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**Single cell profiling of transcriptomic changes during *in vitro* maturation
of human oocytes**

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Abstract

In vitro maturation of human oocytes is widely used for infertility treatment. However, the success rate of maturation varies depending on patients and molecular mechanisms underlying successful maturation remain unclear. Especially, gene expression profiles of oocytes at each maturation stage need to be revealed to understand the differential developmental abilities of oocytes. Here, we show transcriptomes of human oocytes during *in vitro* maturation by single cell RNA-seq analyses. Hundreds of transcripts dynamically altered their expression, and we identify molecular pathways and upstream regulators that may govern oocyte maturation. Furthermore, oocytes that are delayed in their maturation show distinct transcriptomes. Finally, we reveal genes whose transcripts are enriched in each maturation stage and that can be used for selecting an oocyte with a high developmental potential. Taken together, our work uncovers transcriptomic changes during human oocyte maturation and provides a molecular insight into the differential developmental potential of each oocyte.

Keywords

Human oocyte; Single cell RNA-sequencing, Oocyte maturation; Transcriptome

Introduction

The first successful pregnancy of an *in vitro* matured human oocyte after *in vitro* fertilization was reported in 1991¹. Although the clinical application of *in vitro* maturation (IVM) has increased since this report², IVM technology is not widely used in infertility treatment due to its poor outcome. Importantly, it is generally regarded that the quality of embryos generated from IVM oocytes is worse than that of embryos from *in vivo* matured oocytes in multiple aspects, such as developmental competence and subsequent pregnancy rates^{3,4}. Therefore, oocytes other than the metaphase II (MII) stage at the time of oocyte retrieval are discarded in many fertility clinics. However, IVM of immature oocytes offers a unique opportunity. For women diagnosed with polycystic ovary syndrome, IVM of non-MII oocytes can prevent the risk of ovarian hyperstimulation syndrome. In addition, ovarian tissue is cryopreserved prior to cancer treatment for ensuring fertility of patients who might lose gonadal functions due to the treatment. The introduction of this fertility preservation techniques has provided the possibility of obtaining immature oocytes from very small antral follicles and cryopreserving them after *in vitro* maturation⁵. There are reports of pregnancy from embryos obtained from IVM of these immature oocytes^{6,7,8}. Thus, IVM of immature oocytes plays a key role in warranting fertility for some patients.

Despite the fact that IVM of human oocytes is an invaluable choice for human reproductive technologies, the pregnancy rate of IVM-derived oocytes is still low^{3,9}. Moreover, the developmental ability of IVM oocytes is variable³. It is extremely important to learn about the nature of this variability of oocyte quality. The quality of oocytes is profoundly affected by gene expression during oocyte growth^{10,11}. As a result, the gene expression pattern of MII oocytes with a high developmental capacity seems different from that with a low developmental ability^{12,13}. However, it remains unclear when the gene expression pattern of human MII oocytes is established during maturation and how variable transcriptomes are found among different individuals.

Transcriptomes of oocytes at different maturation stages have first been studied by

microarrays^{14,15,16}. One report has revealed that *in vitro*-matured MII oocytes show the over-abundance of detected transcripts, as compared with *in vivo*-derived MII oocytes¹⁵, suggesting a different transcriptome pattern between *in vitro*- and *in vivo*-matured oocytes. It has also suggested that GV and MI oocytes show similar gene expression profiles¹⁵. Recently, single cell RNA-seq has been applied to reveal transcriptomes of human oocytes^{17,18,19}. Zhang et al. reported transcriptomic profiles of human oocytes during folliculogenesis¹⁸. Differential gene expression related to energy metabolism has been identified between *in vitro*- and *in vivo*-matured oocytes¹⁹. However, transcriptomic changes during *in vitro* oocyte maturation from the germinal vesicle (GV) stage till the MII stage have not been well elucidated even though these oocytes are often collected after oocyte retrieval for infertility treatment. Furthermore, transcriptomic variations among different individuals need to be determined.

