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Effect of DNMT inhibitor on bovine parthenogenetic embryo development

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ABSTRACT

DNA methylation catalyzed by DNA methyltransferase (DNMT) family plays an important role during mammal preimplanted embryo development. However, the effects of RG108, a DNMT inhibitor (DNMTi), on DNMT in the development of bovine preimplanted embryos are not fully elucidated. In this study, we investigated the role of RG108 on the development, dynamics of gene-specific DNA methylation and transcription of bovine parthenogenetic preimplantation embryos. We found that *Dnmt1* and *Dnmt3b* showed highly transcription in parthenogenetic 2-cell embryos, and then the transcription levels decreased during the following development stages, whereas *Dnmt3a* was always maintained at a lower transcription level during bovine parthenogenetic preimplantation embryo development. Treatment with RG108 blocked the development of bovine parthenogenetic preimplantation embryos and induced hypomethylation in the embryos. RG108 decreased the methylation level of the *Nanog* gene promoter region, which caused activation of the *Nanog* gene in 8-cell embryos and increased the transcription level. RG108 also induced the hypomethylation of the repeat elements (satellite I and α-satellite), which may cause genome instability, increasing the number of apoptotic cells in the blastocysts and also the transcription level of the apoptotic gene *Bax*. These results indicate that RG108, a DNMT inhibitor (DNMTi), inhibits the development of bovine parthenogenetic preimplantation embryos, suggesting that the DNMT is necessary for bovine parthenogenetic preimplanatation embryo development.

1. Introduction

DNA methylation plays critical roles in the transcriptional silencing of genes and retrotransposons, gene imprinting and X chromosome inactivation [1]. The DNMT family includes three main members, DNMT1, DNMT3a and DNMT3b. DNMT1 is largely responsible for maintaining methylation patterns through DNA replication, whereas both DNMT3a and DNMT3b are *de novo* methyltransferases [2], and act to transfer methyl groups to previously unmethylated CpG dinucleotides within the genome [3]. In mice, the paternal genome is actively demethylated within 6–8 h post fertilization, before the onset of DNA replication, whereas the maternal genome is gradually demethylated until the blastocyst stage [4]. However, recent studies found that both maternal and paternal genomes undergo widespread active and passive demethylation in zygotes before the first mitotic division in mice [5].

The typical pattern of dynamic change in DNA methylation in early mammalian embryos has been demonstrated with anti-5-methylcytosine (5 mC) immunofluorescence staining [6], although the majority of 5 mC immunofluorescence signals are predicted to correspond to multiple-copy repetitive regions [7]. A regulatory and genomic locus-specific DNA methylation reprogramming pattern during mammalian preimplantation

Abbreviations: DNMT, DNA methyltransferase; DNMTi, DNA methyltransferase inhibitor; P2, parthenogenetic 2-cell embryos; P4, parthenogenetic 4-cell embryos; P8, parthenogenetic 8-cell embryos; PB, parthenogenetic blastocyst embryos; RG-8, RG108 (200 μM) parthenogenetic 8-cell embryos; RG-PB, RG108 (200 μM) parthenogenetic blastocyst embryos; BL, blastocyst embryos.

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development has been detected using genome-wide DNA methylation studies [8–11]. Accordingly, some differentially methylated regions (DMRs) at imprinted loci are resistant to this wave of active paternal and passive maternal DNA demethylation in the zygote and early preimplantation embryos [12]. Similarly, some repeat sequences, such as intracisternal A particle (IAPs) elements, are also exempted from complete DNA demethylation [13]. In addition, a number of promoter regions in non-imprinted genes also escape the global DNA methylation reprogramming in mouse preimplantation embryos [9].

RG108, a novel DNMT inhibitor, lacks cytotoxic or genotoxic effects compared to five other DNMT inhibitors, 5-aza-CR, 5-aza-CdR, zebularine, procaine and epigallocatechin-3-gallate, in human cell lines [14,15]. In mice, cloned embryos treated with 500 μM RG108 from the 2-cell to morula/blastocyst stages, show higher *POU5F1* expression and increase number of inner cell mass (ICM) cells.

Collectively, these studies suggest that although DNA demethylation plays an important role during mammalian early embryo development, locus-specific DNA methylation maintenance is also necessary for the normal development of mammalian early embryos. DNMT is responsible for the maintenance of locus-specific DNA methylation [16], however, the role of DNMT in the development of bovine preimplanted embryos is not fully elucidated. In this study, we evaluated a novel DNMT inhibitor (DNMTi), RG108, and investigated its effects on the development, dynamics of gene-specific DNA methylation and transcription of bovine parthenogenetic embryos.

2. Materials and methods

Unless described elsewhere, all chemicals and reagents were purchased from Sigma (St. Louis, MO, USA).

