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Global DNA methylation and related mRNA profiles in sheep oocytes and early embryos derived from pre-pubertal and adult donors

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ABSTRACT

The developmental capacity of *in vitro*-matured oocytes and *in vitro*-fertilized embryos from pre-pubertal sheep is less than that of adult counterparts, and epigenetic mechanisms are thought to be involved. In the present study, germinal vesicle stage oocytes were collected by follicular aspiration from superovulated 4-week-old lambs and 2.5-year-old ewes. There were evaluations of the developmental potential of oocytes and embryos by *in vitro* culture and fertilization, global DNA methylation and hydroxymethylation patterns by immunofluorescence staining, and relative abundance of enzyme mRNA by quantitative real-time polymerase chain reaction analysis in pre-pubertal and adult sheep donors. The results showed that the rates of maturation and cleavage of oocytes as well as pregnancy and lambing rates from the transfer of 2-cell embryos collected from lambs were less than those from adults ($P < 0.05$). The global DNA methylation and hydroxymethylation and relative abundance of Dnmt1, Dnmt3a, and Tet3 mRNA were less at all stages of oocytes, zygotes, and two-cell embryos from lambs compared with those from adults ($P < 0.05$) with no difference in relative abundance of Dnmt3b mRNA. Thus, younger donor age was associated with disturbed DNA methylation processes due to insufficient methyltransferases during gametogenesis and early embryonic development, and this may be responsible for the lesser developmental potential of oocytes and early developing embryos when oocytes are collected from lambs.

1. Introduction

The genetic and economic advantages of using juvenile domestic animals in breeding programs provide ample justification for considering pre-pubertal animals as potential oocyte donors. Despite the ability to select for genetic background and optimize gonadotropin stimulation in pre-pubertal females, embryos, and fetal derived pre-pubertal oocytes frequently fail to develop. Pre-pubertal oocytes fail to mature *in vitro*, but the reasons for this failure remain unclear. Most previous studies reported incomplete or deficient cytoplasmic maturation, altered protein synthesis (Kochhar et al., 2002), reduced oocyte size (Kauffold et al., 2005), and impaired metabolism (O'Brien et al., 1996) in pre-pubertal oocytes, suggesting that these deficiencies are plausible reasons for the reduced

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developmental potential (Palmerini et al., 2014). However, a nuclear transfer study revealed that the nucleus of the pre-pubertal oocyte is primarily responsible for its developmental failure (Ptak et al., 2006). Epigenetic mechanisms, specifically DNA methylation dynamics, have been implicated in the lesser developmental capacity of pre-pubertal oocytes (Zaraza et al., 2010).

DNA methylation, which is the process of adding methyl groups to DNA CpG islands, is an epigenetic event that has an important role in gene regulation. The somatic cell genome is, however, relatively stable undergoing increases in demethylation and remethylation during gametogenesis and early embryogenesis (Dean, 2014). A family of methyltransferases (Dnmts) mediates the establishment and maintenance of the dynamic patterns of global genomic DNA methylation. The Dnmt1 is the most abundant methyltransferase and is thought to be largely responsible for maintaining methylation patterns throughout DNA replication. The Dnmt3a and Dnmt3b are both *de novo* methyltransferases that transfer methyl groups to previously unmethylated CpG dinucleotides (Lee et al., 2015). In addition, 5-hydroxymethylcytosine (5hmC) is obtained through oxidation of 5-methylcytosine (5mC; Tahiliani et al., 2009). This reaction is catalyzed by a family of dioxygenases—the 10-11 translocation (Tet) proteins, which catalyze 5mC to 5hmC by hydroxylating the genome (Pastor et al., 2013).

