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Regulated Mitochondrial DNA Replication During Oocyte Maturation Is Essential for Successful Porcine Embryonic Development

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ABSTRACT

Cellular ATP is mainly generated through mitochondrial oxidative phosphorylation, which is dependent on mitochondrial DNA (mtDNA). We have previously demonstrated the importance of oocyte mtDNA for porcine and human fertilization. However, the role of nuclear-encoded mitochondrial replication factors during oocyte and embryo development is not yet understood. We have analyzed two key factors, mitochondrial transcription factor A (TFAM) and polymerase gamma (POLG), to determine their role in oocyte and early embryo development. Competent and incompetent oocytes, as determined by brilliant cresyl blue (BCB) dye, were assessed intermittently during the maturation process for *TFAM* and *POLG* mRNA using real-time RT-PCR, for TFAM and POLG protein using immunocytochemistry, and for mtDNA copy number using real-time PCR. Analysis was also carried out following treatment of maturing oocytes with the mtDNA replication inhibitor, 2′,3′-dideoxycytidine (ddC). Following in vitro fertilization, preimplantation embryos were also analyzed. Despite increased levels of TFAM and POLG mRNA and protein at the four-cell stage, no increase in mtDNA copy number was observed in early preimplantation development. To compensate for this, mtDNA appeared to be replicated during oocyte maturation. However, significant differences in nuclear-encoded regulatory protein expression were observed between BCB^+ and BCB^- oocytes and between untreated oocytes and those treated with ddC. These changes resulted in delayed mtDNA replication, which correlated to reduced fertilization and embryonic development. We therefore conclude that adherence to the regulation of the timing of mtDNA replication during oocyte maturation is essential for successful embryonic development.

embryo, fertilization, gene regulation, oocyte development

INTRODUCTION

It has previously been demonstrated that the ATP content of individual embryonic blastomeres is correlated to their mitochondrial content [1], suggesting that mitochondrially derived ATP is important during early embryogenesis. Indeed, aggregation of active mitochondria has been reported around the pronuclei of fertilized oocytes and/or around the nuclei of cleavage-stage embryos in many species, including the monkey [2], hamster [3, 4], mouse [5], and human [6]. Clustering of mitochondria around the nuclear material has also been reported during oocyte maturation in the cow [7], mouse [8], and pig [9], and a significant increase in ATP levels during bovine in vitro maturation (IVM) is associated with higher quality oocytes producing significantly higher blastocyst rates [7]. ATP levels have also been linked to fertilization outcome in humans, with an estimated minimum requirement of 2 pmol ATP for successful fertilization [10].

Although ATP can be generated through a variety of processes, the most efficient method is oxidative phosphorylation (OXPHOS) via the mitochondrial electron transfer chain (ETC). This is vital for many cell types, including oocytes [11]. The ETC is located within the inner mitochondrial membrane and consists of five enzyme complexes each containing multiple subunits. Electrons flow through the first four complexes, releasing protons from the mitochondria. Re-entry of the protons down the newly produced gradient results in the production of ATP. The ETC is unique in that its complexes consist of both nuclear- and mitochondrially encoded subunits. In the pig, the mitochondrial genome (mtDNA) is 16.6 kb in size [12] and encodes 13 subunits of the ETC. It also encodes 22 tRNAs and 2 rRNAs that are necessary for mRNA expression.

Replication of mtDNA is dependent on nuclear-encoded transcription and replication factors being translocated to the mitochondria [13]. It utilizes an RNA primer generated through transcription by mitochondrial transcription factor A (TFAM) [14] and the mitochondrial-specific polymerase gamma [15], consisting of catalytic (POLG) and accessory (POLG2) subunits [16]. These two factors regulate mtDNA copy number, which is correlated to the OXPHOS requirements for specific cells [17]. The replication of mtDNA occurs throughout oocyte growth in many species [18, 19], and reports on the frog [20], mouse [21, 22], and shrimp [23] suggest that no mtDNA replication occurs in the early cleavage stages of embryogenesis. Absence of mtDNA replication until the blastocyst stage was recently confirmed in the mouse [24], and has also been reported in cattle [25]. However, one report suggests that a short burst of mtDNA replication takes place in mouse two-cell-only embryos, although there was no increase in mtDNA copy number [26].

Limited mtDNA replication during preimplantation development results in a progressive decrease in mtDNA content in cleaving blastomeres. Blastomeres in later-staged embryos, therefore, contain very low levels of mtDNA from which replication can be propagated. Once gastrulation has taken place, the inner cell mass (ICM) cells clonally expand their mtDNA to match the OXPHOS demands of the cells that they will differentiate into [27]. The segregation process increases the likelihood that adult cells derived from the same blastomere will possess the same mtDNA sequence [28]. The lack of mtDNA replication during preimplantation development suggests that mtDNA copy number needs to be amplified to sufficient levels prior to fertilization. Evidence in various mammalian species suggests that preimplantation embryonic mtDNA composition is dependent on the number of oocyte mtDNA copies present at fertilization, constituting an invest-

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ment until mtDNA replication is initiated postimplantation. A critical threshold of approximately 100 000 copies in the mature oocyte has therefore been proposed for the mouse [22], pig [29], and human [30, 31], suggesting that mtDNA copy number may be an indicator of oocyte competence.

Oocyte developmental competence can be assessed by the activity of glucose-6-phosphate dehydrogenase (G6PD). Loss of expression of G6PD is associated with oocytes reaching competency. These oocytes fail to enzymatically break down the dye, brilliant cresyl blue (BCB) [32, 33], and thus stain positively (BCB^+). Oocytes not having reached competency are colorless (BCB^-) due to the continued expression of G6PD, which breaks down the BCB dye. In the pig, competent BCB^+ oocytes contain more copies of mtDNA, and are more likely to fertilize than incompetent BCB^- oocytes [29]. However, supplementation of BCB^- oocytes with mitochondria from BCB^+ oocytes, and subsequent improved fertilization outcome, again demonstrates the association between mitochondrial number and fertilization outcome [29].

Here, we have investigated the importance of mtDNA replication during pig early embryogenesis through TFAM and POLG expression and mtDNA copy number analysis. Through BCB selection, we have further tested the hypothesis that oocyte mtDNA replication, and, therefore, oocyte competence, is dependent on correct expression of TFAM and POLG. Finally, we have demonstrated the importance of mtDNA replication for successful oocyte maturation using the inhibitor, 2′,3′-dideoxycytidine (ddC) [34].

MATERIALS AND METHODS

All chemicals and reagents used were purchased from Sigma Chemical Company (Poole, UK) unless otherwise stated.

Oocyte Retrieval

Ovaries were collected from a slaughterhouse in PBS supplemented with 75 μg/ml potassium penicillin G and 50 μg/ml streptomycin sulphate. They were rinsed three times with PBS at 39°C and transferred to a laminar-flow hood for cumulus oocyte complex aspiration from follicles with a diameter of 3–6 mm. Aspirated oocytes were transferred to a 15-ml conical tube containing 1 ml basic oocyte culture media consisting of TCM-199 with Earle salts and sodium bicarbonate, supplemented with 25 mM hepes, 3 mM L-glutamine, 0.1% (w/v) BSA, 0.57 mM cysteine, 50 IU/ml penicillin, and 50 μg/ml streptomycin.

In Vitro Maturation of Oocytes

Oocytes were matured in 500 μl of IVM media under equilibrated mineral oil at 39°C, 5% CO_2 in air. IVM media consisted of basic oocyte culture media supplemented with 10 ng/ml epidermal growth factor (EGF), 0.5 μg/ml luteinizing hormone and 50 ng/ml follicle-stimulating hormone [35]. For the first 16 h of IVM, the media was further supplemented with 5 μg/ml cycloheximide (CHX). Oocytes were then washed in basic oocyte culture media and matured in IVM media without CHX for a further 28–30 h before fertilization [35]. Oocytes to be analyzed during maturation were matured in IVM media without CHX for 44–46 h, with one media change.

BCB Test

Oocytes were washed three times in modified Dulbecco PBS (DPBSm; Dulbecco PBS supplemented with 0.4% [w/v] BSA, 0.34 mM pyruvate, 5.5 mM glucose, 50 IU/ml penicillin, and 50 μg/ml streptomycin). Oocytes were treated with 13 μM BCB diluted in DPBSm at 39°C, 5% CO_2 in air for 90 min. After treatment, the oocytes were transferred to DPBSm and washed three times. During the washing procedure, the oocytes were examined under a stereomicroscope and classified as either having stained blue (BCB^+) or remained colorless (BCB^-).

