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Dynamic reprogramming of 5-hydroxymethylcytosine during early porcine embryogenesis

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ABSTRACT

DNA active demethylation is an important epigenetic phenomenon observed in porcine zygotes, yet its molecular origins are unknown. Our results show that 5-methylcytosine (5mC) converts into 5-hydroxymethylcytosine (5hmC) during the first cell cycle in porcine *in vivo* fertilization (IVV), IVF, and SCNT embryos, but not in parthenogenetically activated embryos. Expression of Ten-Eleven Translocation 1 (TET1) correlates with this conversion. Expression of 5mC gradually decreases until the morula stage; it is only expressed in the inner cell mass, but not trophectoderm regions of IVV and IVF blastocysts. Expression of 5mC in SCNT embryos is ectopically distinct from that observed in IVV and IVF embryos. In addition, 5hmC expression was similar to that of 5mC in IVV cleavage-stage embryos. Expression of 5hmC remained constant in IVF and SCNT embryos, and was evenly distributed among the inner cell mass and trophectoderm regions derived from IVV, IVF, and SCNT blastocysts. Ten-Eleven Translocation 3 was highly expressed in two-cell embryos, whereas *TET1* and *TET2* were highly expressed in blastocysts. These data suggest that TET1-catalyzed 5hmC may be involved in active DNA demethylation in porcine early embryos. In addition, 5mC, but not 5hmC, participates in the initial cell lineage specification in porcine IVV and IVF blastocysts. Last, SCNT embryos show aberrant 5mC and 5hmC expression during early porcine embryonic development.

1. Introduction

Since its discovery over 60 years ago, it is now widely accepted that DNA methylation plays a vital role in gene transcription, genomic imprinting and integrity, X chromosome inactivation, cellular differentiation, development, and cancer [1,2]. DNA methylation occurs at the fifth carbon of cytosine residues, predominantly in genomic CpG dinucleotides. Cytosine methylation in all contexts is initially established by the *de novo* methyltransferase (DNMT)3A and (DNMT)3B, and this mechanism is faithfully maintained by DNMT1 after each round of DNA replication in mammalian cells [2]. Until recently, DNA methylation was considered a stable epigenetic modification. However, recent studies indicate that DNA methylation is a dynamically reversible mechanism at specific developmental stages [3,4]. Indeed, active DNA demethylation is well documented during the development of mammalian primordial germ cells [5–7] and in early embryos [8–13].

The mechanisms that regulate the asymmetrical genomic DNA demethylation in parental pronuclei have been under intensive investigation in the last decade. Some models have been suggested, but none have been

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completely proven [3,6,14–17]. Recently, mammalian Ten-Eleven Translocation (TET) proteins including TET1, TET2, and TET3 were identified as homologs of the trypanosome J-binding proteins (JBPs), JBP1 and JBP2, which oxidize the 5-methylthymine into 5-hydroxymethyluracil [18]. These mammalian homologs catalyze the conversion of 5-methylcytosine (5mC) into 5-hydroxymethylcytosine (5hmC) in genomic DNA of human embryonic kidney 293 cells, U2OS cells, and mouse embryonic stem (ES) cells [19–21]. Therefore, these data suggest that 5hmC is an intermediate during the active DNA demethylation process in animals. Furthermore, knockdown of *TET1* in mouse two-cell embryos results in preferentially development of blastomeres to trophectoderm (TE) cells. This is consistent with *in vitro* evidence that knockdown of *TET1* reduces the self-renewal ability of ES cells, supporting the hypothesis that *TET1* is another important player in the "pluripotency network" [20]. Mutant *TET2* impairs the hydroxylation of 5mC and results in myeloid cancers, indicating that *TET2* may suppress some cancers [22]. *TET3* is highly expressed in mouse oocytes and zygotes [23,24], yet TET3 protein is only localized in paternal pronuclei [25]. Several studies show that 5hmC is exclusively catalyzed by TET3, and this process contributes to active DNA demethylation in paternal and pseudo-pronuclei during the development of IVF and SCNT one-cell embryos in mice [23–25].

