGACTACCTG	201
	R-CTGGTACTGATAGGCGGTGAG	
KDM7A	F-GGAGTTCATCTGTGACCCAG	305
	R-TCTCAGTTCTTTTAGGGTTTC	
PHF2	F-AAGCTGGACTCGGCGCTCTAC	349
	R-TCCCACCAGCCCCGTTCTTC	
PHF8	F-GCTCCATGGAGTCCTAAAGCC	270
	R-GGTGAGAAGCTGGGGTGTAG	
KDM8	F-CGTGCCAATTCCAGACGTGAC	228
	R-CACTCCTCGTCCGTGTACCTG	
JMJD6	F-GAGGCTGGTGGCACGTTGTCC	321
	R-TCTTCCTGCGGTGCATTG	
UTY	F-TCTGCTCCAGGATAGATCCAG	200
	R-AATGAGGGCAGTATATCTTC	
KDM4C	F-GATTGGCCATATGTCGTGAAC	239
	R-TCTCGGCTCACAATATCCTC	
KDM1D	F-AGACCAGCAGCTCCCCTACTG	258
	R-TCCACCTCCTGAACGCATCG	
JHDM1C	F-CTTTGGCTGCTGGATCCGACC	205
	R-CGGCGAACCACACCACTAAG	
CYP51A1	F-TTGAATGCCGAGGAAGTCTAC	319
	R-CCAAATCCGCATACAGTTGTG	
JARID2	F-CAATCCCAGCCGAATAGTC	206
	R-TCCTCAACATCCTCCTCGTCC	
JMJD1C	F-GGGAGATGTGTGATGCATGTG	273
	R-TGACAATGGGATTTAATACC	
JMJD4	F-ATGGAGACGTGGTTGTACCTG	243
	R-CGTCCCAGTACTCGTTGAGC	
JMJD7	F-CCTCCACTTCTACCGGGACTG	242
	R-GGCCCTCCAGGACGTCCAG	
JMJD8	F-TTGAGCACCGCCAACACCTAC	293
	R-CGCTTGCGGCCATAGATCAC	

Table 3. Composition of *in vitro* fertilization (IVF) medium.

Component	For 1000 mL
NaCl	6.66 g
KCl	0.238 g
$MgCl_2.6H_2O$	0.1 g
$CaCl_2.2H_2O$	0.294 g
$NaH_2PO_4.2H_2O$	0.062 g
$NaHCO_3$	2.09 g
Sodium pyruvate	27.5 mg
Sodium lactate	1.121 g
Glucose	2.5 g
HEPES	2.4 g
BSA	6 g
Penicillin	100 mg
Streptomycin	40 mg

Table 4. Composition of *in vitro* culture (IVC) medium.

Chemical	10 mL	100 mL	1000 mL
NaCl	0.067	0.67	6.7
KCl	0.0023	0.0231	0.231
$NaHCO_3$	0.022	0.22	2.2
L-glutamine	0.0015	0.015	0.15
PSG	5 µL	50 µL	500 µL
MEM	100 µL	1 mL	10 mL
BME	200 µL	2 mL	20 mL
Pyruvate stock	100 µL	1 mL	10 mL
L(+)-lactate	0.0055	0.055	0.55
BSA	0.03	0.3	3.0

Immunofluorescence for bovine early embryos at various stages

Early embryos at various developmental stages were collected to detect the expression levels of *JMJD1C* at each stage using immunofluorescence. The embryos were fixed in 4% paraformaldehyde at room temperature for 30 min. After a PBS wash, they were transferred to PBS with 1% Triton X-100 and incubated overnight with CO_2. The embryos were then washed with PBS, blocked using a 5% BSA (Sigma) solution for 1 h, and incubated with the anti-JMJD1C primary antibody (ab130922; Abcam, Cambridge, England) at 1:100 dilution in PBS for 1 h in a CO_2 incubator. After a PBS wash, the embryos were incubated for 2 h in a 1:100 solution of FITC-labeled secondary antibody (Boster, Pleasanton, CA, USA) in PBS in a CO_2 incubator. This was followed by treatment with DAPI (Sigma) for 5 min. After washing and mounting, JMJD1C expression was observed under a fluorescence microscope.

Design and synthesis of *JMJD1C* siRNA

The *JMJD1C* siRNAs were formulated by GenePharma (Shanghai, China). The sequences are shown in Table 5.