2′,3′-dideoxycytidine Treatment

Selected untreated oocytes were cultured in IVM media supplemented with 10 μM ddC. Being a nucleoside analogue, ddC can be incorporated into mtDNA molecules and synthesized in place of the normal nucleoside. However, lack of a 3′ OH group prevents addition of subsequent nucleosides, resulting in termination of the DNA chain [36]. Replication is further inhibited by failure of the POLG exonuclease to remove the ddC [37]. Oocytes were treated for the entire maturation period, with one media change on Day 2, as for nontreated oocytes.

Sperm Preparation

Sperm were obtained from purebred boars supplied by JSR (Selby, UK). Samples were centrifuged at 1500 rpm for 5 min. The supernatant was then removed and the pellet resuspended in 3 ml of sperm preparation media (DPBS supplemented with 1 mg/ml BSA, 100 μg/ml potassium penicillin G, and 75 μg/ml streptomycin) followed by centrifugation at 1200 rpm for 5 min. The new pellet was then resuspended in 2 ml of sperm preparation media and layered onto a 95%:45% Percoll gradient before centrifugation at 1500 rpm for 25 min. The 90% fraction was resuspended in 2 ml modified Tris-buffered medium (mTBM) consisting of 113.1 mM NaCl, 3 mM KCl, 11 mM glucose, 7.5 mM $CaCl_2 \cdot 2H_2O$, 20 mM Tris, and 5 mM sodium pyruvate supplemented with 0.1% (w/v) BSA, 20 μM adenosine, and 0.2 mM glutathione [35]. The 90% sperm sample was then centrifuged at 1500 rpm for 5 min and the pellet resuspended in 1 ml mTBM. Sperm counts were performed and samples were diluted to a concentration of 2×10^6/ml.

In Vitro Fertilization

Following IVM, oocytes were washed three times in mTBM and cumulus cells were removed by vortexing for 20 sec followed by pipetting through a fine pipette. Oocytes were then placed in 50 μl drops of mTBM under mineral oil and incubated for approximately 20 min, at which time 50 μl of sperm solution was added to the mTBM containing the mature oocytes, producing a final sperm concentration of 1×10^6/ml. Gametes were coincubated for 4–6 h at 39°C, 5% CO_2 in air.

Embryo Production

After sperm-oocyte coincubation, putative zygotes were washed three times in NCSU-23 media, with glucose replaced by lactate and pyruvate [35, 38], supplemented with 0.4% BSA and cultured at 39°C, 5% CO_2 in 100 μl of the same media under mineral oil for 48 h. Cleaved embryos were then washed and transferred to NCSU-23 media, containing glucose, supplemented with 0.4% BSA for further culture to the blastocyst stage [35].

DNA Extraction from Oocytes and Embryos

Oocytes/embryos were transferred individually to a 0.2-ml tube containing 20 μl ddH_2O. The contents were then freeze–thawed twice to lyse the oocytes/embryos and release the DNA [39]. Samples were diluted 1:5 in sterile ddH_2O before analysis.

RNA Extraction from Oocytes and Embryos and Reverse Transcription

Oocytes/embryos were transferred in groups of 5- to 0.2-ml tubes, and RNA was extracted using the RNAqueous-Micro kit (Ambion, Huntingdon, UK) according to the manufacturer's instructions. RNA was reverse transcribed using the Absolute 2-step QRT-PCR SYBR Green kit (Abgene, Epsom, UK). The resulting cDNA was then diluted 1:5 in sterile ddH_2O before analysis.

Generation of DNA and cDNA Standards for Real-Time PCR

The external standard of the appropriate size for each gene of interest was generated as previously described [29] using primers designed to amplify the specific genes (see Table 1). Reactions were performed in 20 μl using 1× PCR buffer (Bioline, London, UK), 1.5 mM $MgCl_2$ (Bioline), 0.5 μM each primer, 200 μM dNTP mix (Bioline), and 2 U BioTaq polymerase (Bioline). PCR products were run on 2% agarose gels, and DNA was extracted from the excised bands using the QiaQuick Gel Extraction kit (Qiagen, Crawley, West Sussex, UK) according the manufacturer's instructions. Samples were diluted to 0.02 ng/μl and then serially diluted 10 fold for use as standards for real-time PCR.

Real-Time PCR Quantification

Real-time PCR was performed on a Rotorgene 3000 cycler (Corbett Research, UK) using a 72 well rotor. Reactions tubes contained 7.5 μl ABsolute

TABLE 1. Primers used for real-time PCR.

Gene/mRNA	Forward primer	Reverse primer	Annealing temp. (°C)	Extension time (sec)	Primer concentration (μM)	Product size (bp)
TFAM	CAG ACT GGC AGG TGT ACA	CGA GGT CTT TTT GGT TTT CCA	54	15	400	164
POLG	GAG CAT GCA CAT GGC CAT CT	AGC TGC TTT CCA GCA GCA GCT	57	20	330	341
POLG2	GTG CCT TGG AGC ATT ATG TT	GAA GCT TCA GTC TTT TCA CCG	54	15	400	157
mtDNA	CTC AAC CCT AGC AGA AAC CA	TTA GTT GGT CGT ATC GGA ATC G	56	20	330	254

QPCR SYBR Green Mix (Abgene), primers at optimized concentrations (see Table 1) and 2 μl each sample or standard, made up to 15 μl with sterile ddH_2O. The reaction conditions were 1 cycle at 95°C for 15 min followed by 50 cycles of 94°C for 10 sec, the appropriate annealing temperature (see Table 1) for 15 sec and 72°C for the appropriate extension time (see Table 1), with data being acquired at the end of the extension steps. When primer-dimers were produced, an extra cycle and acquisition step was inserted after the extension phase. This consisted of a 15 sec hold at the temperature just higher than that at which the primer-dimers melted, allowing for calculations to be made that excluded fluorescence generated by the primer-dimers [40]. The data were acquired after primer-dimer denaturation on the FAM/SYBR channel, and a melt curve was analyzed to check for the absence of further mispriming. All samples were run twice in triplicate, and absolute mRNA levels per fixed number of oocytes/embryos ($n = 5$) were calculated by the Rotorgene software (Version 6, Corbett Research) and Microsoft Excel using the standards described above. Mean ± SEM expression levels are presented relative to either Day 1 BCB^+ oocytes or two-cell-staged embryos, as previously described [41, 42]. This allowed all three genes to be normalized for direct comparison of changes in expression levels between the three genes at each of the oocyte or embryo stages.

Immunocytochemistry

Antibodies were used against the nuclear-encoded transcription and replication regulators of mtDNA, TFAM (Santa Cruz Biotechnology, Santa Cruz, CA) and POLG (Abcam, Cambridge, UK) and against the mitochondrially encoded cytochrome c oxidase I (MT-CO1) protein to demonstrate colocalization with TFAM and POLG. Oocytes/embryos were fixed in 3.8% formaldehyde (BDH Laboratory Supplies, Poole, Dorset, UK) for 15 min at room temperature, permeabilized with 0.1% (v/v) Triton X100 for 5 min, and blocked with 100 μM glycine and 2 mg/ml BSA in PBS. Primary antibody was added at optimized concentrations (TFAM: 4 μg/ml; POLG: 20 μg/ml; MT-CO1: 2 μg/ml), and the oocytes/embryos were incubated at 37°C for 2 h. Oocytes/embryos were then transferred to 0.1% Triton X100 for washing before being labeled with the appropriate secondary antibodies (TFAM: 1μg/ml Alexa Fluor 593 anti-goat immunoglobulin (Ig) G; POLG: 2 μg/ml Alexa Fluor 488 anti-rabbit IgG or 1 μg/ml Alexa Fluor 593 anti-rabbit IgG; MT-CO1: 2 μg/ml Alexa Fluor 488 anti-mouse IgG; Molecular Probes, Paisley, UK) for 1 h at 37°C. After further washing, oocytes/embryos were mounted on to slides using Vectashield mounting medium (Vectalabs, Burlingame, CA), and viewed using the Leica DM IRE2 confocal microscope with a Leica TCS SP2 scanner (Leica Microsystems Ltd., Buckinghamshire, UK), as described previously [39]. Data were analyzed using the Leica Confocal software (Leica). Fluorescein isothiocyanate (FITC) excitation was at 488 nm, and detection was between 500 nm and 535 nm; rhodamine excitation was at 594 nm, and detection was between 600 nm and 700 nm. To allow for comparisons of staining intensity following confocal microscopy, the auto gain functions were switched off, and the same gain and photomultiplier settings were used for each embryo. Each channel was adjusted before images were taken to remove bleed through from other channels, and images were taken from FITC and rhodamine channels simultaneously from the same section of the oocytes/embryos.

