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Transcriptional insights on the incomplete cytoplasmic maturation and developmental potential of oocytes cultured without granulosa cells in mice

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Abstract

Background Oocyte maturation is crucial for female fertility and embryonic development, encompassing nuclear and cytoplasmic maturation. Supportive cells of follicles, such as granulosa cells, are essential for oocyte growth and maturation. Oocytes can achieve nuclear maturation without granulosa cells during in vitro maturation (IVM). However, there is still a higher chance of incomplete cytoplasmic maturation for these oocytes with mature nuclei compared with oocytes cultured with granulosa cells. Oocytes with incomplete cytoplasmic maturation have lower fertilization rates and developmental potential than mature ones, although underlying mechanisms are poorly understood. Identifying key genes and signaling pathways associated with oocyte cytoplasmic maturation can help further elucidate the maturing process of oocytes and understand the impact of immature oocytes on embryonic development, throwing insights into the strategy to improve the success rate of assisted reproductive technologies.

Results Our study investigated murine oocytes maturing with and without granulosa cells. IVM without granulosa cells yielded oocytes with lower nuclear maturation rates than IVM with granulosa cells and in vivo maturation (IVO). Even though oocytes could achieve nuclear maturation without granulosa cells, they showed incomplete cytoplasmic maturation featuring higher levels of reactive oxygen species, lower mitochondrial density, and higher proportions of cells with abnormal distributions of cortical granules. Of note, oocytes with immature and mature cytoplasm had distinct transcriptional profiles. In the immature oocytes, we observed a deficient mRNA restoration of genes in crucial

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regulatory pathways of cellular growth and division, potentially affecting embryonic development. Differentially expressed genes (DEGs) between immature and mature oocytes were identified to be highly expressed in different pre-implantation stages, such as the MII oocyte, the 8-cell stage, and the ICM stage. Identified DEGs were enriched in key regulatory pathways of fertilization and embryonic development, such as energy and metabolic pathways. These observations indicated that the impeded development potential of oocytes with immature cytoplasm might be the result of abnormal gene expressions during oocyte maturation.

Conclusions We show that granulosa cells are important for both nuclear and cytoplasmic maturation of oocytes. Abnormal gene expression in oocytes with incomplete cytoplasmic maturation may be associated with potential defects in fertilization and embryonic development.

Keywords Oocyte cytoplasmic maturation, In vitro maturation, Granulosa cells, Fertilization, Embryonic development

Introduction

Oocyte maturation is a key factor in female reproduction. High-quality and mature oocytes are essential for maintaining fertility and are typically accomplished by carefully managing meiotic arrest and resumption [1]. Any disruption could lead to oogenesis failure, significantly affecting fertility and female health [2–4]. Mammalian oocyte maturation encompasses nuclear and cytoplasmic maturation, both of which are essential for subsequent fertilization and embryonic development [4]. Numerous biological processes influence oocyte maturation, such as organelle redistribution, epigenetic regulation, the regulation of maternal mRNA, and communications with surrounding somatic cells [5–9].

Follicles are basic functional units in ovaries, composed of oocytes, granulosa cells, and theca cells. The crosstalk between granulosa cells and oocytes is a key factor in regulating the growth and maturation of oocytes [10–12]. Granulosa cells can provide nutrients and signal exchange for oocytes [13–16]. Disrupting physical connections between granulosa cells and oocytes, such as gap junctions, transzonal projections, and microvilli, can impede oocyte maturation [11, 17, 18]. Fully grown germinal vesicle (GV)-stage oocytes during IVM can undergo meiotic resumption from arrest, germinal vesicle breakdown (GVBD), and extrusion of the first polar body (PB1), which are indicators of nuclear maturation [19, 20]. However, their fertilization rates and potential for early embryonic development are significantly lower than oocytes matured in vivo, implying a defective maturation status besides nuclear maturation [6, 20]. IVM oocytes without granulosa cells may not achieve cytoplasmic maturation simultaneously with nuclear maturation.

To understand oocyte cytoplasmic maturation, we collected murine oocytes from IVM and in vivo maturation (IVO) processes. IVM oocytes comprised two subgroups: oocytes in cumulus-oocyte complex (COC) and denuded oocytes (DO), representing oocytes cultured with and without granulosa cells in vitro. DO oocytes had lower nuclear maturation rates compared with COC and IVO oocytes. Unlike nuclear maturation, it remains challenging to discern cytoplasmic maturation based on morphological criteria alone [21]. A recent study revealed that the levels of reactive oxygen species (ROS) and organelle organization could serve as potential indicators of cytoplasmic maturation [21].

Consistent with previous findings, DO oocytes with mature nuclei showed incomplete cytoplasmic maturation compared with COC and IVO oocytes, with excessive ROS levels and disorganized organelles. Smart-seq2 was used to profile the transcriptome of oocytes with mature nuclei from all the groups. Oocytes with immature and mature cytoplasm showed distinct transcriptional profiles, implying a substantial effect of immature cytoplasm on gene expression. Moreover, maternal factors play an important role in regulating fertilization and early embryonic development. In the immature oocytes, insufficient mRNA restoration of genes in key regulatory pathways, such as mitotic DNA replication and tRNA methylation, could hinder subsequent cell growth and differentiation, resulting in fertilization failure and defects in embryonic development.

In summary, we have utilized experiments and single-cell sequencing technology Smart-seq2 to reveal that granulosa cells play important role in regulating the nuclear and cytoplasmic maturation of oocytes. The successful occurrence of nuclear and cytoplasmic maturation is vital for the fertilization of oocytes and subsequent early embryonic development. Therefore, in assisted reproductive technologies (ART), the cytoplasmic maturation of oocytes should be closely monitored. New strategies that enable cytoplasmic maturation to occur in synchrony with nuclear maturation may represent a novel approach to effectively increase the success rate of ART.