In this study, transcriptomes for oocytes at different maturation stages were examined using single cell RNA-seq and differentially expressed genes were identified. Surplus oocytes, which are discarded as medical waste, were subjected to this study. Immature oocytes at the time of oocyte retrieval were used for *in vitro* maturation, and oocytes at the GV, metaphase I (MI), and MII stages were subjected to RNA-seq. Moreover, oocytes that reached each maturation stage with a significant delay were also collected for the comparison. By comparing the transcriptomes of GV, MI, and MII oocytes, we found genes and pathways whose expression was dynamically altered during maturation. Furthermore, we compared the oocyte transcriptomes obtained from different patients and reveal the gene set that tends to be differentially expressed among individuals. Our study deepens the molecular understanding of human oocyte maturation and provides an important platform to search for maternal genes that reflect the oocyte quality.

Results and Discussion

RNA-seq analyses of human oocytes during oocyte maturation.

To reveal transcriptomes of human oocytes during *in vitro* maturation, single cell RNA-seq analyses were performed using oocytes at different maturation stages. In total, 19 oocyte samples, collected from 11 patients, were subjected to RNA-seq analyses. Oocytes were classified into three different maturation stages; the immature GV stage, maturing oocytes from the GV to MII stage, and the matured MII stage (Fig. 1a). Maturing oocytes from the GV to MII stage were described as the metaphase I (MI), and many of them showed condensed chromosomes after DNA staining (Fig. 1a, b). As shown in Fig. 1a, human oocytes were first collected for infertility treatment and oocytes at the MII stage or that reached the MII stage until 0-7 hours after the oocyte retrieval were used for *in vitro* fertilization. Oocytes that did not reach the MII stage by then are routinely discarded as medical wastes in our hospital (Mie university) and these surplus oocytes were analyzed. On the same day of oocyte retrieval, oocytes at the GV, MI and MII stages were morphologically judged at 7.5-9 hours after oocyte retrieval (GV, MI, and MII, respectively) and sampling was carried out (Fig. 1a). Some remaining oocytes were further cultured for 15-16.5 hours, and oocytes that reached each developmental stage were collected. These oocytes were called as delayed GV, MI and MII oocytes (dGV, dMI, and dMII, respectively). Successful collection of oocytes at different maturation stages were confirmed with DNA staining (Fig. 1b). The collected oocytes were subjected to single cell RNA-seq by taking advantage of the SMART-seq procedure²⁰. After sequencing, $13,799,742 \pm 4,127,048$ reads were obtained and $85.6 \pm 2.0\%$ of reads were uniquely mapped to the human hg19 genome (Supplementary Table 1). When the reads and mapping rates were calculated in each oocyte maturation stage, similar statistics were obtained (Supplementary Table 1).

We then compared transcriptomes during oocyte maturation. Generally, oocytes derived from the same maturation stage resemble each other in their transcriptomes (Supplementary Fig. 1). Principal component analysis (PCA) captured transcriptomic transitions during human oocyte maturation (Fig. 1c). Oocytes at different maturation stages were also separated by a hierarchical clustering analysis (Fig. 1d).

Of note, a small number of oocytes were not well classified into the corresponding oocyte stages; GV3 rather exhibited a higher correlation with MI oocytes (Fig. 1c, d and Supplementary Fig. 1) and MII1 showed a closer transcriptome to that of the GV or MI state (Fig. 1c, d and Supplementary Fig. 1). These results suggest that an oocyte classified as the MII stage according to the morphological criteria can contain an improperly matured oocyte at the transcriptome level.