Western Blotting

TFAM antibody specificity was determined using Western blotting both with and without the associated blocking peptide (Santa Cruz Biotechnology). Protein extracted from porcine heart tissue was run on a 12% polyacrylamide gel, alongside an SDS molecular weight marker (range = 6.5–175 kDa; New England Biolabs, Hitchin, Hertfordshire, UK), for 1 h at 140 V. Human protein extracted from umbilical vein endothelial cells was used as a positive control. Proteins were blotted onto Immobilon P transfer membrane (Millipore, Billerica, MA) for 70 min at 44 mA. Membranes were blocked with 5% (w/v) Marvel (Safeway, UK) in TBS (1.5 M NaCl, 0.2 M Tris and dd$H_2$0 to 1 L, pH 7.6) and slowly agitated for 1 h at room temperature. The blot was then incubated with TFAM primary antibody (0.8 μg/ml), either with or without the blocking peptide, overnight at 4°C. After washing in TBS plus 0.1% [v/v] Tween 20 (TBS-T) 5 times for 10 min each, the blot was incubated with the secondary antibody (Rabbit anti-goat IgG conjugated to horseradish peroxidase) for one hour before washing in TBS-T a further 5 times for 10 min each. Visualization of protein bands was through the enhanced chemiluminescence Western blotting system (Pierce Biotechnology Inc, Rockford, IL).

Statistical Analysis

Nonparametric Mann-Whitney *U*-tests were used to determine difference in mtDNA copy number and mRNA levels between stages of oocyte and embryo development, between BCB^+ and BCB^- oocytes, and between ddC-treated and untreated oocytes [25]. Differences in fertilization rates between ddC-treated and untreated oocytes were determined using the Fisher exact test [39]. Groups producing P values < 0.05 were considered significantly different.

RESULTS

Analysis of Preimplantation Porcine Embryos

In order to determine whether mtDNA replication takes place throughout preimplantation development, we analyzed the level of *TFAM*, *POLG*, and *POLG2* mRNA from the two-cell stage through to the expanded blastocyst. At the two-cell stage, all three factors were expressed at extremely low levels (Fig. 1). However, at the 4-cell stage, mRNA levels were increased for all 3 factors, by 43-fold for *TFAM* ($P = 0.02$), 11-fold for *POLG* ($P > 0.05$), and 48-fold for *POLG2* ($P = 0.02$). At the 8-cell stage, *TFAM* mRNA levels were slightly reduced, although *POLG* mRNA levels decreased to lower than those at the 2-cell stage ($P > 0.05$), while *POLG2* mRNA levels rose by 1.8 fold ($P > 0.05$). At the 16-cell stage, *TFAM* mRNA levels continued to decrease, representing a 2.6-fold decrease from the 4-cell to

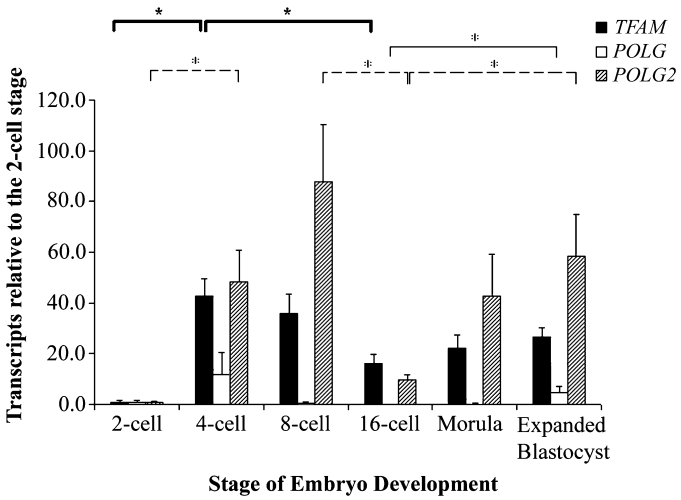

FIG. 1. Expression of mtDNA replication-associated genes during preimplantation development. Bars represent the number (mean ± SEM) of *TFAM, POLG,* and *POLG2* mRNA levels in two-cell- to expanded blastocyst-staged embryos as a proportion of those in two-cell embryos. * Differences between the various stages of development are significant ($P \leq 0.02$).

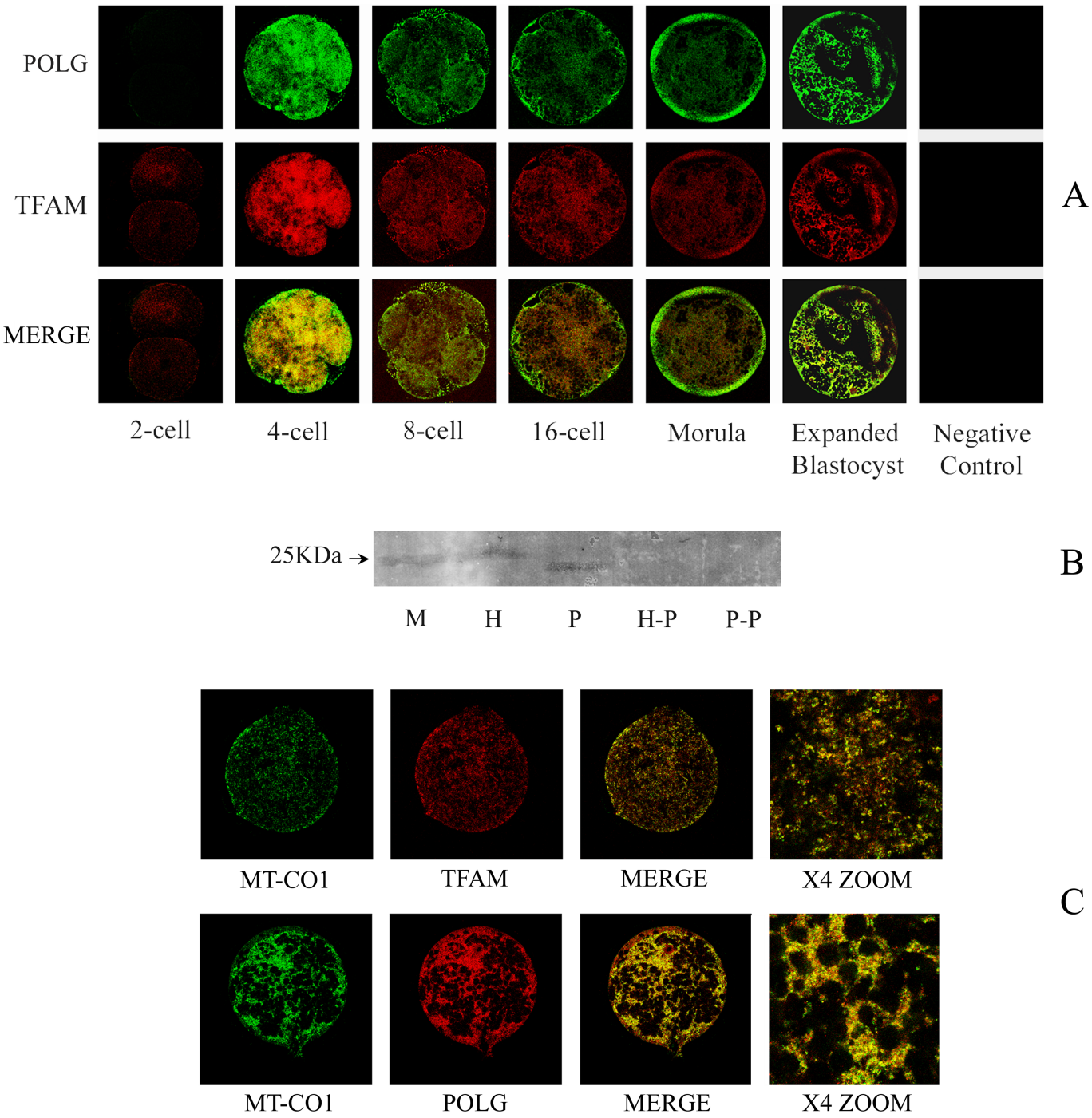

FIG. 2. Expression of TFAM and POLG proteins during preimplantation development. **A**) POLG (green) and TFAM (red) protein staining patterns in preimplantation embryos from the two-cell to the expanded blastocyst stage. Magnification ×630. **B**) Western blot showing molecular weight marker (M), human (H) and porcine (P) protein incubated with the TFAM antibody, and human (H-P) and porcine (P-P) protein incubated with the TFAM antibody and its associated blocking peptide. **C**) Green staining in the far left panels shows MT-CO1 localization in porcine oocytes. Adjacent panels with red staining show TFAM (top) and POLG (bottom) localization in the same oocytes. Merged images, both in full (third from left; magnification ×630) and in more detail (far right; magnification ×4 digital zoom) demonstrate colocalization of TFAM and POLG with MT-CO1.