Results

The absence of granulosa cells impairs the nuclear and cytoplasmic maturation of oocytes

For the IVM model, fully grown GV-stage oocytes were cultured with (COC) and without (DO) granulosa cells separately (Fig. 1A). For the IVO model, oocytes were

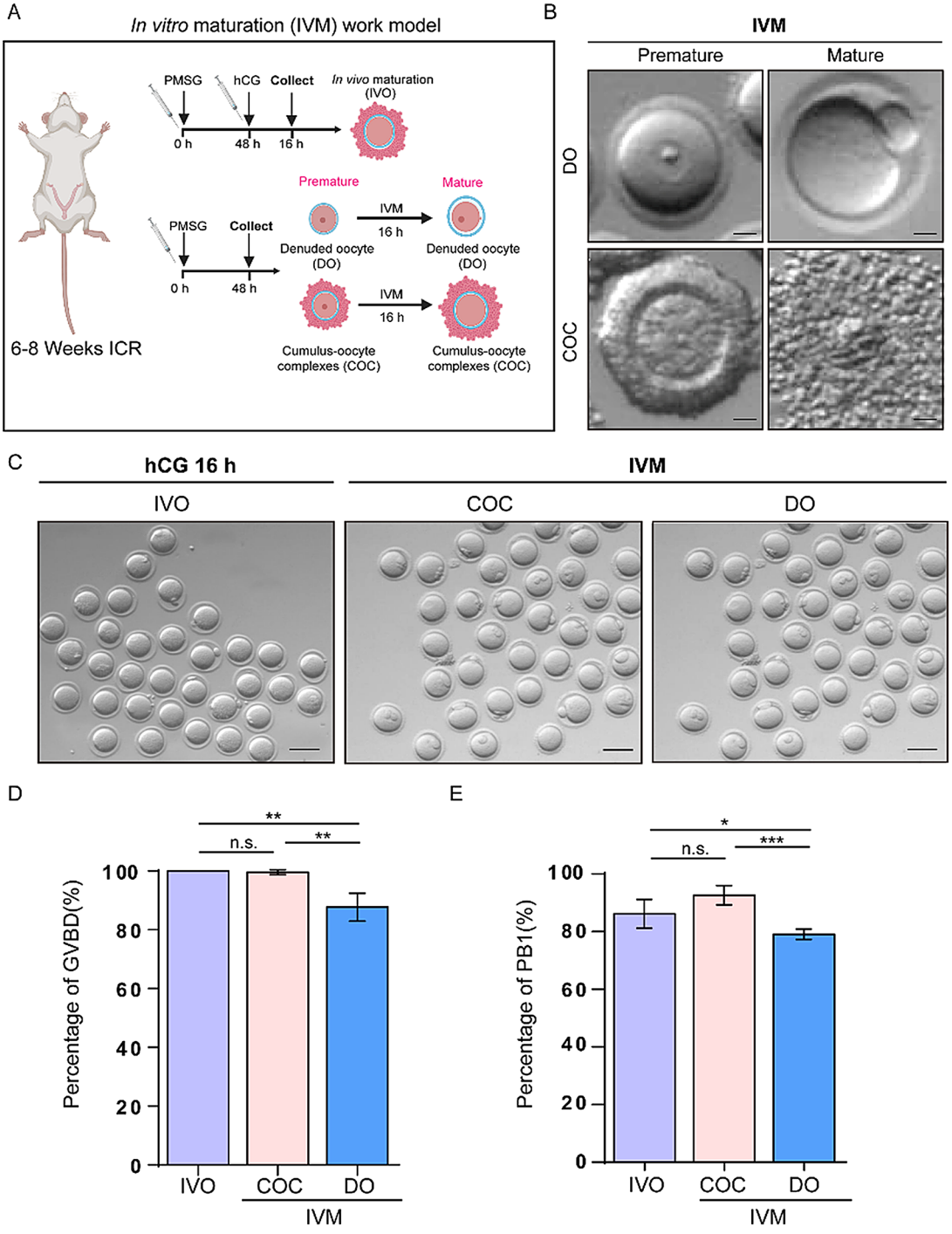

Fig. 1 Differences in maturation of oocytes with different maturation methods. (**A**) The schematic diagram illustrates the workflow of oocyte collection and in vitro maturation (IVM) in mice, with the in vivo maturation (IVO) group serving as a control. Oocytes of the IVM group, including denuded oocytes (DO) and cumulus-oocyte complexes (COC), were collected from ovaries 48 h after PMSG treatment. Oocytes of the IVO group were collected 16 h after hCG treatment. (**B**) Representative bright field images show the morphology of premature and mature oocytes from the DO and COC groups after IVM, with mature oocytes exhibiting germinal vesical breakdown (GVBD) and polar body extrusion (PB1). (**C**) Bright field images display oocytes from IVO, COC, and DO groups after cumulus cell removal with hyaluronidase from groups of IVO and COC. (**D**) Statistical analysis of the germinal vesicle breakdown (GVBD) rates in oocytes from the IVO, COC, and DO groups ($n = 5$ per group). (**E**) Statistical analysis of the PB1 rates in oocytes from the IVO, COC, and DO groups ($n = 5$ for each group). The data in panels D and E are shown as the mean ± SD. Statistical significance: n.s., not significant; *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$. Statistical analysis was performed by a two-tailed unpaired Student's t-test. Scale bar in panel B: 15 μm. Scale bar in panel C: 100 μm

collected 16 h after human chorionic gonadotropin (hCG) treatment (Fig. 1A). Both IVM protocols could yield oocytes that have expelled PB1 (Fig. 1B). However, the absence of granulosa cells could result in a lower nuclear maturation rate (Fig. 1C-E). The proportion of oocytes with GVBD and PB1 in the DO group was significantly lower than that in the COC and IVO groups (Fig. 1D-E).

Besides the nuclear maturation rate, we further evaluated the cytoplasmic maturation of oocytes with mature nuclei in all groups. Cytoplasmic maturation was assessed by measuring cytoplasmic ROS levels, as well as the density and distribution of mitochondria and cortical granules (CGs) (Fig. 2) [20, 22]. Compared with IVO oocytes, DO oocytes had significantly higher cytoplasmic ROS levels (Fig. 2A-B), lower mitochondrial densities (Fig. 2C-E), and higher proportions of cells with an abnormal distribution of CGs (Fig. 2F-H), indicating an overall defective status of cytoplasmic maturation. COC oocytes also had suboptimal cytoplasmic maturation compared with the IVO group in almost all the indicators (Fig. 2) but better than the DO group, implying other factors besides granulosa cells may also be important to the cytoplasmic maturation. Our results suggested that granulosa cells are important to both the nuclear and cytoplasmic maturation of oocytes.