We next sought to identify differentially expressed genes (DEGs) among different maturation stages. When a stringent criterion is used ($\text{padj} < 0.05$, 4-fold difference in FRKM values), 1, 8, and 8 DEGs were identified by comparing GV vs MI, MI vs MII, and GV vs MII stage oocytes, respectively. This is probably due to the variable transcriptome of some individual oocytes such as MII1 and GV3 (Fig. 1c, 1d and Supplementary Fig. 1). Then, we used another condition to find DEGs between different oocyte maturation stages ($P < 0.05$, 4-fold difference in FRKM values), and 241, 428, and 523 DEGs were identified by comparing GV vs MI, MI vs MII, and GV vs MII stage oocytes, respectively. DEGs were further grouped into upregulated and downregulated genes in each comparison (Fig. 1e, f); 77 (GV vs MI), 317 (MI vs MII), and 360 (GV vs MII) upregulated genes were found, while 164 (GV vs MI), 111 (MI vs MII), and 163 (GV vs MII) genes were downregulated (Supplementary Table 2). Genes that were previously shown to be involved in oocyte meiosis and subsequent embryonic development such as *ASF1B* and *DPPA3* were indeed upregulated in MII oocytes (Table S2)^{21,22}. GO analyses were performed using these gene sets (Supplementary Fig. 2a-c). Interestingly, many specific terms were found by comparing to MII transcriptomes. Some GO terms such as MAP kinase activity were also found in the previous study in which human GV and MII transcriptome were compared²³. Expression of genes related to lipoprotein metabolism was significantly altered between maturing MI and MII stages (Supplementary Fig. 2b). Abnormal lipoprotein metabolism is relevant to female infertility²⁴, suggesting that expression of important genes for subsequent development is dynamically changed at this maturation stage. Moreover, when DEGs found between GV and MII were analyzed, myosin-related

terms were found, in good agreement with the previous report that Myosin IIA, IIB and microfilaments are dynamically reorganized during oocyte maturation²⁵. Our DEG list contains myosins (MYH3, MYH4, MYH8) and tubulins (TUBA1A, TUBA1B, TUBA1C, TUBA3E, TUBA3D) as upregulated genes in MII oocytes (Fig. 1g). Translational regulation of these genes might be key for *in vitro* oocyte maturation.

To further characterize transcriptomic changes during human oocyte maturation, DEGs identified by comparing each maturation stage were subjected to Ingenuity Pathway Analysis (IPA). IPA of DEGs revealed the significantly changed canonical pathways and predicted upstream regulators. Many canonical pathways were found as significantly altered by comparing GV vs MI or GV vs MII stage oocytes (Fig. 2a). As upstream regulators, AKT (also called protein kinase B) was identified by comparing GV and MI transcriptomes (Fig. 2b). The previous report also showed a dynamic change of AKT activity for the meiotic progression of GV oocytes to the MI stage²⁶. When GV and MII transcriptomes were compared, NOBOX, TEAD3, PSEN1 were identified as top upstream regulators (Supplementary Table 3), suggesting that genes regulated by these transcription factors might dynamically change their expression during *in vitro* maturation. Moreover, the lipid metabolic pathway was again identified also by IPA as significantly affected during maturation (Supplementary Fig. 3). Interestingly, hypoxia-inducible factor 1a (HIF1A) target genes were largely inhibited presumably due to the effect of maternal HIF1A (Fig. 2c). HIF1A is involved in the hypoxia-induced dormant state of oocytes²⁷ and is upregulated from the primary oocytes stage¹⁸. Injection of an inhibitor that suppresses HIF1A activity into mice reduces the quality of oocytes by impairing the meiotic apparatus, suggesting a defect in spindle assembly and actin dynamics²⁸. In good agreement with this paper²⁸, we have also identified genes related to actin dynamics such as VASP and ACTA2 as downstream targets for HIF1A (Fig. 2c). HIF is a key player for metabolic reprogramming in concert with Sirtuin²⁹. Sirt1-Nrf2-Cyclin B1/CDK1 signaling pathway is important for oocyte meiosis by regulating spindle/chromosome

organization³⁰. Importantly, canonical pathway analyses showed that Sirtuin signaling genes were enriched in DEGs between GV and MII (Fig. 2a; GV vs MII, green arrow). Taken together, HIF1A and Sirtuin signaling pathway might be an important player for *in vitro* oocyte maturation in human.