16-cell stages ($P = 0.02$). *POLG2* mRNA followed suit, with an 8.8-fold decrease between 8-cell and 16-cell embryos ($P = 0.02$), while *POLG* mRNA levels remained low. All three factors, particularly *POLG* ($P = 0.01$) and *POLG2* ($P = 0.02$), then increased their mRNA levels between 16-cell and expanded blastocyst-staged embryos. Changes in mRNA levels were mirrored by levels of antibody staining for POLG and TFAM protein (Fig. 2A). This was especially so at the four-cell stage, with increases for both TFAM and POLG proteins, and at the morula and expanded blastocyst stages, with increases for POLG protein. Interestingly, POLG protein was concentrated at the outer edge of the embryo in the morulae and then throughout the entire embryo in expanded blastocysts.

In order to confirm the specificity of the TFAM antibody, a Western blot was carried out in which porcine (test) and human (control) protein was incubated with the TFAM antibody,

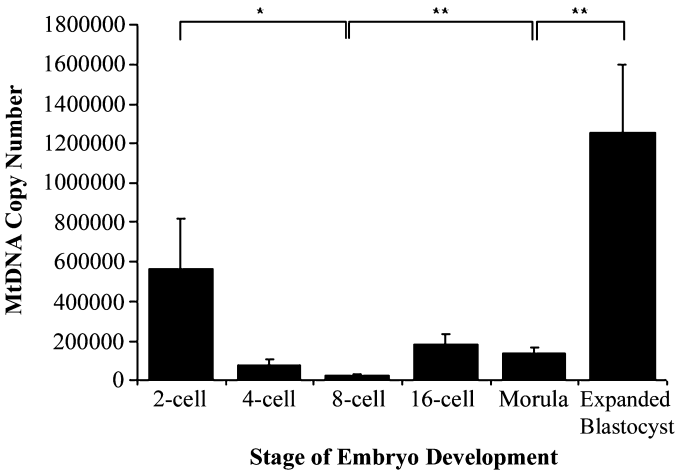

FIG. 3. MtDNA copy number in preimplantation embryos. Bars represent mean ± SEM mtDNA copy number in two-cell to expanded blastocyst staged embryos. Significant differences between developmental stages are indicated (* $P \leq 0.01$; ** $P < 0.005$).

either with or without the associated blocking peptide. A band of approximately 24 kDa, as expected for TFAM [43], was observed in the porcine protein sample (Fig. 2B), but this was not visible in the sample containing the blocking peptide. Costaining of POLG and TFAM antibodies with an antibody against MT-CO1 was also carried out. The predominantly yellow staining in the merged images suggests that both TFAM and POLG staining is localized to the mitochondria, further indicating the specificity of the antibodies against these proteins (Fig. 2C).

In order to determine whether the expression of these proteins had a functional role, mtDNA copy number was analyzed in whole embryos at each stage of development (Fig. 3). Mean values of 564 283 ± 253 833 copies were detected in 2-cell embryos. However, a progressive decrease was then observed, resulting in 73 561 ± 28 106 copies in 4-cell embryos ($P > 0.05$), and 21 104 ± 8249 in 8-cell embryos ($P = 0.01$). This was followed by an increase in mtDNA copy number to 175 454 ± 55 981 in 16-cell embryos ($P = 0.009$ compared with 8-cell embryos), and 131 238 ± 34 498 in morulae ($P = 0.001$ compared with 8-cell embryos). A further increase then occurred resulting in 1 254 604 ± 345 329 copies at the expanded blastocyst stage ($P = 0.002$ compared with morulae).

Analysis of Maturing Oocytes

In order to elucidate the mechanisms that ensure sufficient mtDNA copy numbers are present prior to fertilization, the expression of *TFAM*, *POLG*, and *POLG2* mRNA was analyzed in selected BCB^+- and BCB^--selected oocytes throughout maturation. On Day 1 of maturation, *TFAM* and *POLG* mRNAs were present at significantly higher levels in BCB^+ oocytes than in BCB^- oocytes ($P \leq 0.001$ and $P \leq 0.001$, respectively; Fig. 4) and at slightly higher levels for *POLG2* mRNA (Fig. 4). Throughout the maturation process, BCB^+ oocytes demonstrated a decrease to 33% by Day 2 ($P = 0.004$) and to 11% by Day 3 ($P = 0.004$) for *TFAM* mRNA, and to 18% by Day 2 ($P = 0.004$) and to 13% by Day 3 ($P = 0.004$) for *POLG* mRNA. A similar pattern was then observed for *POLG2* mRNA, with reductions to 58% by Day 2 ($P > 0.05$) and to 10% by Day 3 ($P = 0.02$ compared with Day 2).

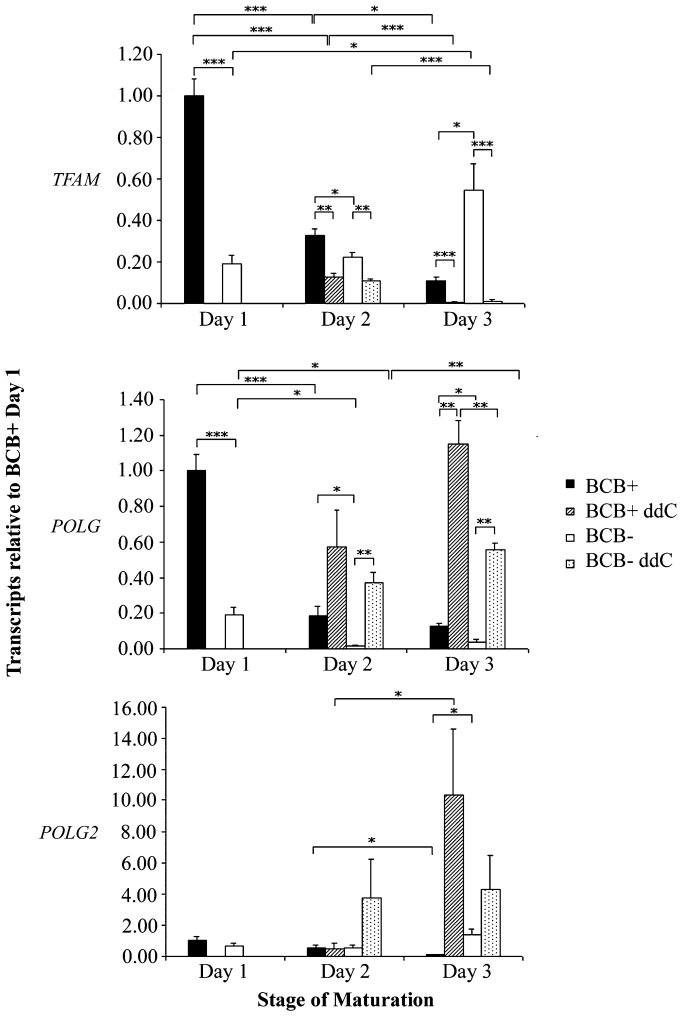

FIG. 4. Expression of mtDNA replication-associated genes during oocyte IVM. Bars represent the number (mean ± SEM) of *TFAM*, *POLG*, and *POLG2* mRNA copies in oocytes matured with and without ddC. Significant differences between groups are indicated (* $P < 0.05$, ** $P < 0.01$, *** $P \leq 0.005$).

