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Reduced nucleic acid methylation impairs meiotic maturation and developmental potency of pig oocytes

Yan-Kui Wang [1], Xiao-Xia Yu [1], Yun-Hua Liu, Xuan Li, Xiao-Man Liu, Pei-Chao Wang, Shuai Liu, Jia-Kun Miao, Zhi-Qiang Du, Cai-Xia Yang*

College of Animal Science and Technology, Northeast Agricultural University, Harbin, 150030, Heilongjiang, China

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ABSTRACT

Oocyte meiosis is a complex process coordinated by multiple endocrinal and molecular circuits. Recently, N^6-methyladenosine (m^6A) epigenetic modification on RNA is revealed to be important for meiotic maturation. However, the molecular mechanism of how m^6A modification exerts its effect on oocyte maturation is largely unknown. Here, we showed that endogenous m^6A writers (Mettl3 and Wtap) and eraser (Fto) elevated their transcript levels during meiotic maturation of pig oocytes. From germinal vesicle (GV) to metaphase II (MII) stages, global m^6A level significantly increased, and existed mostly in ooplasm. Methyl donor (betaine, 16 mM) treatment of porcine cumulus-oocyte complexes (COCs) during *in vitro* maturation (IVM) significantly boosted nucleic acid m^6A level within oocytes, but unchanged meiotic process and oocyte subsequent development. By contrast, methylation inhibitor (cycloleucine, 20 mM) reduced nucleic acid m^6A level, and significantly decreased the germinal vesicle breakdown (GVBD) rate, the extrusion rate of the first polar body, and the cleavage and blastocyst rates of parthenotes. In addition, in cycloleucine-treated oocytes Wtap increased but Lin28 decreased their abundances significantly, along with the higher incidence of spindle defects and chromosome misalignment. Furthermore, pT161-CDK1 protein level in pig oocytes was confirmed to be decreased after cycloleucine treatment for 24 h. Taken together, chemical induced reduction of nucleic acid m^6A methylation during pig oocyte meiosis could impair meiotic maturation and subsequent development potency, possibly through down-regulating pluripotency marker Lin28 mRNA abundance and disturbing MPF-regulated chromosome/spindle organization.

1. Introduction

In mammals, oocytes arrest at prophase of meiosis I until puberty. Then, stimulated by combined neuronal, hormonal and molecular signals, fully-grown germinal vesicle (GV) oocytes resume meiosis, and germinal vesicle breakdown (GVBD) occurs [1]. Subsequently, homologous chromosomes form pairs, and microtubule assembles into bipolar spindles at the metaphase stage of meiosis I (MI). Later on, one set of homologous chromosomes extrudes out of the oocyte as the first polar body via the pull-force of attached spindle, to complete the first cycle of meiosis. Afterwards, oocytes enter the second cycle of meiosis, and arrest again at the metaphase stage II (MII) [2]. Until fertilized by sperm or stimulated by parthenogenetic manipulation, oocytes resume again and complete the second meiosis, and go on with the early embryo development [3].

The successful execution of oocyte meiotic program is orchestrated by a complex signaling network [4]. Two principal regulatory pathways, the maturation-promoting factor (MPF) complex and the mitogen-activated protein kinase (MAPK) cascade, constitute important nodes on the network [5]. MPF, consisting of the catalytic (cyclin-dependent kinase 1, CDK1) and the regulatory (cyclin B1) subunits, could be activated when cyclin B1 binds to CDK1, causing dephosphorylation of Thr-14/Tyr-15 residues and phosphorylation of Thr-161 residue of CDK1 unit [5]. MPF activity is required for chromosome condensation, nuclear envelope breakdown [6], spindle formation and MII arrest [7]. As for the MAPK family members, the extracellular signal-regulated kinase-1 (ERK1)

* Corresponding author. College of Animal Science and Technology, Northeast Agricultural University, 600 Changjiang Road, XiangFang District, Harbin, 150030, Heilongjiang, China.
E-mail address: caixiayang@neau.edu.cn (C.-X. Yang).
[1] Contributed equally.

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and −2 (ERK2) are serine/threonine protein kinases that can be activated via phosphorylation. Activated ERK1/2 phosphorylate downstream target proteins to regulate microtubulin organization into spindle [8] and meiotic MII arrest [9,10]. At MI/MII stages, normal-shaped bipolar spindle can ensure the correct segregation of chromosomes and avoid aneuploidy, which is vital for oocyte maturation and subsequent development [11,12].

N^6-methyladenosine (m^6A) modification has been proven to exist in eukaryotic DNA [13,14] and RNA [15,16]. The most common and abundant RNA m^6A modification in eukaryotic cells has been shown to regulate a variety of RNA function, including splicing [17], translation [18], stability [19] and degradation [20]. The m^6A writers organize into the METTL3-METTL14-WTAP methyltransferase complex, and join together with the erasers, FTO (fat mass and obesity-associated protein) and ALKBH5 (AlkB homologue 5), to regulate dynamically the level of m^6A modification [21] [22]; [23] [24] [25]. m^6A modification underlies multiple cellular events, such as cellular reprogramming [15], stem cell self-renewal [26], oocyte meiosis [27] and maternal-to-zygotic transition in zebrafish [28]. In yeast, RNA m^6A modification mediated by Ime4p, an ortholog of METTL3, is important for sporulation (meiosis) [29]. In *Xenopus laevis*, m^6A regulated translation of maternal mRNAs is involved in meiosis during oocyte maturation [27]. In zebrafish, loss of METTL3 induces the decrease of overall m^6A modification, leading to failed oocyte maturation and reduced fertility [30]. In mice, m^6A reader YTHDF2 is required to maintain oocyte competence for early zygotic development by regulating transcript dosage of genes during oocyte maturation [31]. METTL3, METTL14 and ALKBH5 were highly expressed in mouse oocytes [32]. However in pigs, it remains unclear whether m^6A modification exists, how its level dynamically changes and if it functions on meiotic process and developmental competence of oocytes.

