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Polymerase subunit gamma 2 affects porcine oocyte maturation and subsequent embryonic development

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ABSTRACT

Deoxyribonucleic acid polymerase subunit gamma (POLG) is an enzyme encoded by the mitochondrial *Polg* gene. Polymerase (DNA directed), gamma 2, accessory subunit, also known as POLG2, is involved in mitochondrial replication. In the present study, we examined the role of *Polg2* in the maturation of porcine oocytes. After *Polg2* knockdown, the mitochondrial DNA copy number was significantly ($P < 0.05$) lower than that in the control group. However, there was no decrease in mitochondrial membrane potential. The decrease in mitochondrial DNA copy number led to reductions in adenosine-5′-triphosphate content ($P < 0.05$) and the maturation rate ($P < 0.05$) of oocytes. Furthermore, in the *Polg2*-knockdown group, maturation-promoting factor activity was decreased ($P < 0.05$) and the percentage of oocytes displaying abnormal actin filaments and microtubules was significantly increased ($P < 0.05$). This likely led to the reduced development rate and number of cells per blastocyst in this group ($P < 0.05$). In conclusion, *Polg2* seems to be critical for mitochondrial replication and regulation of adenosine-5′-triphosphate content and affects porcine oocyte maturation and subsequent embryonic development.

1. Introduction

In most mammals, oocytes are arrested at the diplotene stage, also called prophase I and the germinal vesicle (GV) stage, until gonadotropins (particularly luteinizing hormone) released from the pituitary gland stimulate immature oocytes to resume meiosis and undergo ovulation. The completion of meiosis depends on the extent of phosphorylation and dephosphorylation as well as the energy supply. Mitochondria are the “power factory” of the cell, providing adenosine-5′-triphosphate (ATP) to meet the cell’s energy needs. Glycolysis is limited during early developmental stages in oocytes; therefore, mitochondria are the primary source of ATP during oocyte maturation and are essential for the fertilization and preimplantation steps of embryo development [1].

In humans, point mutations, duplications, or deletions in any of the mitochondrial genes can cause disease. Therefore, any mutations in the genes that encode DNA polymerase subunit gamma (*Polg*) and affect its targeting, transport, polymerase activity, or proofreading activity may manifest as a defect in oxidative phosphorylation. Mitochondrial DNA (mtDNA) in pig is 16.6 kb long [2] and encodes 13 subunits of the electron transfer chain, which is essential for energy production in cells [3]. Additionally, the number of mtDNA copies found in a cell is directly proportional to the amount of ATP synthesized [3,4]. Mammalian oocytes typically contain approximately 160,000 mitochondria at the GV stage, which increases at metaphase (M) II stage to 100,000 in mouse and 300,000 in pig [5]. Replication of mtDNA is

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controlled by several replisomes, which are composed of several nuclear-encoded mitochondrial factors including transcription factor A, mitochondrial [6], general transcription factor IIH, polypeptide 1 (transcription factor B1) [7], POLG, single-stranded DNA-binding [8], and TWINKLE [9]. In the absence of mtDNA replication, embryonic cells would be expected to be largely depleted of mtDNA between embryonic Days 7.5 and 8.5 in mouse. Conversely, a high mtDNA copy number improves the quality and competence of mature mammalian oocytes [10].

Polymerase subunit gamma, POLG, is a trimeric protein complex composed of a catalytic subunit of 140 kDa, which is encoded by the *Polg1* gene, and a dimeric accessory subunit of 55 kDa, which is encoded by the *Polg2* gene, both of which are located in mitochondria [11]. The porcine *Polg* complementary DNA (cDNA) and gene were mapped to sus scrofa chromosome (SSC)7 (NC_010449.4). In eukaryotic cells, *Polg* is the only polymerase involved in the replication of the mitochondrial genome [12]. Consequently, perturbation of this polymerase will affect ATP production, causing oocyte maturation failure and poor embryonic development. Over 100 mutations in the POLG1 gene have been recorded [13], whereas only one heterozygous missense mutation in a progressive external ophthalmoplegia patient has been reported for the POLG2 gene [14]. Moreover, the function of *Polg2* in mammalian oocyte maturation and development is unknown.

In the present study, we systemically evaluated the function of *Polg2* in porcine oocyte maturation and development. To this end, we knocked down the expression of *Polg2* in oocytes, which were subsequently matured *in vitro*. The knockdown of *Polg2* perturbed ATP synthesis and reduced the oocyte maturation rate. This knockdown also reduced porcine oocyte parthenogenetic activation. We conclude that *Polg2* is essential for porcine oocyte maturation *in vitro*.