Differentially expressed genes between normal and delayed oocytes

We next examined transcriptomic profiles of oocytes that were significantly delayed to reach MI and MII stages (Fig. 1a; dMI and dMII, respectively) or GV oocytes that remained immature (Fig. 1a; dGV). These types of oocytes are not used for human infertility treatment, but their developmental ability and similarity to the normally obtained oocytes have not been elucidated well. PCA analyses showed that transcriptomes of dGV were similar to those of GV oocytes (Fig. 3a, +dGV), suggesting that transcripts are relatively stable during the overnight incubation of GV oocytes. In contrast, dMI samples were well separated from MI (Fig. 3a, +dMI). Regarding transcriptomes of dMII, two of them were close to MII, but one of dMII samples (dMII3) showed a distinct transcriptomic pattern (Fig. 3a, +dMII), suggesting that delayed MII oocytes include those close to MII oocytes at least in a transcriptomic level. A hierarchical clustering analysis also supported these notions (Supplementary Fig. 4a).

We then identified DEGs specifically up- or down-regulated in delayed oocytes. When a stringent condition is used ($\text{padj} < 0.05$, 4-fold difference in FRKM values), 1, 7, and 28 DEGs were identified by comparing GV vs dGV, MI vs dMI, and MII vs dMII stage oocytes, respectively. Then, we used another condition to find DEGs between different oocyte maturation stages ($P < 0.05$, 4-fold difference in FRKM values), and 180, 568, and 414 DEGs were identified by comparing GV vs dGV, MI vs dMI, and MII vs dMII stage oocytes, respectively. DEGs were further grouped into upregulated and downregulated genes in each comparison (Fig. 3b, c, and Supplementary Table 4); 114 (GV vs dGV), 430 (MI vs dMI), and 119 (MII vs dMII) upregulated genes were found, while 66 (GV vs dGV), 138 (MI vs dMI), and 295 (MII vs dMII) genes were downregulated. GO analyses were performed using these

gene sets (Supplementary Fig. 4b-d). Many specific terms were identified in downregulated genes in dMI samples, and especially genes involved in ubiquitin proteasome pathways were misregulated (Supplementary Fig. 4c). Moreover, the comparison between MII and dMII shows that apoptosis-related genes were misregulated in delayed oocytes (Supplementary Fig. 4d). IPA of DEGs revealed canonical pathways and predicted upstream regulators, which were significantly affected by delayed maturation. In canonical pathway analysis, altered pathways between MII and dMII (Fig. 4a) were significantly overlapped with the molecular pathways identified by comparing GV and MII (Fig. 2a). This result suggests that genes and related pathways, which are activated during *in vitro* maturation, tend to be misregulated in delayed MII oocytes. The term, G1/S Checkpoint Regulation, was only found when MII and dMII sample were compared. *CDKN1A* gene is a part of the G1/S Checkpoint Regulation, and has been shown to play a key role for oocyte meiosis³¹. *CDKN1A* was downregulated in delayed MII oocytes (Supplementary Table 4), and thus might be used as a marker for selecting normally maturing oocytes. Furthermore, genes related to Sirtuin Signaling Pathway were identified by comparing MII vs dMII stage oocytes (Fig. 4a, green arrow); Of note, *CYCI*, *SDHD*, and tubulin genes (*TUBA1A*, *TUBA1B*, *TUBA3C/TUBA3D*, *TUBA3E*, *TUBA4A*), all involved in Sirtuin Signaling Pathway, were downregulated in delayed MII oocytes (Fig. 4b and Supplementary Table 4). As the upstream regulators, SMARCA4 was detected in the comparison between MII and dMII (Supplementary Table 5) and target genes of SMARCA4 were downregulated (Fig. 4b). In good line with our observation, SMARCA4 was shown to be accumulated in aged preovulatory oocytes³². Oocytes of delayed maturation might accumulate SMARCA4, which in turn results in misregulated gene expression.