Oocytes with mature and immature cytoplasm show distinct transcriptional profiles

To understand oocyte cytoplasmic maturation, we applied Smart-seq2 to profile the transcriptome of oocytes with mature nuclei in IVO, COC, and DO groups (Fig. 3A). To determine cytoplasmic maturation level of oocytes, we selected 13 pathways involved in the transcriptional regulation, cell cycle, DNA repair, RNA metabolism, as well as oocyte maturation and development [22, 23]. Among those 13 pathways, 11 of them were positively correlated with cytoplasmic maturation, and 2 of them were negatively correlated. Of note, DO oocytes showed the lowest enrichment scores in most positively correlated pathways and the highest scores in most negatively correlated ones (Fig. S1). The cytoplasmic maturation score was calculated based on these 13 pathways to evaluate the cytoplasmic maturation of each oocyte (see Methods for details). All DO oocytes except DO5 had the lowest cytoplasmic maturation scores, confirming their defects in cytoplasmic maturation (Fig. S2).

We further selected oocytes with the highest and lowest cytoplasmic maturation scores to characterize the transcriptomic changes along with oocyte cytoplasmic maturation. The mature group consisted of three IVO samples and two COC samples, while all the oocytes in the immature group were DO samples (Fig. S2). We observed significant transcriptional differences between the immature and mature groups (Fig. 3B-D; Fig. S3). In total, we determined 870 differentially expressed genes (DEGs), including 651 up-regulated and 219 down-regulated DEGs in the immature group (Table S1). Identified DEGs were enriched in pathways related to oocyte maturation, metabolism, organelle, and embryonic development, confirming the immature status of DO oocytes and suggesting that the dysregulated genes due to immature cytoplasm may impair subsequent biological processes such as embryonic development (Fig. 3E).

Oocytes with immature cytoplasm fail to reserve the necessary mRNAs for subsequent developmental processes

Fully grown GV-stage oocytes are transcriptionally silent and must stably reserve mRNAs for oocyte maturation and early embryonic development [24]. We thus questioned whether oocytes with immature cytoplasm successfully reserve the necessary mRNA. Gene set enrichment analysis (GSEA) identified several important pathways for early embryonic development, such as mitotic DNA replication, tRNA methylation, and mitotic spindle pole, whose expression was down-regulated in the immature group (Fig. 4), implying deficient mRNA storage. DNA replication, tRNA modification, and spindle migration are essential molecular processes for cell growth and division [25–27]. Abnormalities in these signaling pathways often cause abnormal embryonic division, apoptosis, and aneuploidy, ultimately leading to the failure of early embryonic development [25–27]. These findings suggested immature oocytes fail to make the necessary preparations for subsequent development, which could partially explain their lower fertilization rates and developmental potential.

Abnormal expression of stage-specific high-expression genes in oocytes with immature cytoplasm may affect fertilization and early embryonic development

We next compared DEGs between the immature and mature groups with highly expressed genes in each preimplantation stage in mice (see Methods for details). From tens of DEGs to hundreds of DEGs were found to be highly expressed in each stage (Fig. 5A; Fig. S4; Table S2).

We first focused on fertilization, which is the first step in initiating embryonic development. Abnormal fertilization during IVM is a leading factor in ART failure [28, 29]. Maternal genes that are highly expressed in MII oocytes were reported to play important roles in fertilization and embryonic development [30, 31]. In total, 130 DEGs showed the highest expression in MII oocytes (Fig. 5A). These DEGs were primarily involved in the regulation of oocyte fertilization (Fig. 5B), suggesting that the lower fertilization rate of oocytes with incomplete