Oocyte microinjection

The MII oocytes were cleaned, randomly divided into two groups, and placed in IVC droplets that contained Cytochalasin B (CB). Subsequently, 1 µL each of *JMJ1C*-bos-2149 siRNA

and *JMJD1C*-bos-NC siRNA, both at 20 μM concentration, were added to the plate. A holding pipette (Beijing Zheng Tian Yi Science and Trade, Beijing, China) with an outer diameter of 100-150 μm was bent to an angle of approximately 30° to hold the oocytes and a glass capillary tube (GD-1; Narishige, Tokyo, Japan) was used as the injection needle. The needle was used to aspirate the siRNA, pierce the oocyte membrane, and inject the siRNA (10 picoliter) into the cytosol. The arm of the microinjector was adjusted to inject each of the oocytes individually.

Table 5. *JMJD1C* siRNA sequences.

Interference site	Sequence (5'-3')
JMJD1C-bos-2149	Sense strand: GCACUUAUUGGGUCAGAUATT Antisense strand: UAUCUGACCCAAUAAGUGCTT
Negative Control	Sense strand: UUCUCCGAACGUGUCACGUTT Antisense strand: ACGUGACACGUUCGGAGAATT

Statistical analysis

SPSS17.0 was used to analyze the data on zygote cleavage and blastocyst rates.

RESULTS

Gene expression of HDM family members in GV and MII bovine oocytes (*JMJD1C* screening)

qRT-PCR was employed to compare the mRNA expression levels of HDM family genes in GV and MII bovine oocytes. We found that the relative expression of *JMJD1C* was the highest in GV and MII bovine oocytes while *KDM1A, KDM1B, KDM2A, KDM3A, KDM4A, KDM5A, KDM5C, KDM6B, and KDM7* also had considerably higher levels of expression (Figure 1).

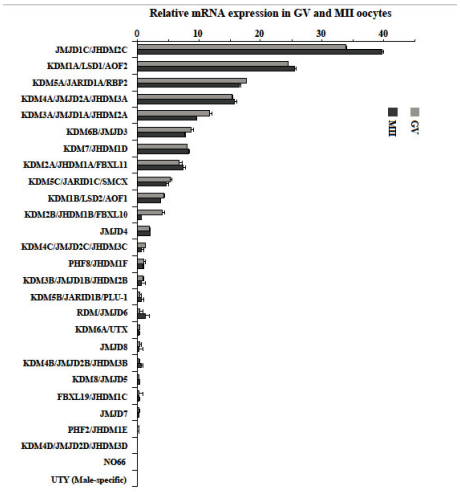

Figure 1. Expression of HDM genes in GV and MII bovine oocytes. Relative mRNA expression levels of HDM genes in GV and in MII bovine oocytes detected by qRT-PCR. GAPDH gene was used as the internal control. The values are representative of three replicates and indicated as mean ± standard error.

JMJD1C expression during early bovine embryonic development

Since *JMJD1C* showed the highest relative expression in bovine oocytes, early bovine embryos were collected at different developmental stages after IVF and analyzed for expression and localization of JMJD1C using immunofluorescence. JMJD1C was found to be expressed primarily in the nucleus at all early embryonic stages (Figure 2).