On the other hand, BCB^- oocytes continued to contain significantly lower levels of *TFAM* mRNA than BCB^+ oocytes on Day 2 ($P = 0.04$), but there was a 2.4-fold increase by Day 3 ($P = 0.02$). For *POLG* mRNA, there was a decrease to 31% by Day 2 ($P = 0.02$), followed by a 2.8-fold increase between Days 2 and 3 ($P > 0.05$). For *POLG2*, mRNA levels on Day 2 remained similar to those on Day 1, although there was a 2.4-fold increase in expression on Day 3, resulting in BCB^- oocytes containing significantly more *POLG2* mRNA than BCB^+ oocytes ($P = 0.02$). These changes in mRNA levels for BCB^+ oocytes were matched by changes in protein staining intensity for POLG (Fig. 5) and TFAM (data not shown). However, for BCB^- oocytes, POLG protein synthesis appeared to be delayed until Day 3 (Fig. 5).

ddC Inhibition

In order to determine the importance of the last stages of mtDNA replication for oocyte maturation, BCB^+ and BCB^- oocytes were treated with ddC, a nucleoside analogue causing inhibition of mtDNA replication. As can be seen in Figure 4, ddC-treated BCB^+ oocytes had higher levels of *POLG* mRNA than untreated oocytes and, by Day 3, treated oocytes

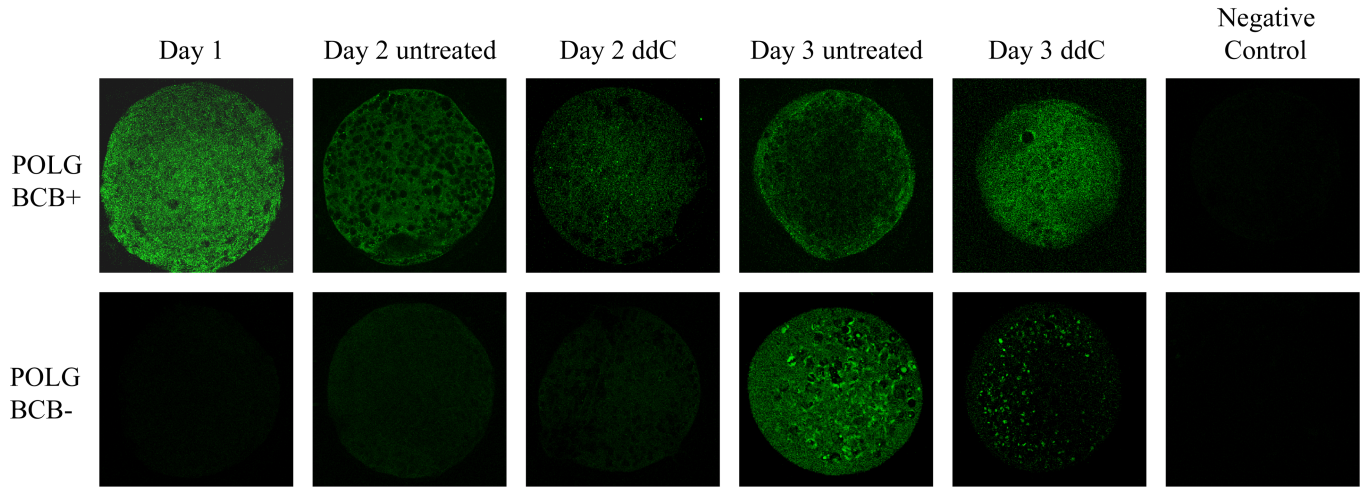

FIG. 5. Localization of POLG protein during oocyte maturation. Images show the localization of POLG protein in BCB^+ (top row) and BCB^- (bottom row) oocytes on Day 1, Day 2 (untreated and ddC treated), and Day 3 (untreated and ddC treated). Magnification ×630.

contained significantly more *POLG* mRNA than untreated oocytes ($P = 0.007$; Fig. 4). However, in ddC-treated BCB^- oocytes, *POLG* mRNA levels began to increase earlier than in the untreated BCB^- oocytes, resulting in significantly higher mRNA levels on Day 2 ($P = 0.006$) and Day 3 ($P = 0.006$) than in untreated oocytes. The effect of ddC treatment was also reflected in POLG protein expression patterns (Fig. 5). Interestingly though, ddC-treated BCB^+ oocytes maintained their expression of POLG protein throughout the oocyte from Days 1 to 3, while the untreated oocytes localized to the outer edges of the oocyte on Day 3 (Fig. 5). Unexpectedly, ddC-treated BCB^- oocytes on Day 3 did not demonstrate an increase in POLG protein to the extent observed in the untreated oocytes, despite higher levels of mRNA being present.

Treatment with ddC on maturing BCB^+ oocytes also resulted in a significantly greater reduction in *TFAM* mRNA levels throughout the maturation process than that observed in untreated BCB^+ oocytes (Day 2: $P = 0.006$; Day 3: $P = 0.004$; Fig. 4). In contrast, ddC-treated BCB^- oocytes demonstrated no significant change in *TFAM* mRNA levels between Day 1 and Day 2, as was the case for untreated BCB^- oocytes. However, by Day 3, there was a significant decrease in *TFAM* mRNA ($P = 0.006$) to 8% of the level present on Day 1. This is in contrast to the increase observed in untreated oocytes at this stage. Day 3 ddC-treated BCB^- oocytes also lacked the increased TFAM protein observed in the untreated oocytes (data not shown).

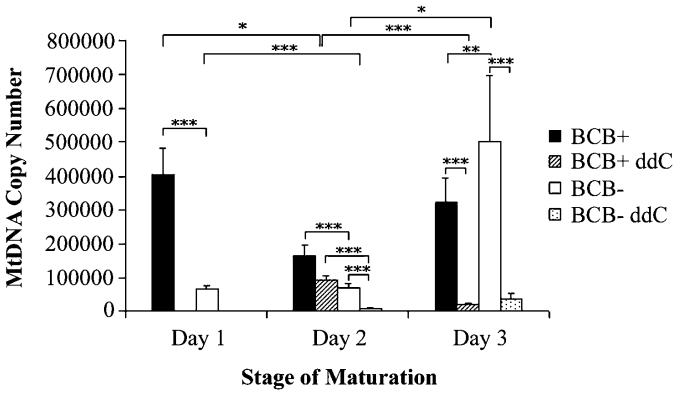

FIG. 6. MtDNA copy number in maturing oocytes. Bars represent mean ± SEM mtDNA copy number in maturing oocytes. Significant difference in mtDNA between two groups are indicated (* $P < 0.05$; ** $P < 0.01$; *** $P \leq 0.001$).

POLG2 mRNA in BCB^+ oocytes treated with ddC followed a similar pattern to that of the untreated oocytes on Day 2. However, by Day 3, there was a 22-fold increase in *POLG2* mRNA levels ($P = 0.04$), resulting in these oocytes containing more *POLG2* mRNA than the untreated oocytes ($P > 0.05$). BCB^- oocytes treated with ddC throughout the maturation process also demonstrated slightly increased levels of *POLG2* mRNA compared with untreated BCB^- oocytes.

Analysis of mtDNA copy number was carried out to determine whether oocyte mtDNA content was affected. BCB^+ oocytes contained 403 113 ± 80 314 mtDNA copies on Day 1 (Fig. 6), which decreased to 165 362 ± 30 461 on Day 2 ($P > 0.05$), followed by an increase to 323 038 ± 71 650 on Day 3 ($P > 0.05$). BCB^- oocytes contained significantly fewer mtDNA copies on Day 1 (66 781 ± 8587; $P < 0.001$) and Day 2 (68 406 ± 13 733; $P = 0.001$). However, a 7-fold increase resulted in 503 263 ± 193 295 copies being present on Day 3 ($P = 0.02$). BCB^+ oocytes treated with ddC decreased their mtDNA copy number on Day 2 to 92 857 ± 20 160 ($P = 0.04$), and then to 20 160 ± 4625 on Day 3 ($P < 0.001$), resulting in these oocytes containing significantly fewer mtDNA molecules than the untreated oocytes ($P < 0.001$). BCB^- oocytes treated with ddC also decreased their mtDNA copy number during maturation, with 9925 ± 2949 copies present on Day 2 ($P < 0.001$) and 35 848 ± 15 803 on Day 3 ($P < 0.001$), again with Day 3 treated oocytes containing significantly fewer mtDNA copies than the untreated oocytes ($P < 0.001$).