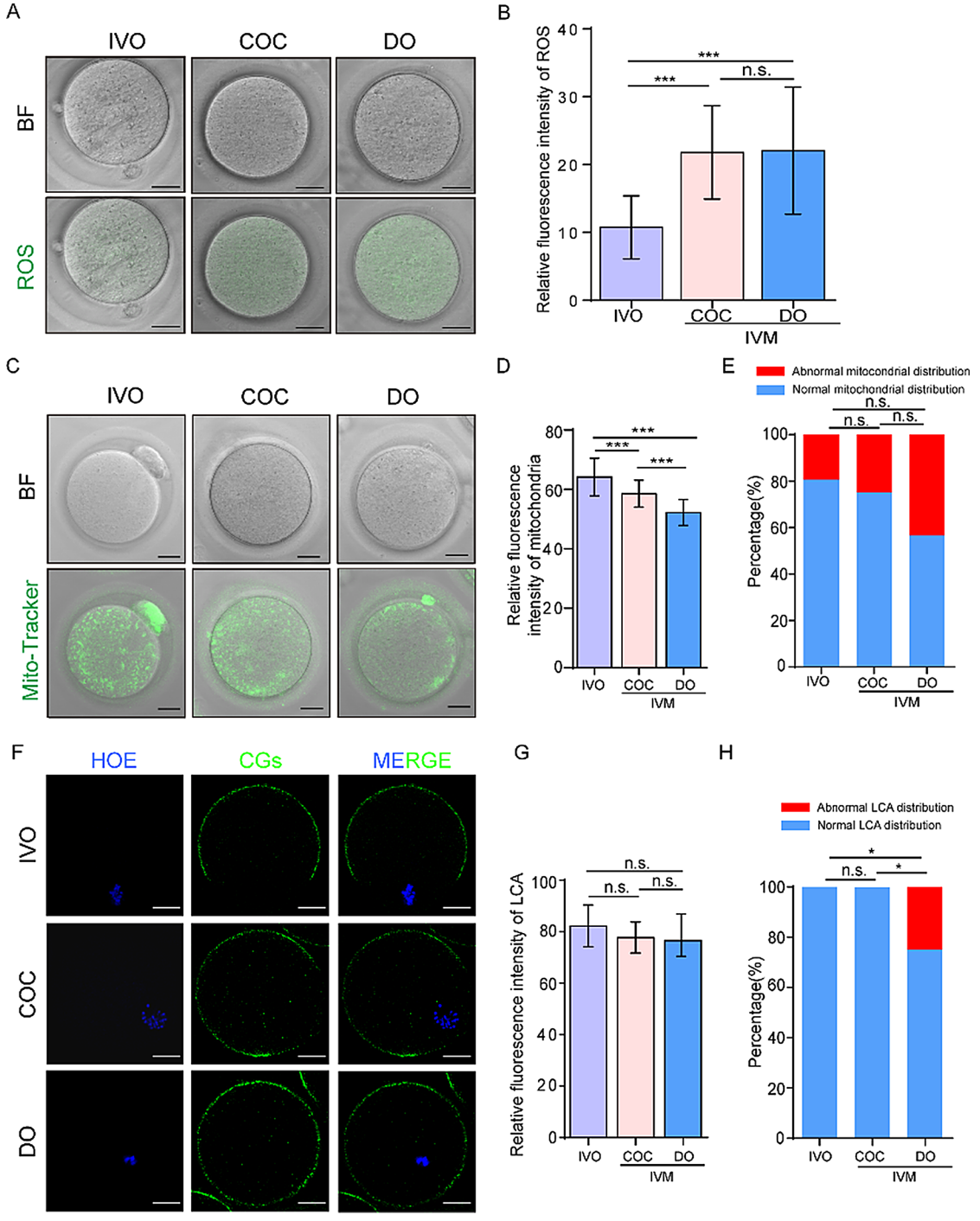

Fig. 2 Differences in cytoplasmic maturation of oocytes with different maturation methods. (**A**) Reactive oxygen species (ROS) levels (green) were detected in oocytes with nuclear maturation from the IVO, COC, and DO groups. BF, bright field. (**B**) Fluorescence intensity quantification of ROS was measured in oocytes from the IVO, COC, and DO groups after maturation ($n = 10–16$ oocytes per group). (**C**) Mitochondrial distribution (green) was detected in oocytes with nuclear maturation from the IVO, COC, and DO groups staining with MitoTracker. BF, bright field. (**D**) Fluorescence intensity and statistical analysis of mitochondrial signals were performed in oocytes from the IVO, COC, and DO groups after maturation ($n = 22–33$ oocytes per group). (**E**) Analysis of abnormal mitochondrial distribution rates in oocytes from the IVO, COC, and DO groups after maturation ($n = 22–33$ oocytes per group). (**F**) Distribution of cortical granules (CGs, green) was detected in oocytes with nuclear maturation from the IVO, COC, and DO groups. HOE, Hoechst. (**G-H**) Analysis of fluorescence intensity quantification and mislocalization rates of CGs were detected in oocytes from the IVO, COC, and DO groups after maturation ($n = 15–25$ oocytes per group). The data in panels B, D, E, G, and H are shown as the mean ± SD. Statistical significance: n.s., not significant; *, $p < 0.05$; ***, $p < 0.001$. Statistical analysis was performed by a two-tailed unpaired Student's t-test or Fisher's exact test. Scale bar: 15 μm