Pre-pubertal oocytes are epigenetically immature, and epigenetics has been proposed to be involved in the acquisition of full developmental competence by pre-pubertal oocytes. Previous studies have shown that DNA methylation is less in pre-pubertal sheep germinal vesicle (GV)-stage oocytes compared with that of adult counterparts (Ptak et al., 2006), and that methylation status of the BTS sequence changes are associated with donor age in cows (Mike et al., 2012). Epigenetically immature oocytes can result in epigenetic mosaicism or a loss of methylation imprinting of maternal alleles in embryos (Obata et al., 2011). Thus, the correct epigenetic modifications of the maternal genome necessary for full-term development have an important role during oocyte maturation (Katari et al., 2009). It was, therefore, hypothesized that pre-pubertal oocytes were epigenetically immature because DNA methylation dynamics did not result in methylation events during *in vitro* maturation so as to have adult-like oocyte methylation patterns.

Thus, the present study investigated potential epigenetic mechanisms leading to the loss of oocyte development potential in lambs by determining whether global genomic methylation changes are associated with donor age. Specifically, relative abundance was determined for 5mC and 5hmC, as well as the abundance of Dnmt1, Dnmt3a, Dnmt3b, and Tet3 mRNA in various oocyte stages and during early embryonic development.

2. Materials and methods

All procedures involving animals were approved by the Chinese Academy of Science Animal Care and Use Committee. All chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA).

2.1. Experimental design

GV-stage oocytes were collected from Small-tail Han and Dorper crossbred 4-week-old lamb donors ($n = 24$) and 2.5-year-old ewes ($n = 34$) produced by natural mating. Samples of oocytes at the GV, germinal vesicle breakdown (GVBD), metaphase I (MI), and metaphase II (MII) stages, as well as zygotes and two-cell embryos were collected during *in vitro* maturation (IVM) and *in vitro* fertilization (IVF) for immunofluorescence staining, quantitative real-time polymerase chain reaction (qRT-PCR), and the embryos were transferred to recipient ewes.

A total of 1380 oocytes were obtained by superovulation in the lamb group; 286 were used to assess oocyte developmental potential, 683 were used for immunofluorescence staining (Table 1), and 411 were used to detect relative abundance of Dnmts and Tet3 mRNA.

A total of 1097 oocytes were obtained by superovulation in the adult group; 204 were used to assess oocyte developmental potential, 513 were used for immunofluorescence staining (Table 1), and 380 were used to detect relative abundance of Dnmts and Tet3 mRNA.

2.2. In vivo oocyte collection

All donors were subjected to a superovulation protocol. Adult donors were treated with an intravaginally controlled progesterone release device (CIDR) (Pharmacia and Upjohn Co., Rydalmere, NSW, Australia) and follicle-stimulating hormone (FSH; Sansheng, Ningbo, China). The CIDR was inserted into the vagina on Day 0 (Beginning of estrus synchronization treatment). The FSH (total dose, 300 IU) was administered intramuscularly every 12 h for 4 days beginning on Day 9. The CIDR was removed on Day 12, and pregnant mare serum gonadotropin (PMSG; Sansheng) was administered intramuscularly (total dose, 360 IU). Cumulus oocyte complexes (COC) were collected 54 h after removing the CIDR.

The donor lambs were treated with FSH injected intramuscularly every 12 for 2 days (total dose, 250 IU). The PMSG (total dose, 250 IU) was administered intramuscularly at the time of the last FSH treatment. The COC were collected 14 h after the PMSG administration. These are standard hormone doses to superovulate healthy adult and lamb donors.

The COC were collected by follicular aspiration from ovaries. Briefly, the donors were anaesthetized with acepromazine maleate (0.05 mg/kg body weight) and sodium pentothal (10 mg/kg body weight). The ovaries were exposed after opening the abdominal cavity. The COC were aspirated from visible follicles (2–5 mm) using a 10 ml syringe equipped with an 18 gauge needle and seeded in M199 medium supplemented with 25 mM HEPES, 10 μg/mL heparin, and 0.4% fatty acid-free bovine serum albumin (BSA). The reproductive organs were washed extensively with saline after puncturing the follicles to avoid adhesions.