Analysis of Fertilization Ability of ddC-Treated Oocytes

There was no significant difference in fertilization outcome between ddC-treated and untreated BCB^+ oocytes. However, those ddC-treated oocytes that were able to fertilize did not develop beyond the six-cell stage. MtDNA copy number was, therefore, analyzed in these early embryos in order to determine whether a lack of mtDNA copies was responsible for their developmental arrest. Two-cell embryos derived from ddC-treated oocytes contained 185 234 ± 49 874 copies of mtDNA, only slightly less than 2-cell embryos derived from

untreated oocytes ($P > 0.05$). However, this was significantly higher than in Day 3 oocytes treated with ddC ($P < 0.001$), indicating that mtDNA replication had occurred at fertilization. As with embryos derived from untreated oocytes, mtDNA copy number then decreased to 106 692 ± 28 497 for 4-cell embryos ($P > 0.05$) and 4804 ± 1092 for 6-cell embryos ($P < 0.001$). These values were not significantly different from their respective, untreated four-cell and eight-cell counterparts.

DISCUSSION

ATP is vital for preimplantation development. In mammals, such as the pig [44], cow [45], rat [46], mouse [47], and human [48], the requirement for ATP generated through OXPHOS decreases as the preimplantation embryo develops. Consequently, there is a greater dependence on glycolysis [49]. Once at the blastocyst stage, the requirement for OXPHOS then increases [50], coinciding with the increased mitochondrial metabolism detected in mouse trophectoderm (TE) cells [51]. It is apparent from both this study in the pig, and in cattle [25], that the requirement for OXPHOS-derived ATP mirrors the initial decline and subsequent increases in mtDNA copy number. Similarly, somatic cells with reduced mtDNA content, such as aged cells with accumulated mtDNA deletions [52], various cancer cells [53], and cells lacking TFAM [54], demonstrate reduced mitochondrial ATP production and increased dependence on glycolysis. This clearly demonstrates the relationship between mtDNA copy number and mitochondrial ATP production.

The marked increase in mtDNA copy number observed at the expanded blastocyst stage (Fig. 3) coincided with the increased transcription of *POLG* and *POLG2* genes (Fig. 1) and increased translation of POLG (Fig. 2A). Increased POLG protein was observed in morulae localized to the outer edges of the embryos, the region likely representing cells destined to become TE (Fig. 2A). Increased POLG protein was not detected in the inner embryo, representing cells of the ICM, until the expanded blastocyst stage. This might suggest that mtDNA replication occurs first in the TE and then in the ICM. Again, this is consistent with the recent analysis of mouse blastocyst metabolism, where TE cells were determined to have increased oxygen consumption, increased ATP production, and increased mitochondrial number relative to cells of the ICM [51]. This would account for the high mtDNA copy numbers detected in our study and those observed in the mouse [24] and cattle [25]. Indeed, undifferentiated embryonic stem cells, which are derived from the ICM, are known to contain low numbers of mitochondria [27, 55] and low levels of mtDNA transcription and replication factor expression [27]. Despite a small but consistent increase in *TFAM* gene transcription from morula to expanded blastocyst, TFAM protein was present at very low levels throughout the cytoplasm, even at the expanded blastocyst stage. This would suggest that only low levels are necessary for mtDNA replication. The levels of porcine *TFAM* mRNA are consistent with increases observed at the morula and blastocyst stages in mouse [24] and cow [25] embryos, although these studies did not include analysis of the TFAM protein.

The discreet early burst of POLG expression observed here at the four-cell stage and the mtDNA replication observed at the two-cell stage in the mouse [26] coincides with embryonic genome activation (EGA), which occurs at the four-cell stage in pigs [56] and the two-cell stage in mouse [57]. In cattle, upregulation of *TFAM* and *NRF1* expression at the 8- to 16-cell stage [25] also coincides with EGA taking place at the 16-cell stage [58]. Although no cumulative increase in mtDNA copy number was recorded as a result of these activities, this mechanism could act to replenish degraded copies [26]. However, this activity might merely be explained by the generic expression of a whole host of genes at this one particular stage in development [59] rather than a specific mechanism to drive mtDNA replication.

As embryo mtDNA copy number decreased by 60% from the 2-cell to the 4- to 8-cell stage in cattle [25], and by 96% from the 2-cell to the 8-cell stage in the pig, this suggests the presence of an active mtDNA degradation process. A decrease in mtDNA copy number to critical levels at the eight-cell stage suggests that the mtDNA copy number present at fertilization is an important investment for subsequent developmental success. This is demonstrated by the decreased fertilization outcome of BCB^- oocytes and ddC-treated oocytes, both of which contained decreased mtDNA levels compared with untreated BCB^+ oocytes. This supports the hypothesis that a minimum mtDNA threshold exists for development to ensue [30]. This would further support the rationale behind cytoplasmic supplementation, instigated to overcome developmental arrest in oocytes from older women [60], although its clinical use is confounded by other outcomes [61, 62]. Indeed, supplementation with pure populations of mitochondria from BCB^+ oocytes into BCB^- oocytes again suggests that appropriate numbers of mtDNA copies are a determiner of oocyte competence [29]. Consequently, prefertilization mitochondrial biogenesis is critical. This is best exemplified by the large increase in mtDNA replication during oogenesis observed in many species in preparation for fertilization. For example, there is a 45-fold increase in cattle mtDNA copy number from the primordial germ cell stage to preovulating oocytes [19]. In the human, premigratory primordial germ cells contain approximately 10 mitochondria per cell [18], and increase to 700 000 in mature oocytes [30, 31], with similar results having been reported in pigs [29].

We have now carried out the first analysis of mtDNA copy number throughout IVM, using both competent (BCB^+) and less competent (BCB^-) oocytes, to test the hypothesis that mtDNA replication is either delayed or compromised in less competent oocytes. Previous studies have indicated that BCB^+ oocytes tend to fertilize more readily [29, 33] and also have higher numbers of mtDNA copies at Day 2 of maturation [29]. In this study, less competent BCB^- oocytes exhibited a delay in mtDNA replication due to the delayed onset of expression of their nuclear-encoded replication factors. For example, replication factor expression decreased during IVM in the BCB^+ oocytes, and mtDNA copy number was higher in these oocytes than in BCB^- oocytes on Days 1 and 2, suggesting that the major increase in mtDNA copy number had already occurred by this time. However, BCB^- oocytes increased their expression of replication factors during oocyte maturation, and a sharp increase in mtDNA copy number was observed on Day 3, suggesting that the majority of mtDNA replication in these oocytes was occurring during the IVM period.

Although we observed a decrease in mtDNA copy number in BCB^+ oocytes from Day 1 to Day 2, followed by an increase to Day 3, these differences were not statistically significant. To determine whether an active replication process was taking place, oocytes were treated with ddC, the antiretroviral nucleoside analogue that inhibits mtDNA replication [37]. The initial decline in mtDNA copy number between Days 1 and 2 was more severe than for untreated oocytes, and the tendency for an increase in mtDNA copy number between Days 2 and 3 was lost. This resulted in a further 75% decrease in mtDNA copy number over the maturation period in those oocytes treated with ddC. Consequently, this would suggest

that, in untreated oocytes, mtDNA replication was still taking place during the later stages of maturation in order to replenish degraded mtDNA molecules. Indeed, accumulation of mtDNA deletions has also been demonstrated during oocyte maturation in the rhesus macaque [63], again supporting the concept of discreet levels of mtDNA replication taking place.

The loss of mtDNA copies following ddC treatment is comparable to human MOLT T-lymphoblastic cells which, following 48 h of ddC treatment, decreased their mtDNA copy number 5-fold [64]. To compensate, these cells upregulated glycolysis following the mtDNA loss, producing increased levels of lactate after 72 h. Increased lactate production was also noted in rat PC12 cells after 48 h of ddC treatment [65]. However, at the transcriptional and translational level, we noted a further response to ddC inhibition of mtDNA replication. This involves the increased transcription of the *POLG* and *POLG2* genes, and also the maintenance of the POLG protein in BCB^+ oocytes. Similarly, BCB^- oocytes increased expression of TFAM and POLG protein in the later stages of IVM and, as a consequence, increased mtDNA copy number late in the maturation process. This suggests the presence of a feedback mechanism for maintaining sufficient mtDNA copies to support fertilization. MtDNA depletion due to ddC has previously been reported to initiate a feedback increase in TFAM expression [66], although effects on POLG expression were not analyzed.