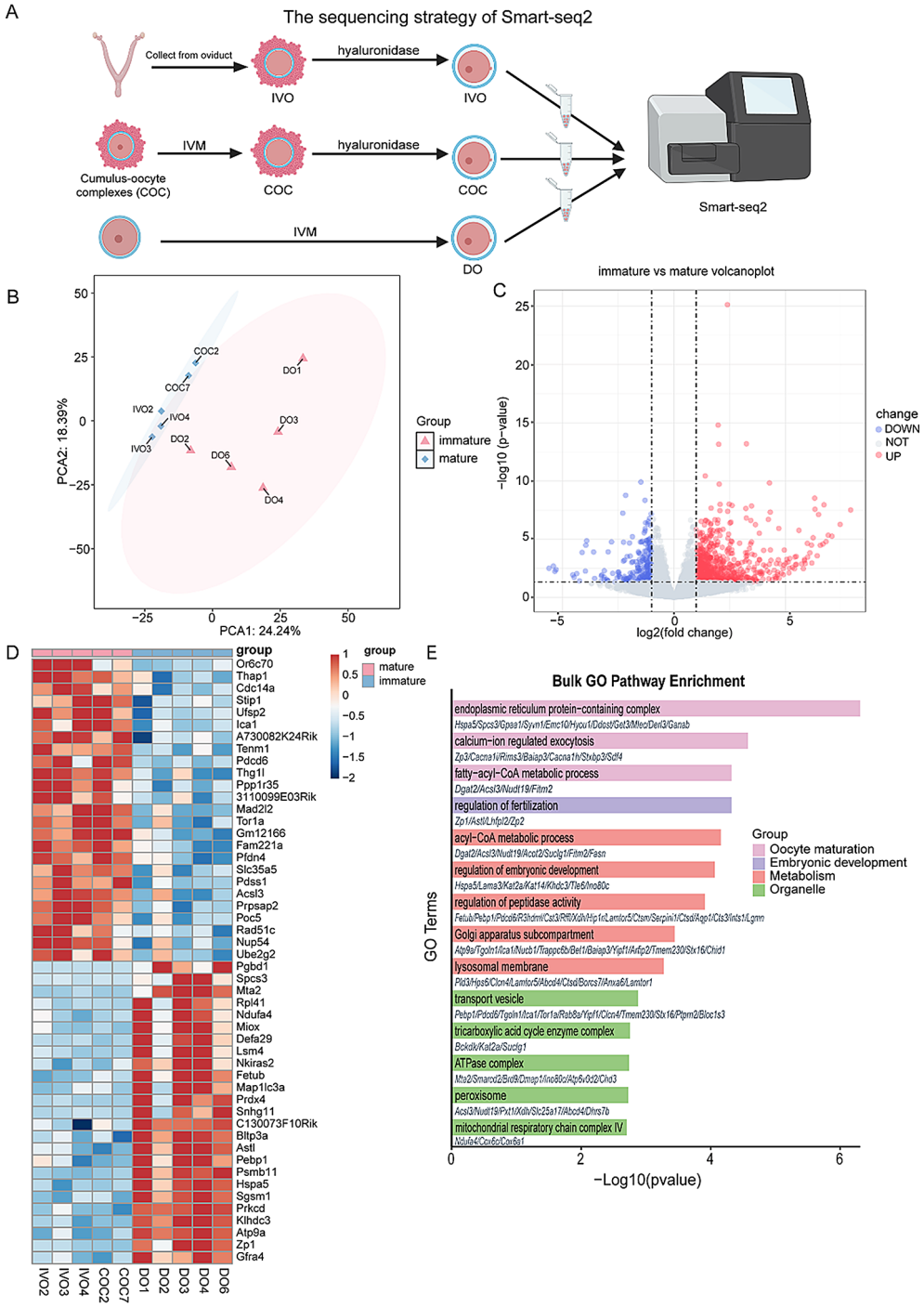

Fig. 3 Differentially expressed genes (DEGs) and gene ontology (GO) enrichment analysis in mature and immature mouse oocytes. (**A**) Schematic diagram of sample collection and Smart-seq2 single-oocyte sequencing of IVO, COC, and DO oocytes in mice. Oocytes were collected from 6–8 week-old mice (IVO, $n=7$; COC, $n=7$; DO, $n=6$). All obtained oocytes were with extrusion of PB1. (**B**) Principal component analysis (PCA) plot of single-oocyte RNA transcriptomes from mature (IVO2, IVO3, IVO4, COC2, COC7) and immature oocytes (DO6, DO4, DO3, DO2, DO1). (**C**) Volcano plot of differentially expressed genes (DEGs) showing the downregulated genes (DOWN), upregulated genes (UP), and unchanged genes (NOT) between mature and immature oocytes. DEGs were defined as genes with an adjusted p-value < 0.05 and $|\log_2 FC| > 1$. (**D**) Heatmap of the top 50 DEGs (25 upregulated genes and 25 downregulated genes) between mature and immature mouse oocytes. DEGs were defined as genes with an adjusted p-value < 0.05 and $|\log_2 FC| > 1$, ranked by ascending p-value and descending $|\log_2 FC|$. (**E**) Enrichment analysis of DEGs and representative Gene Ontology (GO) terms in mature and immature mouse oocytes. Enriched pathways were significantly related to oocyte maturation, embryonic development, metabolism, and organelles

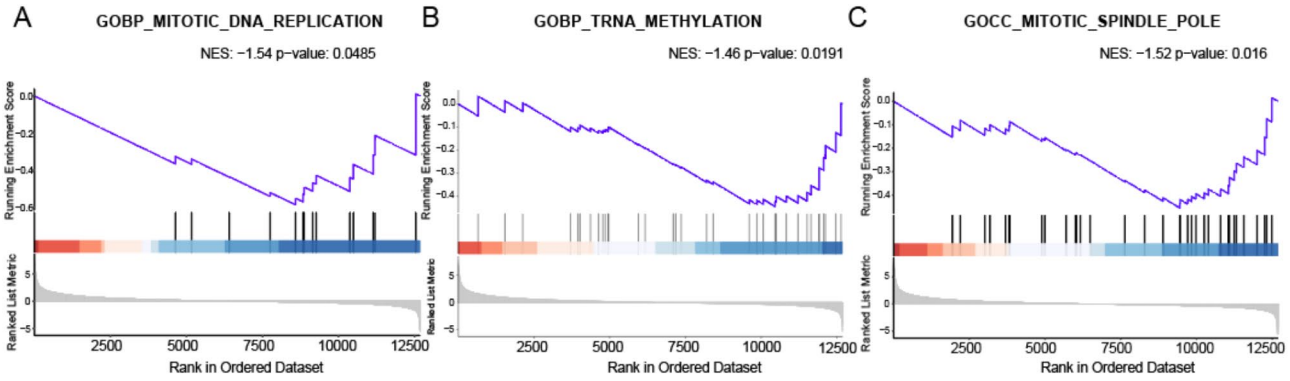

Fig. 4 Gene set enrichment analysis (GSEA) reveals important roles of nucleic acid stability and spindle pole in cytoplasmic maturation and early embryonic development. (**A**-**C**) GSEA enrichment showing significantly downregulated of the pathways: "Mitotic DNA replication", "tRNA methylation" and "Mitotic spindle pole" in immature oocytes

cytoplasmic maturation might be the result of abnormal functions of these genes. For instance, zona pellucida proteins ZP1, ZP2, and ZP3 were highly expressed in the immature group (Table S1). Overexpression of zona pellucida proteins was observed in GV oocytes of polycystic ovary syndrome (PCOS) rats, which typically have impaired oocyte qualities [32]. We also observed a downregulation of *Ppp3ca* in the immature group (Fig. 5C), which encodes the subunit alpha of calcineurin and is activated by increased cytoplasmic calcium concentrations [33]. We speculated that the cytoplasmic calcium levels could not be properly maintained in the immature oocytes, which are essential for oocyte maturation and subsequent calcium-dependent zona pellucida reaction at fertilization [34].

Besides DEGs highly expressed in MII oocytes, we also investigated DEGs highly expressed in other stages, such as the 8-cell and ICM stages (Fig. S4). Both of these two groups of DEGs were enriched in pathways related to embryonic development (Fig. 5D-E). DEGs highly expressed in the 8-cell stage were primarily involved in pathways of gene expression regulation, protein synthesis, RNA processing, and metabolism in cells, which are extremely important for cellular function and development (Fig. 5D). For DEGs highly expressed in the ICM stage, their enriched pathways were related to energy production, such as the oxidative phosphorylation and the mitochondrial ATP synthesis coupled electron transport (Fig. 5E), which support developmental processes of embryos.

Collectively, abnormal gene expression during oocyte cytoplasmic maturation may be associated with subsequent fertilization and embryonic development by disrupting key regulatory genes and signaling pathways.

Discussion

IVM of human immature oocytes is a viable option for patients at risk of ovarian hyperstimulation syndrome and can be combined with ovarian tissue cryobanking to assist the conception of cancer survivors [35]. Even though IVM provides several benefits, its lower success rates compared with the traditional in vitro fertilization cycles indicate that advanced techniques are needed to further improve the efficiency and effectiveness of IVM [36, 21]. During IVM, nuclear and cytoplasmic maturation must occur for the oocyte to be fertilized [37]. Unlike nuclear maturation, it remains challenging to discern cytoplasmic maturation based on morphological criteria alone. Therefore, we speculated that incomplete cytoplasmic maturation of oocytes might be an important reason for the lower success rate of IVM.

To confirm our hypothesis, we collected IVM oocytes cultured with and without granulosa cells as well as IVO oocytes. Granulosa cells are known to play important roles in supporting oocyte development via signal transduction and nutrient exchange with oocytes [12, 16, 39]. They participate in the regulation of meiotic arrest, meiotic resumption, cytoplasmic maturation, and global suppression of transcriptional activity in oocytes [4, 40–43]. Intervention of the interaction between oocytes and granulosa cells has a side effect on fertilization and early embryonic development [13]. Consistent with previous findings, we observed incomplete cytoplasmic maturation in IVM oocytes cultured without granulosa cells, featuring excessive cytoplasmic ROS levels and abnormalities in organelles, confirming the indispensable role of granulosa cells in oocyte cytoplasmic maturation.