2.1. In vitro maturation of bovine oocytes

Bovine ovaries were collected from a local abattoir and transported to the laboratory at 37 °C within 3 h. The ovaries were washed three times in 0.9% NaCl supplemented with both 100 IU/ml penicillin and 100 IU/ml streptomycin. Cumulus-oocyte complexes (COCs) were aspirated from the follicles (2–8 mm) using an 18-guage needle attached to a 10-ml syringe. Then, COCs with intact, unexpanded cumulus cells were divided into two groups. One group of 20–25 COCs was cultured for 20–22 h at 38.5 °C in 100 μl of maturation medium [17] in a humidified 5% CO_2 incubator. The second group was cultured in 100 μl of maturation medium supplemented with 200 μM RG108. The oocyte maturation rates were determined by the presence of first polar body (PB1).

2.2. Parthenogenetic activation of bovine oocytes

The mature oocytes were treated with 5 mM ionomycin for 5 min, followed by treatment with 2 mM 6-DMAP in synthetic oviduct fluid (Sofaa) [17] containing 10% FBS for 3.5 h at 38.5 °C in 5% CO_2 and 95% humidified air. Treated oocytes were then washed in Sofaa three times and divided into two groups. One group of 20–25 mature oocytes was cultured at 38.5 °C in 100 μl droplets of Sofaa in a humidified 5% CO_2 incubator. The second group was cultured in 100 μl droplets of Sofaa supplemented with RG108 (100 μM, 200 μM, and 400 μM). The cleavage rates of the parthenogenetic embryos were determined at 48 h after culturing. The blastocyst rates were calculated at 7 days after parthenogenetic activation.

2.3. RNA isolation, cDNA preparation and qRT-PCR

All RNA from bovine oocytes or parthenogenetic embryos was extracted using the RNeasy Mini kit (Qiagen, Hilden, Germany). The 1st-Strand cDNA Synthesis kit (Promega, Madison, WI, USA) was used to synthesize the 1st-strand cDNA. The primers used for *Dnmt1*, *Dnmt3a*, *Dnmt3b*, *Nanog, Bax* and 18S rRNA are listed in Table 2. The qRT-PCR mix (20 μl) consisted of 2 μl of cDNA, 10 μl of SYBR green master mix, 6.4 μl of RNase-free water and 0.8 μl each of forward and reverse primers (10 pmol) for each gene. The program used for the amplification of all genes consisted of a denaturing cycle of 3 min at 95 °C, 40 cycles of PCR (95 °C for 10 s, 55 °C for 45 s, and 95 °C for 1 min), a melting curve analysis consisting of 95 °C for 1 min followed by 55 °C for 1 min, and a step cycle starting at 55 °C for 10 s with a 0.5 °C/s transition rate, and cooling at 4 °C. Relative gene expression data were analyzed using Quantitative Real-Time PCR (qRT-PCR) and the $2^{-\triangle\triangle CT}$ method.

2.4. Sodium bisulfite genomic sequencing

Bisulfite sequencing was used to analyze the locus-specific DNA methylation of parthenogenetic blastocysts as described previously [18]. Briefly, at least ten parthenogenetic blastocysts were treated with lysis solution (10 mM Tris–HCl, pH7.6, 10 mM EDTA, 1% SDS, and 20 μg/μl of proteinase K in ddH_2O) for 1.5 h at 37 °C. Then, the samples were boiled for 5 min in a water bath, chilled on ice and spine down. A volume of 4 μl of 2 M N_aOH (final concentration 0.3 M N_aOH) was added and incubated for 15 min at 50 °C. Samples were mixed with 2 volumes of 2% low melting point agarose and pipetted into chilled mineral oil to form beads. Then, the beads were treated with freshly prepared bisulfite solution (2.5 M sodium metabisulfite and 125 mM hydroquinone, pH 5) for 5 h in the dark and covered with mineral oil at 50 °C. The reactions were stopped by equilibration against 1 ml Tris–EDTA buffer (pH 8.0) for 4 × 15 min. After desulfonation in 0.5 ml 0.2 M N_aOH for 2 × 15 min, the beads were washed with 1 ml Tris–EDTA buffer for 3 × 10 min and H_2O for 2 × 15 min, and then used for PCR. The PCR primer sequences are listed in Table 2.

2.5. Statistical analysis

Data were analyzed with Statistics Production for Service Solution (Version 16.0; SPSS, Chicago, IL, USA) by one-way ANOVA. A value of $p < 0.05$ was considered different, and $p < 0.01$ was considered significantly different.

3. Results

3.1. Expressions of the Dnmt gene family in bovine parthenogenetic preimplantation embryos

The quantitative real-time PCR (qRT-PCR) results revealed that transcripts of *Dnmt1*, *Dnmt3a* and *Dnmt3b* were differentially expressed in bovine parthenogenetic preimplantation embryos. *Dnmt1* and *Dnmt3b* showed high expression in the parthenogenetic 2-cell stage embryo, then, the expression levels of *Dnmt1* and *Dnmt3b* decreased during the following developmental stages (Fig. 1). The *Dnmt3a* showed lower expression compared to *Dnmt1* and *Dnmt3b* during bovine parthenogenetic preimplantation embryo development (Fig. 1).

3.2. The effect of RG108 on the development of bovine preimplantation parthenogenetic embryos

To determine the optimum dosages of RG108, we first treated