Due to be more similar to human in size, physiology, and genetics than rodents and dogs, pig is regarded as an ideal larger animal model for human health and diseases [33]. We hypothesized that m^6A modification exists and plays vital role during maturation of porcine oocytes. Thus in the present study, using the *in vitro* maturation system for pig oocytes, we aimed to examine the endogenous expression levels of m^6A writers and erasers, and the global m^6A level during oocyte maturation; to observe the regulatory effects of m^6A modification on oocyte meiotic maturation by altering the global m^6A level using methyl donor (betaine) and methylation inhibitor (cycloleucine) [34]; and to study the molecular signaling events underlying oocyte meiosis disturbed by changed m^6A level.

2. Materials and methods

2.1. Ethics statement and chemicals

All experimental materials and procedures used in the present study were reviewed and approved by the Animal Care Commission and Ethics Committee of Northeast Agricultural University. All reagents were purchased from Sigma (St. Louis, MO, USA), unless stated otherwise.

2.2. In vitro maturation of cumulus-oocyte complexes

Ovaries were obtained from a local slaughter-house, and transported to the laboratory within 2 h in 0.9% saline maintained at 30–35 °C. Cumulus-oocyte complexes (COCs) were aspirated from antral follicles (3–6 mm in diameter) with an 18-gauge needle fixed to a 10 ml disposable syringe, and rinsed three times with TL-HEPES-PVA [35]. Then, the recovered COCs with at least three layers of compact cumulus cell and uniform ooplasm were selected for *in vitro* maturation (IVM). Degenerated oocytes with few or devoid of cumulus cells were discarded. Fifty porcine COCs were put in one well of 24-well dish with 500 μl of maturation medium (TCM 199 medium (Gibco BRL, Grand Island, NY) supplemented with 0.1% PVA, 3.05 mM D-glucose, 0.91 mM sodium pyruvate, 1 μg/ml gentamicin, 0.57 mM cysteine, 0.5 μg/ml luteinizing hormone (LH), 0.5 μg/ml follicle stimulating hormone (FSH), 10 ng/ml epidermal growth factor (EGF)), covered with paraffin oil and cultured in an incubator at 39 °C, 5% CO_2 and saturated humidity in air [36] for 24 h or 44hto collect oocytes at different stages [35].

2.3. Evaluation of oocyte nuclear status

In order to estimate the nuclear status, the cumulus cells were separated from oocytes by gentle vortexing in 0.1% hyaluronidase in HEPES-buffered Tyrode medium containing 0.01% PVA. Then, denuded oocytes were fixed in 4% paraformaldehyde in phosphate buffer solution (PBS) for 40 min at room temperature (RT) and then stained with 10 μg/ml Hoechst33342 in PBS for 10 min at RT. After washed in PBS once, oocytes were mounted onto glass slides in ProLong Diamond Antifade Mountant reagent (Life Technologies, USA), and observed under an inverted fluorescence microscope (Olympus, Tokyo, Japan). According to the stages of the oocyte nuclei, they were classified into five different types: germinal vesicle (GV), metaphase I (MI), anaphase I (AI)+telophase I (TI), MII, and abnormal, respectively [37].

2.4. Cycloleucine or betaine treatment

Porcine COCs were *in vitro* cultured in maturation medium supplemented with cycloleucine (CL) (A48105, Sigma) (10 mM, 20 mM, 40 mM) and betaine (B2629, Sigma) (4 mM, 8 mM, 16 mM) for 24 h or 44 h. The concentrations of chemical reagents were chosen first based on a previously published report [34], and then preliminary experiments were performed to determine further the optimal concentrations, which were then used in subsequent experiments.

2.5. Immunocytochemistry staining

The denuded oocytes were fixed in 4% paraformaldehyde/PBS for 40 min at RT, permeabilized with 1% Triton X-100/PBS overnight at 4 °C, and then blocked with 1% bovine serum albumin (BSA)/PBS for 1 h at RT. Then, samples were incubated with rabbit anti-α-tubulin polyclonal antibody (1:50; Abclonal, Nanjing, China) and rabbit anti-N6-methyladenosine antibody (m^6A) monoclonal antibody (1:75; Abcam, Shanghai, China) overnight at 4 °C, respectively. Following three washes in PBS supplemented with 0.1% Triton X-100 and 0.01% Tween-20 (PBST, 10min each), oocytes were incubated in second antibody (1:150 FITC conjugated goat anti-rabbit IgG (H + L)) for 1 h at RT. Then after washing three times with PBST (10min each), samples were stained with 10 μg/ml Hoechst33342 in PBS for 10 min at RT. Finally, oocytes were mounted onto glass slides in ProLong Diamond Antifade Mountant reagent (Life Technologies, USA). Samples incubated without the primary or secondary antibodies were treated as negative controls. A Nikon fluorescence microscope (at 100 × magnification, 80i, Japan) was used to take fluorescent images for α-tubulin to assess chromosomal alignment and spindle assembly. An Olympus fluorescence microscope (at 40 × magnification, I×71, Tokyo, Japan) was used to take all m^6A fluorescent images by setting the same parameters for all groups. To quantify the m^6A fluorescent intensity, Image J software (National Institutes of Health) was used to convert a colour image into gray one, circle the whole area of each oocyte along the membrane and measure the average gray value of circled