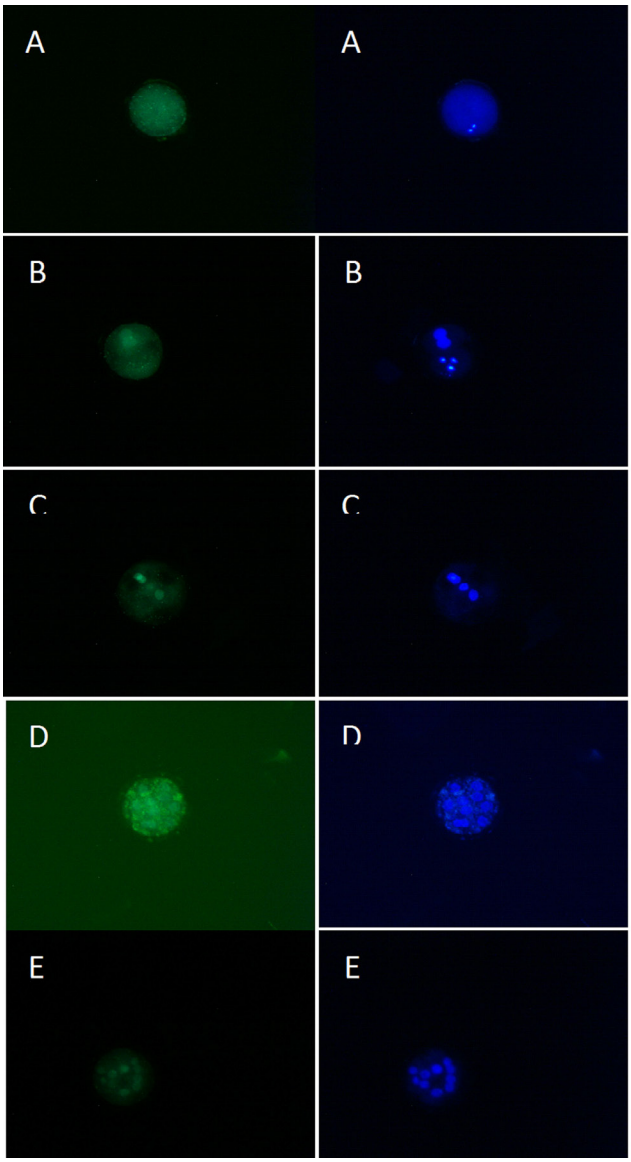

Figure 2. JMJD1C protein localization in early developmental stages of bovine embryos. (**A**) 2-cell stage, (**B**) 4-cell stage, (**C**) 8-cell stage, (**D**) morula, (**E**) blastocyst; FITC (left column) represents JMJD1C expression sites and DAPI (right column) represents DNA loci.

siRNA screening and the effect of *JMJD1C* silencing in early bovine embryos

The *JMJD1C* siRNA knockdown sites in GV oocytes and the fragments amplified by qRT-PCR primers are shown in Figure 3A. qRT-PCR detected relatively high levels of expression of *JMJD1C* mRNA even after interference by different siRNAs. Figure 3B shows that the *JMJD1C*-bos-2149 siRNA had a *JMJD1C* knockdown efficiency of approximately 80% compared to the blank control group ($P < 0.01$) and 60% compared to the negative control group ($P < 0.01$) (Figure 3B), which prompted its use in subsequent trials in zygotes.

After microinjection of siRNA, the MII oocytes were fertilized *in vitro* and the zygotic cleavage and development of early embryos were observed. The *JMJD1C*-bos-2149 siRNA-treated group showed a significant reduction in the zygote cleavage rate (57.1 ± 0.07%) and blastocyst rate (50 ± 0.01%) after IVF compared to the control siRNA-treated group. Both the results were statistically significant ($P < 0.05$) (Figure 3C).

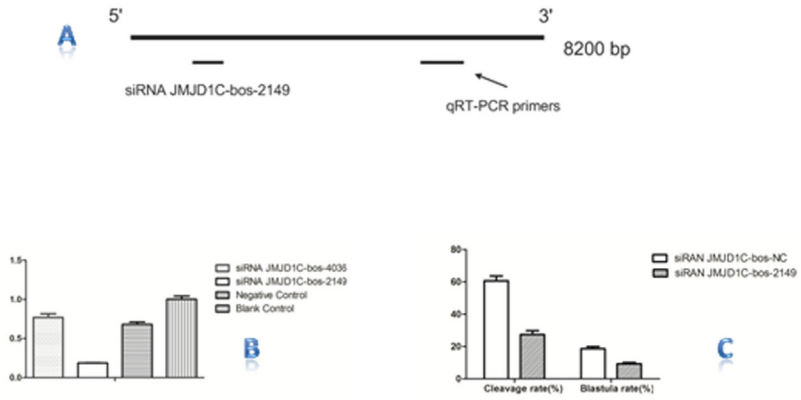

Figure 3. Effect of JMJD1C silencing on zygote cleavage and blastocyst formation in early bovine embryos. (**A**) siRNA knockdown sites and fragments amplified by qRT-PCR primers. (**B**) Effects of transfection of JMJD1C siRNAs that targeted different interference sites in bovine oocytes. The knockdown efficiency of the JMJD1C-bos-2149 siRNA was approximately 80% compared with the blank control group ($P < 0.01$) and 60% compared with the negative control (NC) group ($P < 0.01$); (**C**) Effects of siRNA on zygote cleavage rate and blastocyst rate of early bovine embryos ($P < 0.05$).

DISCUSSION

Our results show that, of all known HDM genes, the mRNA levels of *KDM1A, KDM2A, KDM3A, JMJD1C, KDM4A, KDM5A, KDM5C, KDM6B,* and KDM7 were high in both GV and *in vitro*-matured MII bovine oocytes. Canovas et al. have previously reported that the high expression of *KDM6B* (*JMJD3*) mRNA in MII oocytes affected the blastocyst rate after the initiation of zygotic cleavage (Canovas et al., 2012). Antony et al. used mouse stem cell lines to stably overexpress the H3K9 trimethylation (H3K9me3)-specific demethylase *KDM4B (JMJD2B)* and found that it could significantly increase the blastocyst rate of reconstructed embryos after nuclear transfer. However, they did not investigate the underlying molecular mechanisms further (Antony et al., 2013).