Transcriptomic studies comparing COCs versus IVOs and comparing COCs versus DOs have been conducted in different species [44–46]. However, comparing three different maturation processes directly in the same species is still helpful in extending our understanding of oocyte cytoplasmic maturation. Smart-seq2 was used to

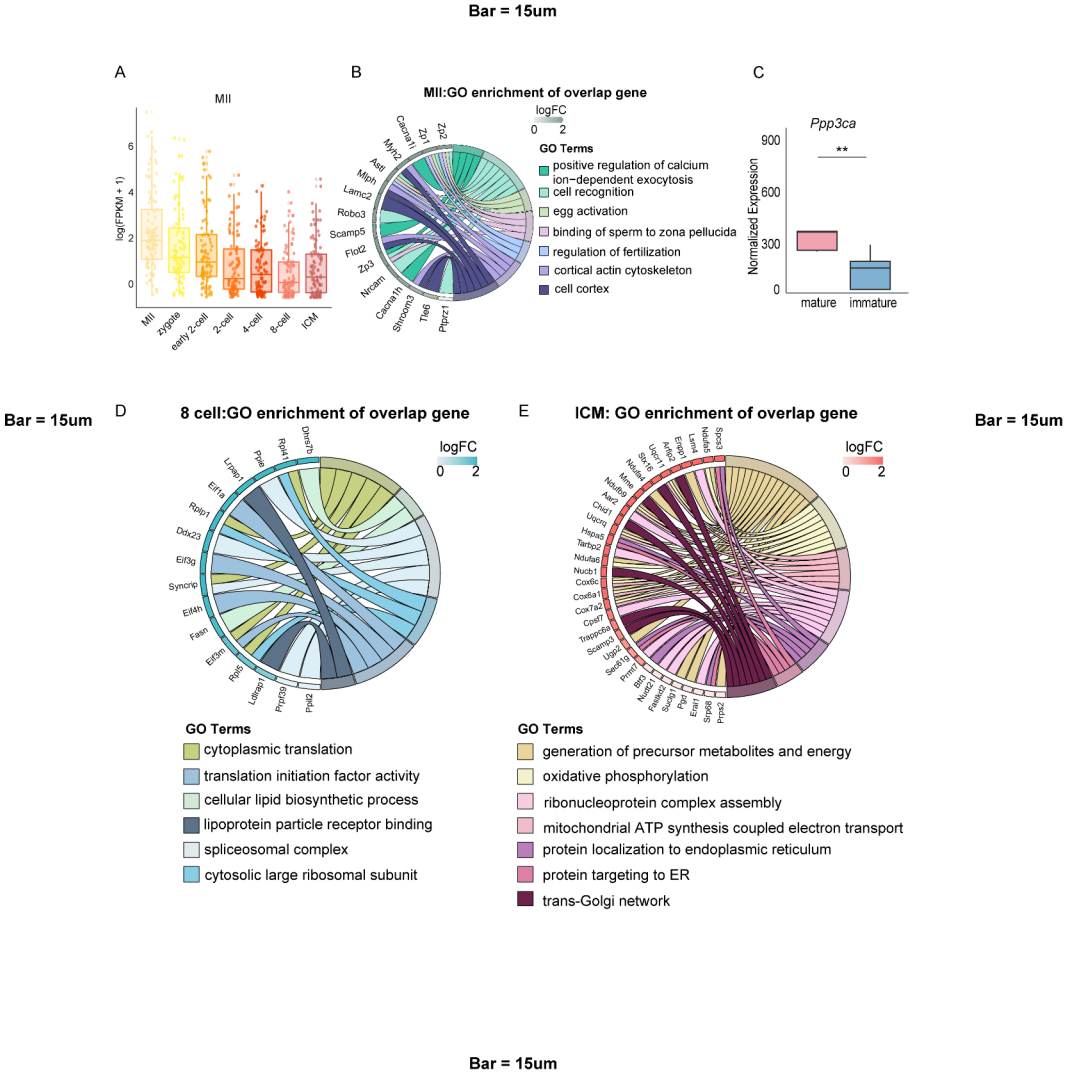

Fig. 5 Incomplete maturation of oocytes affects the fertilization and pre-implantation embryonic development process in mice. (**A**) Boxplots showing gene expression of DEGs that are highly expressed in the MII oocyte stage ($n = 130$ genes) across different early embryonic stages. (**B**) Chord plot displaying selected GO terms enriched of DEGs highly expressed in the MII oocyte stage. Genes are ordered by $\log_2$FC, with green indicating upregulation. (**C**) Boxplots showing the differential expression of *Ppp3ca* between mature and immature oocytes. (**D**) Chord plot displaying selected GO terms enriched of DEGs highly expressed in the 8-cell stage. Genes are ordered by $\log_2$FC, with blue indicating upregulation. (**E**) Chord plot displaying selected GO terms enriched of DEGs highly expressed in the ICM stage. Genes are ordered by $\log_2$FC, with orange indicating upregulation. Statistical significance: **, $p < 0.01$. *P*-value was calculated using a Wald test based on a negative binomial distribution

profile the transcriptome along with cytoplasmic maturation. We designed a transcriptional cytoplasmic maturation score and confirmed that it is positively correlated with the cytoplasmic maturation level, with the lowest numbers in the DO group. Comparison between oocytes with the highest (the mature group) and lowest (the immature group) scores identified a series of pathways that could explain incomplete cytoplasmic maturation or serve as potential indicators, such as pathways related to energy metabolism and organelle organization [47–51]. Here exists the new question of how the abnormal gene expression associated with immature oocyte cytoplasm would affect the subsequent fertilization and embryonic development. We observed a deficient mRNA restoration of genes in key regulatory pathways for cell proliferation and differentiation, including mitotic DNA replication, tRNA methylation, and mitotic spindle poles. Dysfunctions in these pathways are closely associated with abnormal embryonic cell division, cell death, and aneuploidy, which may ultimately lead to impaired embryonic development [52–57]. For example, the deceleration and halting of replication forks are common occurrences in the early developmental stages of mammals, which can result in genomic instability, aneuploidy, and developmental deficiency [52].

By comparing DEGs between the immature and mature groups with highly expressed genes in each pre-implantation stage, we found that dysregulated genes in the immature oocytes were extensively associated with different developmental stages. For instance, DEGs highly expressed in MII oocytes were enriched in pathways such as the regulation of fertilization and the positive regulation of calcium ion-dependent exocytosis, implying that the defective fertilization might be due to the unsuccessful maintenance of cytoplasmic calcium levels during oocyte maturation. As a potential indicator of cytoplasmic calcium concentrations [33], the encoding gene of the subunit alpha of calcineurin (*Ppp3ca*) was significantly down-regulated in the immature group. The abnormal calcium concentrations in the immature oocytes may impede subsequent calcium-dependent zona pellucida reaction at fertilization and induced polyspermy together with the abnormal distributions of CGs [58].