Due to insufficient mtDNA levels on Day 3, we had anticipated that ddC-treated oocytes would have reduced fertilization rates. However, fertilization rates for ddC-treated and untreated oocytes were similar ($P > 0.05$). Furthermore, analysis of mtDNA copy number in early embryos showed similar copy numbers to those in embryos derived from untreated BCB^+ oocytes. As the ddC-treated oocytes contained significantly fewer mtDNA copies than untreated oocytes at insemination, significantly increased rates of mtDNA replication must have occurred just prior to fertilization, when the ddC was removed. This was most likely facilitated by the increased production of *POLG* and *POLG2* mRNA in ddC-treated oocytes during the later stages of maturation. Interestingly, we did not observe an increase in *TFAM* mRNA following ddC treatment, again suggesting that minimum levels of TFAM protein were sufficient for increased replication to take place. However, despite the apparently normal fertilization ability of ddC-treated oocytes, the resulting embryos did not develop beyond the four- to six-cell stage. This provides further evidence for a minimum mtDNA copy number requirement [29, 30] at a checkpoint during oocyte maturation, and for the necessity of subsequent time-specific downregulation of mtDNA replication to allow for successful preimplantation development.

In conclusion, we propose that the majority of mtDNA replication and the expression of the relevant transcription and replication factors are terminated prior to Days 1–3 of IVM in those oocytes likely to fertilize and develop. In incompetent oocytes, mtDNA replication is delayed, and the oocyte attempts to rescue this during the final stages of maturation. Consequently, oocyte competence in terms of mtDNA replication and composition is not fully synchronized, and will result in either failed fertilization or developmental arrest. This further indicates that mtDNA differentiation is one of the final markers of cellular differentiation and is critical to cellular function. Such outcomes have implications for those investigators choosing oocytes for a variety of reconstruction protocols.

REFERENCES

1. Van Blerkom J, Davis P, Alexander S. Differential mitochondrial distribution in human pronuclear embryos leads to disproportionate inheritance between blastomeres: relationship to microtubular organization, ATP content and competence. Hum Reprod 2000; 15:2621–2633.
2. Squirrell JM, Schramm RD, Paprocki AM, Wokosin DL, Bavister BD. Imaging mitochondrial organization in living primate oocytes and embryos using multiphoton microscopy. Microsc Microanal 2003; 9: 190–201.
3. Barnett DK, Kimura J, Bavister BD. Translocation of active mitochondria during hamster preimplantation embryo development studied by confocal laser scanning microscopy. Dev Dyn 1996; 205:64–72.
4. Bavister BD, Squirrell JM. Mitochondrial distribution and function in oocytes and early embryos. Hum Reprod 2000; 15(suppl 2):189–198.
5. Batten BE, Albertini DF, Ducibella T. Patterns of organelle distribution in mouse embryos during preimplantation development. Am J Anat 1987; 178:204–213.
6. Wilding M, Dale B, Marino M, di Matteo L, Alviggi C, Pisaturo ML, Lombardi L, De Placido G. Mitochondrial aggregation patterns and activity in human oocytes and preimplantation embryos. Hum Reprod 2001; 16:909–917.
7. Stojkovic M, Machado SA, Stojkovic P, Zakhartchenko V, Hutzler P, Goncalves PB, Wolf E. Mitochondrial distribution and adenosine triphosphate content of bovine oocytes before and after in vitro maturation: correlation with morphological criteria and developmental capacity after in vitro fertilization and culture. Biol Reprod 2001; 64:904–909.
8. Nishi Y, Takeshita T, Sato K, Araki T. Change of the mitochondrial distribution in mouse ooplasm during in vitro maturation. J Nippon Med Sch 2003; 70:408–415.
9. Sun QY, Wu GM, Lai L, Park KW, Cabot R, Cheong HT, Day BN, Prather RS, Schatten H. Translocation of active mitochondria during pig oocyte maturation, fertilization and early embryo development in vitro. Reproduction 2001; 122:155–163.
10. Van Blerkom J, Davis PW, Lee J. ATP content of human oocytes and developmental potential and outcome after in-vitro fertilization and embryo transfer. Hum Reprod 1995; 10:415–424.
11. Dumollard R, Marangos P, Fitzharris G, Swann K, Duchen M, Carroll J. Sperm-triggered $[Ca^{2+}]$ oscillations and Ca^{2+} homeostasis in the mouse egg have an absolute requirement for mitochondrial ATP production. Development 2004; 131:3057–3067.
12. Ursing B, Arnason U. The complete mitochondrial DNA sequence of the pig (*Sus scrofa*). J Mol Evol 1998; 47:302–306.
13. Clayton DA. Transcription and replication of mitochondrial DNA. Hum Reprod 2000; 15(suppl 2):11–17.
14. Larsson NG, Wang J, Wilhelmsson H, Oldfors A, Rustin P, Lewandoski M, Barsh GS, Clayton DA. Mitochondrial transcription factor A is necessary for mtDNA maintenance and embryogenesis in mice. Nat Genet 1998; 18:231–236.
15. Hubscher U, Kuenzle CC, Spadari S. Functional roles of DNA polymerases beta and gamma. Proc Natl Acad Sci U S A 1979; 76: 2316–2320.
16. Gray H, Wong TW. Purification and identification of subunit structure of the human mitochondrial DNA polymerase. J Biol Chem 1992; 267:5835–5841.
17. Moyes CD, Battersby BJ, Leary SC. Regulation of muscle mitochondrial design. J Exp Biol 1998; 201:299–307.
18. Jansen RP, de Boer K. The bottleneck: mitochondrial imperatives in oogenesis and ovarian follicular fate. Mol Cell Endocrinol 1998; 145:81–88.
19. Smith LC, Alcivar AA. Cytoplasmic inheritance and its effects on development and performance. J Reprod Fertil Suppl 1993; 48:31–43.
20. El Meziane A, Callen JC, Mounolou JC. Mitochondrial gene expression during *Xenopus laevis* development: a molecular study. EMBO J 1989; 8: 1649–1655.
21. Ebert KM, Liem H, Hecht NB. Mitochondrial DNA in the mouse preimplantation embryo. J Reprod Fertil 1988; 82:145–149.
22. Piko L, Taylor KD. Amounts of mitochondrial DNA and abundance of some mitochondrial gene transcripts in early mouse embryos. Dev Biol 1987; 123:364–374.
23. Vallejo CG, Lopez M, Ochoa P, Manzanares M, Garesse R. Mitochondrial differentiation during the early development of the brine shrimp *Artemia franciscana*. Biochem J 1996; 314:505–510.
24. Thundathil J, Filion F, Smith LC. Molecular control of mitochondrial function in preimplantation mouse embryos. Mol Reprod Dev 2005; 71: 405–413.
25. May-Panloup P, Vignon X, Chretien MF, Heyman Y, Tamassia M, Malthiery Y, Reynier P. Increase of mitochondrial DNA content and transcripts in early bovine embryogenesis associated with upregulation of mtTFA and NRF1 transcription factors. Reprod Biol Endocrinol 2005; 3: 65–72.
26. McConnell JM, Petrie L. Mitochondrial DNA turnover occurs during