Most of the HDM genes analyzed in this study belong to the KDM family. The KDM family of proteins reduces the level of histone lysine methylation and these proteins are also known as histone lysine demethylases. At different sites, lysine has different forms of methylation, which result

in significant differences in gene regulation. There have been relatively few detailed studies on H3K9 methylation, which is an important epigenetic marker for heterochromatin. In the process of cell division, heterochromatin can be inherited stably and plays important roles in genomic stability, centromere function, silencing of repetitive DNA fragments, regulation of gene expression and cell differentiation.

All the JMJD1 family members contain the JMJC domain and are capable of specifically removing H3K9me1 and H3K9me2 methylation modifications but not H3K9me3 (Okada et al., 2007; Kim et al., 2010; Kim et al., 2012). It is now generally accepted that *JMJD1C* is the third member of the KDM3 family, i.e., KDM3C. In addition to the common JMJC domain that is shared by the three members, JMJD1C also contains a C2HC4-type zinc finger-like motif and a thyroid hormone receptor β-binding domain. JMJD1C has unique functions compared to the other two members, KDM3A and KDM3B. Studies have shown that the promoter region of *JMJD1C* gene contains binding sites for POU5F1/OCT-4, the key transcription factor in stem cells for maintaining pluripotency. Thus, POU5F1/OCT-4-mediated *JMJD1C* transcription might play an important role in the regulation of gene expression in undifferentiated stem cells (Chen et al., 2013). JMJD1A is a histone H3K9 demethylase that can interact with genes associated with embryonic stem cell pluripotency, such as Nanog, Tcl1, Tcfcp2l1, and Zfp57. *JMJD1A* is an essential regulator in the development of embryonic stem cells (Loh et al., 2007).

Kuroki et al. (2013) found that *JMJD1C* plays an important role in mouse spermatogenesis using *JMJD1C* knockout male mice model. They showed gradual reduction in germ cells at 3 months, leading to infertility and testicular fine tubule malformation and failed to produce normal primordial germ cells. *JMJD1C* is expressed at high levels in mouse spermatogonia and is essential for male germ cell development.

At the 8-cell stage of bovine zygotes, large-scale chromatin remodeling including changes in the level of DNA methylation and post-transcription chemical modification of histone tails occurs in the genomes of male and female pronuclei (Memili and First 1998). This affects the chromatin structure and gene transcriptional activities (Peterson and Laniel 2004) thus promoting zygotic genome activation (Worrad et al., 1995). Histone lysine methylation plays a key regulatory role in the cell differentiation processes and its dynamic changes are related to the transcriptional status of genes (Surani et al., 2007). Such epigenetic elements can specifically inhibit gene expression in embryonic stem cells to maintain pluripotency (Erhardt et al., 2003; Pan et al., 2007). Previous studies with *JMJD3* knocked down in bovine oocytes found that JMJD3 completed transcription and translation before the activation of the zygotic genome. Knocking down *JMJD3* down-regulated H3K27me3 and significantly decreased the early embryonic blastocyst rate (Canovas et al., 2012).

JMJD1C, KDM3A, and KDM3B are members of the KDM3 demethylase family. JMJD1C was originally identified as the thyroid hormone receptor-binding protein Trip8 (Lee et al., 1995). It also has a splice variant, s-JMJD1C, which is an auxiliary activator of androgen receptors (Wolf et al., 2007). In this study, *JMJD1C* expression was detected mainly in the nucleus at 2-cell, 4-cell, 8-cell, 16-cell, morula, and blastocyst stages of early bovine embryos after IVF. After knocking down *JMJD1C* using siRNA, the early embryo cleavage rate and the blastocyst rate were significantly decreased ($P < 0.05$). These results suggest that *JMJD1C* plays a key role in the normal development of early bovine embryos; however, the exact mechanism is unclear. DNA methylation and histone chemical modifications, particularly H3K9 methylation, interact with each other during germ cell production and spatio-temporally regulate the expression of the genome. H3K9 methylation is a sign of inhibition of gene transcription (Seki et al., 2007; Sasaki and Matsui 2008). Studies have shown that in meiosis, H3K9me1 and H3K9me2 show temporary dynamic changes and have different characteristics in male and female germ cells (Tachibana et al.,

2007). Additionally, studies have shown that in *JMJD1C* knockout male mice, the number of germ cells gradually decreases with age leading to infertility, and that JMJD1C does not affect H3K9 methylation level of the whole genome but only at specific sites and during specific stages (Kuroki et al., 2013). Therefore, our future research focus will be on the specific functions of JMJD1C in early embryonic development. and the effects of microRNAs regulated by JMJD1C on oocytes.

Conflicts of interest

The authors declare no conflict of interest.

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