2. Materials and methods

Unless otherwise indicated, all chemicals were purchased from Sigma Chemical Company (Sigma-Aldrich, St. Louis, MO, USA).

2.1. Oocyte collection and small interfering RNA treatment

Prepubertal porcine ovaries were obtained from a local slaughterhouse. Cumulus oocyte complexes were isolated and cumulus cells were removed by repeated pipetting in the presence of 1 mg/mL of hyaluronidase for 2 to 3 minutes. Scrambled or *Polg2*-targeting small interfering RNA (siRNA) (approximately 10 pL) was injected into the cytoplasm of GV-stage denuded oocytes under a Nikon TE2000-U inverted microscope (Nikon Corporation, Tokyo, Japan) using a FemtoJet microinjector (Eppendorf, Hamburg, Germany). Each siRNA was diluted in Deoxyribonuclease (DNase)- and Ribonuclease (RNase)-free water (distilled water [DW]; Bioneer, Daejean, Korea) to a final concentration of 100 nM and stored at −80 °C.

2.2. In vitro maturation, parthenogenetic activation, and culture of embryos

After siRNA treatment, GV-stage oocytes were treated with 4 mM dibutyryl cyclic-adenosine monophosphate (dbcAMP) for 18 hours to inhibit germinal vesicle breakdown (GVBD) [15]. To confirm that GVBD had been inhibited, oocytes were stained with 5 μg/mL Hoechst 33342, and the meiotic stage was determined by examining oocytes under a fluorescence microscope at ×400 magnification. After treatment with dbcAMP for 18 hours, oocytes were cultured in dbcAMP-free IVM medium. This medium comprised tissue culture medium 199 (Gibco, Grand Island, NY, USA) supplemented with 0.57 mM of cysteine, 10 ng/mL of epidermal growth factor, 10 IU/mL of pregnant mare's serum gonadotropin, and 10 IU/mL of human chorionic gonadotropin. After maturation for 44 hours, oocytes were parthenogenetically activated with the calcium ionophore A23187 (50 μM) for 5 minutes, and then incubated in porcine zygote medium (PZM)-5 [16] containing 7.5 μg/mL of cytochalasin B for 3 hours. Finally, embryos were cultured in PZM-5 medium supplemented with 0.4% BSA (wt/vol) under light mineral oil for 7 days at 38.5 °C and in 5% CO_2 (v:v).

2.3. Real-time reverse transcriptase–polymerase chain reaction

Messenger RNA was extracted and cDNA was synthesized as previously described [17]. Briefly, mRNA was extracted from 10 oocytes per group using the Dynabeads mRNA Direct Kit (Dynal Asa, Oslo, Norway). Thereafter, cDNA was synthesized by reverse transcription of RNA using an oligo $(dT)_{12\text{-}18}$ primer and superscript reverse transcriptase (Invitrogen, Grand Island, NY, USA) according to the manufacturer's instructions.

Sequence-specific primers were designed using Primer3 software (http://www.broad.mit.edu/cgibin/primer/primer3.cgi). The sequences and product sizes of all primers are listed in Table 1. Polymerase chain reaction was conducted in a Bio-Rad PCR machine (Bio-Rad, Hercules, CA, USA). Products were detected with SYBR green, a double-stranded DNA-specific fluorescent dye present in the SYBR Green qPCR premix (Finnzymes). The following amplification cycle was used: preincubation at 95 °C for 1 minute to activate the hot-start DNA polymerase, annealing at 55 °C for 1 minute, elongation at 72 °C for 1 minute, and acquisition of fluorescence at 72 °C for 1 second for 40 cycles. After the last cycle, a melting curve was generated by measuring fluorescence acquisition from 65 °C to 95 °C, taking measurements every 0.2 °C. Before quantification, optimization procedures were performed by running PCR reactions (with or without purified template) to identify the melting temperatures of primer dimers and specific products using various annealing temperatures. Product sizes were confirmed by electrophoresis on a standard 1.5% agarose gel, which was stained with RedSafe and exposed to ultraviolet light.

Relative gene expression was analyzed using the $2^{-\Delta\Delta Ct}$ method [18]. *Glyceraldehyde 3-phosphate dehydrogenase (Gapdh)* mRNA was used as an internal control. Three independent experiments were performed, each of which used triplicate samples.

2.4. Polymerase subunit gamma 2–targeting siRNA selection

Three independent siRNAs (siRNA1, siRNA2, and siRNA3) were designed to silence the porcine *Polg2* gene. The