While we believe this work offers novel insights into the cytoplasmic maturation of oocytes, further work will be needed to validate our conclusions. For instance, the IVM media used for DOs and COCs were different, which may introduce potential variations in gene expression. The IVM medium for COC culture consists of unphysiological doses of EGF and FSH, which might also affect gene expression. A more sophisticated study design is also needed in the future. The model of DOs here did not mimic the clinical practice, where DOs subjected to IVM always failed to respond to the trigger of maturation. Their transcriptional abnormalities might be severer compared to DOs that can mature, which could provide extra valuable insights for improving the clinical IVM practice. Accumulating evidence suggests that co-culturing DOs with granulosa-like support cells has the promise to promote oocyte maturation and early embryonic development in the rescue IVM model [59, 60]. It would be informative to include an additional group where DOs are co-cultured with granulosa cells to further understand the transcriptional mechanism for the improved performance of this rescue IVM model. Lastly, the association between abnormal gene expression during oocyte cytoplasmic maturation and subsequent fertilization and embryonic development needs stronger supporting evidence. Sequencing data of the same mouse strain is needed for more concrete conclusions. Also, the association should be validated based on maternal genes instead of all genes, which requires further exploration.

In conclusion, our study helps to further understand how abnormalities during oocyte maturation could affect subsequent fertilization and embryonic development. We also provide a series of genes and pathways that could be disrupted upon incomplete cytoplasmic maturation, which would exert adverse effects on early embryonic development. However, due to the limited sample sizes and the lack of experimental validations, the causal relationships between their abnormal expression in immature oocytes and embryonic deficiencies require extra work in the future. Nonetheless, our work confirms the indispensable role of granulosa cells in both nuclear and cytoplasmic maturation, highlights the necessity of oocyte cytoplasmic maturation for fertilization and early embryonic development, and throws insights into the evaluation of oocyte cytoplasmic maturation. Novel strategies enabling cytoplasmic maturation along with nuclear maturation could effectively improve the success rate of ART in the future.

Materials and methods

Animals and ethics statement

The 6–8 week-old ICR male and female mice were obtained from the Experimental Animal Center of the Peking University Health Science Center Animal Laboratory Center and housed in a temperature- (22 ± 0.5 °C) and light-controlled environment (12 h light-12 h dark cycle) and provided with food and water ad libitum.

Collection of GV stage denuded oocytes (DOs) and cumulus-oocyte complexes (COCs) and in vitro maturation (IVM)

Female mice were stimulated by an intraperitoneal injection of 10 IU pregnant mare serum gonadotropin (PMSG, Ningbo Second Hormone Factory, X-001) and sacrificed by cervical dislocation 48 h later. The ovaries were collected and placed in the preheated M2 medium (Sigma, M7167). For denuded oocytes (DOs), Milrinone (1:1000, Selleck, S2484) was additionally added to the medium to prevent spontaneous maturation. Well-developed Graafian follicles were punctured with a 10 ml syringe and needle to collect DOs and COCs, and only those with appropriate size, morphological integrity, and a distinct germinal vesicle (GV) were selected under a stereomicroscope.

DOs were washed with the M2 medium and cultured in the M16 medium (Sigma, M7292). COCs were washed with the M2 medium and cultured in the IVM medium (Nanjing Aibei Biotechnology, M211). The IVM medium consists of the following components: 5% Fetal Bovine Serum (FBS) to provide essential growth factors and nutrients; 3 ng/ml Epidermal Growth Factor (EGF) to stimulate cell proliferation and differentiation; 50 mIU/ml Recombinant Follicle Stimulating Hormone (rhFSH) to mimic the natural hormone that promotes oocyte maturation; 0.25 mM Sodium Pyruvate as an energy substrate to support metabolism; 0.5% Penicillin and 0.5% Streptomycin to ensure sterility and prevent contamination; α-MEM as the base culture medium, which provides a balanced environment for cell growth. All GV oocytes

were cultured at 37 °C under an atmosphere of 5% CO2 in air with maximum humidity for 16–18 h. Granulosa cells of COCs were removed using hyaluronidase (Nanjing Aibei Biotechnology, M2215). The maturation rate of oocytes and the proportion of first polar body (PB1) extrusion were assessed under the microscope.

In vivo maturation (IVO)

Female mice were stimulated by an intraperitoneal injection of 5–10 IU PMSG, and mice were intraperitoneally injected with 5–10 IU of human chorionic gonadotropin (hCG, Ningbo Second Hormone Factory, D-001). Sixteen hours later, the mice were sacrificed with cervical dislocation. Their fallopian tubes were exposed under sterile conditions and placed into the preheated M2 medium, rinsed twice, and quickly torn using a sterile syringe needle. IVO oocytes were selected under the stereo microscope. Impurities such as granulosa cells were removed using hyaluronidase (Nanjing Aibei Biotechnology, M2215). The maturation rate of oocytes and the proportion of PB1 extrusion were assessed under the stereo microscope.

Lens culinaris agglutinin (LCA)-FITC staining

Oocytes were treated with Acidic Tyrode's Solution (Millipore, MR-004-D) to remove the zona pellucida, then incubated with Lens culinaris agglutinin (LCA)-FITC staining (Shanghai Jiwei Biotechnology, G&VS14052.2). Specifically, oocytes were first incubated with 500 μL of fixative (Reagent A) at room temperature for 30 min, washed three times with Reagent B, and permeabilized with 500 μL of Reagent C for 5 min. After washing twice with Reagent B, 100 μL of LCA-FITC staining solution (Reagent D) was added, and the oocytes were incubated at room temperature for 30 min. Samples were then counterstained with Hoechst (1:100, Beyotime Biotechnology, C1027) for 5 min at 37 °C in 5% CO2. After washing 3 times with PBS supplemented with BSA, the oocytes were observed by laser scanning confocal microscopy, and the fluorescence intensity of the oocytes was calculated with ImageJ (ImageJ software, Bethesda, MD, USA).

MitoTracker staining

Oocytes were incubated with MitoTracker (1:1000, ThermoFisher, C10600) in M2 medium for 30 min at 37 °C in a 5% CO2 and saturated humidity. Then, the oocytes were counterstained with Hoechst (1:100, Beyotime Biotechnology, C1027) for 5 min at 37 °C in a 5% CO2 and saturated humidity, and finally, the samples were washed three times with M2 medium and examined with an inverted confocal microscope (LSM 880). The fluorescence intensity of the oocytes was calculated with ImageJ (ImageJ software, Bethesda, MD, USA).