Previous studies indicate that active DNA demethylation can also occur in paternal genomes of porcine pronuclear embryos [11,26,27]. However, it is not known if 5hmC is also involved in the dynamic reprogramming of active DNA demethylation during the development of early porcine embryos derived from *in vivo* fertilization (IVV), IVF, SCNT, and parthenogenetic activation (PA). To address this question, we followed 5hmC expression in differently derived porcine embryos. A better understanding of this mechanism will greatly improve the efficiency of porcine SCNT and the induction of pluripotent stem cells (iPS).

2. Materials and methods

All chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA), unless stated otherwise.

2.1. Animals

All pig experiments in the present study were approved by the Animal Care and Use Committee of China Agricultural University. All procedures were carried out in strict accordance with the recommendations made in the Guide for the Care and Use of Laboratory Animals of the National Veterinary Quarantine Service.

2.2. In vivo–derived embryos

Sows (Meishan breed, n = 20) were checked daily for the onset of estrus (Day 0). The unstimulated sows displaying estrus were naturally mated two successive times with two sexually mature and healthy boars. The sows were euthanized at a local abattoir at specific postmating time points, and their reproductive tracts including oviducts and uteri were excised. The following were the postmating time points (after the onset of estrus) for recovering the embryos: 16 to 18 hours for pronuclear stage embryos, 36 hours for two-cell stage embryos; 48 hours for four-cell stage embryos; 72 hours for eight-cell stage embryos; 96 hours for the morula stage embryos; and 120 to 168 hours for early, expanded, and hatched blastocyst stage embryos. The embryos at all different developmental stages were quickly flushed out with prewarmed 0.9% NaCl solution from the oviducts or uteri. The embryos were carefully selected under the stereomicroscope (Nikon, SMZ1000, Japan) and only normal-looking embryos were used for immunocytochemical analysis. In addition, zonae pellucidae were removed from zygotes or developing embryos by pronase treatment (0.5% in Dulbecco's phosphate buffered saline [DPBS]), and at least 15 embryos per embryo stage were fixed for 15 minutes in paraformaldehyde (4% in DPBS) at room temperature, washed several times in DPBS plus 0.3% polyvinylpyrrolidone (PVP), and stored short term at 4 °C in DPBS for immunocytochemical analyses.

2.3. In vitro maturation

This experiment was performed as described previously [28]. Briefly, ovaries were taken from prepubertal gilts and sows at a local slaughterhouse. These were placed at 28 °C to 35 °C in a physiological saline solution containing penicillin (0.2 IU/mL) and streptomycin sulfate (0.2 IU/mL), and were taken to the laboratory within 2 hours and washed with saline. Immediately upon arrival, ovarian follicles 3 to 6 mm in diameter were aspirated using a sterile 10-mL syringe with an 18-ga needle. The aspirated follicular fluid was slowly injected into a 38.5 °C, preincubated, 15-mL centrifuge tube to sediment the cumulus-oocyte complexes (COCs). After approximately 15 minutes of sedimentation, a clear interface was visible. After the removal of the supernatant, the cell pellets were diluted with oocyte-washing medium (DPBS plus 0.01% polyvinyl alcohol) and aspirated gently. Using a stereomicroscope, COCs that had more than two layers of compact cumulus investment and a dense, homogeneous cytoplasm were rapidly selected. They were washed three times with oocyte-washing medium (0.01% polyvinyl alcohol in DPBS), then three more times with IVM medium (tissue culture medium-199 supplemented with 15% fetal bovine serum, 10% porcine follicular fluid, 10 IU/mL of eCG [Suigonan Vet; Denmark], 5 IU/mL of hCG [Suigonan Vet], 0.8 mmol/L L-glutamine and 0.05 mg/mL gentamicin). Subsequently, 50 of the washed COCs were matured in 400 μL IVM at 38.5 °C with 5% CO_2 and saturated humidity for 42 to 44 hours. The COCs were treated with DPBS without Ca^{2+} and Mg^{2+} (Gibco, Grand Isle, NY) containing 1 mg/mL hyaluronidase to remove the surrounding cumulus cells. Finally, the oocytes with clear perivitelline spaces, intact cell membranes, and extruded first polar bodies (pb1) were selected for use.

2.4. In vitro fertilization

Fresh semen was washed three times with DPBS supplemented with 0.1% BSA, 75 μg/mL penicillin G, and 50 μg/mL streptomycin, and centrifugation at ×100*g* for 3