preimplantation development and can be modulated by environmental factors. Reprod Biomed Online 2004; 9:418–424.
27. St John JC, Ramalho-Santos J, Gray HL, Petrosko P, Rawe VY, Navara CS, Simerly CR, Schatten GP. The expression of mitochondrial DNA transcription factors during early cardiomyocyte in vitro differentiation from human embryonic stem cells. Cloning Stem Cells 2005; 7:141–153.
28. Shoubridge EA. Mitochondrial DNA segregation in the developing embryo. Hum Reprod 2000; 15(suppl 2):229–234.
29. El Shourbagy SH, Spikings EC, Freitas M, St John JC. Mitochondria directly influence fertilisation outcome in the pig. Reproduction 2006; 131:233–245.
30. Reynier P, May-Panloup P, Chretien MF, Morgan CJ, Jean M, Savagner F, Barriere P, Malthiery Y. Mitochondrial DNA content affects the fertilizability of human oocytes. Mol Hum Reprod 2001; 7:425–429.
31. Almeida Santos T, El Shourbagy S, St John JC. Mitochondrial content reflects oocyte variability and fertilization outcome. Fertil Steril 2006; 85: 584–591.
32. Roca J, Martinez E, Vazquez JM, Lucas X. Selection of immature pig oocytes for homologous in vitro penetration assays with the brilliant cresyl blue test. Reprod Fertil Dev 1998; 10:479–485.
33. Rodríguez-Gonzáles E, López-Béjar M, Velilla E, Paramio MT. Selection of prepubertal goat oocytes using the brilliant cresyl blue test. Theriogenology 2002; 57:1397–1409.
34. Walker U, Venhoff N. Uridine in the prevention and treatment of NRTI-related mitochondrial toxicity. Antivir Ther 2005; 10:M117–M123.
35. Ye J, Campbell KH, Craigon J, Luck MR. Dynamic changes in meiotic progression and improvement of developmental competence of pig oocytes in vitro by follicle-stimulating hormone and cycloheximide. Biol Reprod 2005; 72:399–406.
36. Brinkman K, Kakuda TN. Mitochondrial toxicity of nucleoside analogue reverse transcriptase inhibitors: a looming obstacle for long-term antiretroviral therapy? Curr Opin Infect Dis 2000; 13:5–11.
37. Feng JY, Johnson AA, Johnson KA, Anderson KS. Insights into the molecular mechanism of mitochondrial toxicity by AIDS drugs. J Biol Chem 2001; 276:23832–23837.
38. Park Y, Hong J, Yong H, Lim J, Lee E. Effect of exogenous carbohydrates in a serum-free culture medium on the development of in vitro matured and fertilized porcine embryos. Zygote 2005; 13:269–275.
39. Lloyd RE, Lee JH, Alberio R, Bowles EJ, Ramalho-Santos J, Campbell KH, St John JC. Aberrant nucleo-cytoplasmic cross-talk results in donor cell mtDNA persistence in cloned embryos. Genetics 2006; 172:2515–2527.
40. Pfaffl MW, Georgieva TM, Georgiev IP, Ontsouka E, Hageleit M, Blum JW. Real-time RT-PCR quantification of insulin-like growth factor (IGF)-1, IGF-1 receptor, IGF-2, IGF-2 receptor, insulin receptor, growth hormone receptor, IGF-binding proteins 1, 2 and 3 in the bovine species. Domest Anim Endocrin 2002; 22:91–102.
41. Pennetier S, Perreau C, Uzbekova S, Thelie A, Delaleu B, Mermillod P, Dalbies-Tran R. MATER protein expression and intracellular localization throughout folliculogenesis and preimplantation embryo development in the bovine. BMC Dev Biol 2006; 6:26.
42. Hayashi S, Yang J, Christenson L, Yanagimachi R, Hecht NB. Mouse preimplantation embryos developed from oocytes injected with round spermatids or spermatozoa have similar but distinct patterns of early messenger RNA expression. Biol Reprod 2003; 69:1170–1176.
43. Ide T, Tsutsui H, Hayashidani S, Kang D, Suematsu N, Makamura K, Utsumi H, Hamasaki N, Takeshita A. Mitochaondrial DNA damage and dysfunction associated with oxidative stress in failing hearts after myocardial infarction. Circ Res 2001; 88:529–535.
44. Sturmey RG, Leese HJ. Energy metabolism in pig oocytes and early embryos. Reproduction 2003; 126:197–204.
45. Thompson JG, Partridge RJ, Houghton FD, Cox CI, Leese HJ. Oxygen uptake and carbohydrate metabolism by in vitro derived bovine embryos. J Reprod Fertil 1996; 106:299–306.
46. Brison DR, Leese HJ. Energy metabolism in late preimplantation rat embryos. J Reprod Fertil 1991; 93:245–251.
47. Houghton FD, Thompson JG, Kennedy CJ, Leese HJ. Oxygen consumption and energy metabolism of the early mouse embryo. Mol Reprod Dev 1996; 44:476–485.
48. Leese HJ, Conaghan J, Martin KL, Hardy K. Early human embryo metabolism. Bioessays 1993; 15:259–264.
49. Van Blerkom J, Davis P, Mathwig V, Alexander S. Domains of high-polarized and low-polarized mitochondria may occur in mouse and human oocytes and early embryos. Hum Reprod 2002; 17:393–406.
50. Trimarchi J, Liu L, Porterfield D, Smith P, Keefe D. Oxidative phosphorylation-dependent and -independent oxygen consumption by individual preimplantation mouse embryos. Biol Reprod 2000; 62:1866–1874.
51. Houghton FD. Energy metabolism of the inner cell mass and trophectoderm of the mouse blastocyst. Differentiation 2006; 74:11–18.
52. Kopsidas G, Kovalenko SA, Heffernan DR, Yarovaya N, Kramarova L, Stojanovski D, Borg J, Islam MM, Caragounis A, Linnane AW. Tissue mitochondrial DNA changes: a stochastic system. Ann N Y Acad Sci 2000; 908:226–243.
53. Lee HC, Yin PH, Lin JC, Wu CC, Chen CY, Wu CW, Chi CW, Tam TN, Wei YH. Mitochondrial genome instability and mtDNA depletion in human cancers. Ann N Y Acad Sci 2005; 1042:109–122.
54. Hansson A, Hance N, Dufour E, Rantanen A, Hultenby K, Clayton DA, Wibom R, Larsson NG. A switch in metabolism precedes increased mitochondrial biogenesis in respiratory chain-deficient mouse hearts. Proc Natl Acad Sci U S A 2004; 101:3136–3141.
55. Sathananthan H, Pera M, Trounson A. The fine structure of human embryonic stem cells. Reprod Biomed Online 2002; 4:56–61.
56. Jarrell VL, Day BN, Prather RS. The transition from maternal to zygotic control of development occurs during the 4-cell stage in the domestic pig, *Sus scrofa*: quantitative and qualitative aspects of protein synthesis. Biol Reprod 1991; 44:62–68.
57. Bolton VN, Oades PJ, Johnson MH. The relationship between cleavage, DNA replication, and gene expression in the mouse 2-cell embryo. J Embryol Exp Morphol 1984; 79:139–163.
58. Camous S, Kopecny V, Flechon JE. Autoradiographic detection of the earliest stage of [^{3}H]-uridine incorporation into the cow embryo. Biol Cell 1986; 58:195–200.
59. Ma J, Svoboda P, Schultz RM, Stein P. Regulation of zygotic gene activation in the preimplantation mouse embryo: global activation and repression of gene expression. Biol Reprod 2001; 64:1713–1721.
60. Cohen J, Scott R, Schimmel T, Levron J, Willadsen S. Birth of infant after transfer of anucleate donor oocyte cytoplasm into recipient eggs. Lancet 1997; 350:186–187.
61. Barritt JA, Willadsen S, Brenner C, Cohen J. Epigenetic and experimental modifications in early mammalian development. Part II: cytoplasmic transfer in assisted reproduction. Hum Reprod Update 2001; 7:428–435.
62. St John JC. Ooplasm donation in humans: the need to investigate the transmission of mitochondrial DNA following cytoplasmic transfer. Hum Reprod 2002; 17:1954–1958.
63. Gibson TC, Kubisch HM, Brenner CA. Mitochondrial DNA deletions in rhesus macaque oocytes and embryos. Mol Hum Reprod 2005; 11:785–789.
64. Chen CH, Cheng YC. Delayed cytotoxicity and selective loss of mitochondrial DNA in cells treated with the anti-human immunodeficiency virus compound 2′,3′-dideoxycytidine. J Biol Chem 1989; 264:11934–11937.
65. Keilbaugh SA, Hobbs GA, Simpson MV. Effect of 2′,3′-dideoxycytidine on oxidative phosphorylation in the PC12 cell, a neuronal model. Biochem Pharmacol 1997; 53:1485–1492.
66. Poulton J, Morten K, Freeman-Emmerson C, Potter C, Sewry C, Dubowitz V, Kidd H, Stephenson J, Whitehouse W, Hansen FJ, Parisi M, Brown G. Deficiency of the human mitochondrial transcription factor h-mtTFA in infantile mitochondrial myopathy is associated with mtDNA depletion. Hum Mol Genet 1994; 3:1763–1769.