Monitoring of reactive oxygen species (ROS) levels in oocytes

The amount of ROS in oocytes was processed with 10 μM oxidation-sensitive fluorescent probe DCFH (Beyotime Biotechnology, S0033S) for 30 min at 37℃ in M2 medium. Then oocytes were washed three times with M2 medium and placed on glass slides for image capture under an inverted confocal microscope (LSM 880). And the fluorescence intensity of the oocytes was calculated with ImageJ (ImageJ software, Bethesda, MD, USA).

Library preparation, sequencing, and data preprocessing of Smart-seq2

IVO ($n=7$), COC ($n=7$), and DO ($n=6$) oocytes were collected from three mice separately. The mRNA was isolated from each oocyte and reverse transcribed using oligo-dT primers, followed by template switching for full-length cDNA synthesis. The cDNA was then amplified using PCR. After amplification, the cDNA was processed for library construction, including end-repair, adapter ligation, and further PCR amplification. The library quality was confirmed and sequencing was performed on the Illumina platform with PE150.

Transcriptional determination of oocytes cytoplasmic maturation

To evaluate the cytoplasmic maturation of oocytes, 13 GO terms reported to be highly associated with oocyte cytoplasmic maturation were selected (eleven terms were positively correlated, and two terms were negatively correlated). The *bitr* function in the R package clusterProfiler v4.8.3 was used to map GO terms to gene sets. Pseudogenes and genes expressed in fewer than ten samples (half of all the 20 oocytes) were excluded. Gene expression data were normalized using the *vst* function in the R package DESeq2 v1.40.2. Single-sample Gene Set Enrichment Analysis (ssGSEA) was applied to calculate enrichment scores of all the oocytes in 18 gene sets via the R package GSVA v1.48.3. Z-scores of enrichment scores were calculated across all the 20 oocytes in each gene set. The cytoplasmic maturation score of each oocyte was determined by the average enrichment scores of gene sets positively correlated with cytoplasmic maturation minus the average enrichment scores of gene sets negatively correlated with cytoplasmic maturation. The correlation score with cytoplasmic maturation of each gene set was determined by the enrichment score difference between the top five and bottom five oocytes ranked by the cytoplasmic maturation score.

Identification of differentially expressed genes (DEGs) between the immature and mature groups

Pseudogenes and genes expressed in fewer than five samples (half of all the ten oocytes in the immature and

mature groups) were excluded. DEGs were determined by R package DESeq2 v1.40.2. Up-regulated genes in the immature group were decided by log2 fold change > 1 and p-value < 0.05 while down-regulated genes were decided by log2 fold change < -1 and p-value < 0.05.

Gene ontology (GO) enrichment analysis of DEGs

Pathway enrichment analysis was performed by *enrichGO* function in the R package clusterProfiler v4.8.3.

Gene set enrichment analysis (GSEA)

Preranked GSEA was performed by the R package clusterProfiler v4.8.3 using the log2 fold change of gene expression in the immature group versus the mature group.

Determination of highly expressed genes in different pre-implantation stages

Gene expression data of pre-implantation embryos from MII oocyte to inner cell mass (ICM) in mice were retrieved from the public dataset GSE66582. For each stage, gene expression was averaged across replicates. Genes expressed in fewer than four stages (half of seven stages in total) were excluded. Each gene was assigned to highly expressed genes of a specific stage when it expressed highest in this stage compared with all the others.

Statistical analysis

All data are presented as mean ± SD. GraphPad Prism 6 Software (CA) was used for statistical analysis. A two-sided Student's t-test was used for comparisons between the continuous values of the two groups. Fisher's exact test was used for comparisons of categorical data. Statistical significance: n.s., not significant; *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; ****, $p < 0.0001$.

Abbreviations

ART	Assisted Reproductive Technology
IVM	In Vitro Maturation
IVO	In Vivo Maturation
IVF	In Vitro Fertilization
ICSI	Intracytoplasmic Sperm Injection
GV	Germinal Vesicle
GVBD	Germinal Vesicle Breakdown
PB1	First Polar Body
COC	Cumulus-Oocyte Complex
DO	Denuded Oocyte
ROS	Reactive Oxygen Species
CGs	Cortical Granules
DEGs	Differentially Expressed Genes
MII	Metaphase II (stage of oocyte)
ICM	Inner Cell Mass
ATP	Adenosine Triphosphate
PCA	Principal Component Analysis
GEO	Gene Expression Omnibus
LCA	Lens Culinaris Agglutinin
FITC	Fluorescein Isothiocyanate
PMSG	Pregnant Mare Serum Gonadotropin
hCG	Human Chorionic Gonadotropin
ICR	Refers to a mouse strain
GO	Gene Ontology
GSEA	Gene Set Enrichment Analysis
ssGSEA	Single-sample Gene Set Enrichment Analysis

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12864-025-11455-7.

Supplementary Material 1: Supplementary Table 1. DEGs between the immature and mature groups

Supplementary Material 2: Supplementary Table 2. DEGs between the immature and mature groups that are highly expressed in each pre-implantation stage.

Supplementary Material 3

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Author contributions

This project was conceived and coordinated by H.P., Y.F., and Y.Y. The experiments were carried out by Y.B.W., Y.C.Z., T.J.L., Y.R., Y.X.L, Y.N.Q., and H.C.Z. Data analyses were performed by Y.C.Z., Y.R., P.Z., L.F., C.X.X., Z.Y.H., H.J.H., and W.F.X. The paper was written and revised by Y.B.W., Y.C.Z., T.J.L., Y.R., Y.Y., Y.F., and H.P.

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Data availability

The public dataset is available at GSE66582. Derived data supporting the findings of this study are available at https://dataview.ncbi.nlm.nih.gov/object/PRJNA1208207?reviewer=qnfplh85d9jpc63ffsvsje128d.

Declarations

Ethics approval and consent to participate

This study is in accordance with ARRIVE guidelines. All procedures executed in mice were approved by the Institutional Animal Care and Use Committee (IACUC) of Peking University Health Science Center Animal Laboratory Center (Beijing, China). Ethical approval for animal survival was given by the Animal Ethics Committee of Peking University Third Hospital (Beijing, China). All mice were sacrificed before the removal of organs based upon the IACUC Guidelines for the euthanasia of experimental animals (Permission No. A2023007).

Consent for publication

Not applicable.

Competing interests

The authors declare no competing interests.

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