A specific set of genes are differentially expressed among different patients during oocyte maturation

We next sought to characterize genes that are variably expressed among MII oocytes of different patients.

By comparing MII1 vs MII2, MII2 vs MII3, and MII3 vs MII1 oocytes, 475, 134, and 469 DEGs were identified, respectively ($P < 0.05$, 4-fold difference in FRKM values). DEGs were further grouped into upregulated and downregulated genes in each comparison (Fig. 5a, b); 357 (MII1 vs MII2), 44 (MII2 vs MII3), and 123 (MII3 vs MII1) upregulated genes were found, while 118 (MII1 vs MII2), 90 (MII2 vs MII3), and 346 (MII3 vs MII1) genes were downregulated (Supplementary Table 6). PCA showed that the MII1 transcriptome was distinct from other two MII samples (Fig. 1c). The heatmap also supports the notion that the MII1 transcriptome was different from MII2 and MII3 (Fig. 5a, b), and rather similar to delayed oocyte samples, dMII1 and dMII2. When all DEGs were compared on heatmap, the different transcriptomic pattern of MII1 was obvious (Supplementary Fig. 5a). IPA revealed that genes related to Sirtuin Signaling Pathway and Nucleotide Excision Repair (NER) Pathway were commonly misregulated in MII1 oocytes (Fig. 5c: green arrows; Supplementary Table 7). Furthermore, KLF3 and TET2 were identified as candidate upstream regulators for explaining this difference in transcriptomes (Supplementary Fig. 5b, c) KLF3 controls the accumulation of fats and lipids³³. Lipids are accumulated in the oocyte cytoplasm during maturation³⁴ and the synthesis and maintenance of lipid droplets are key for the quality of the oocyte and subsequent embryonic development³⁵. *Tet2* knockout mice shows defects in polar body extrusion, mitotic spindle structure, and chromosomal alignment during meiosis, all of which become apparent with aging, eventually resulting in the reduced oocyte quality and embryonic development³⁶. Taken together, specific gene pathways are disturbed in some of the *in vitro* matured MII oocytes.

Potential marker genes for each oocyte maturation stage and for selecting a high quality oocyte

We next sought to find marker genes that were specifically or abundantly expressed in each maturation stage of oocytes, focusing on GV and MII stages. We first performed unsupervised clustering of genes based on their expression levels and 20 clusters were generated (Fig. 6a). For identifying GV-enriched

transcripts, we focused on the cluster 5, which showed strong expression in GV samples, especially in GV1 and GV2 (Fig. 6a). These genes were compared to our upregulated DEG list in GV samples (GV vs MI and GV vs MII), and 40 genes were identified (Fig. 6b and Supplementary Table 8). EIF4EBP1 is included in this list, which is known to be associated with maternal mRNA in mouse oocytes³⁷.

We next searched for transcripts uniquely expressed in matured MII oocytes. Clusters 9, 12, and 16 showed strong expression in MII oocytes, especially in MII2 and MII3 (Fig. 6a). When genes belonging to these clusters were compared to our upregulated DEG list in MII samples (GV vs MII and MI vs MII), 58 genes were identified (Fig. 6c, 32+15+11 genes). Alternatively, our MII DEGs were compared to human MII oocyte-specific genes previously identified by Zhao et al.²³, and 8 overlapping genes were detected (Supplementary Fig. 6a and Supplementary Table 9). Taken together, these genes (Supplementary Table 9) can serve as MII-enriched markers during oocyte maturation. We then examined expression of these marker genes in delayed MII samples, which are supposed to show a lower developmental ability than normally matured MII oocytes. Interestingly, all of these marker genes were downregulated in delayed MII oocytes (Fig. 6d), suggesting that these are good candidates as a marker to select MII oocytes with a high quality. Especially, *ASF1B* and *BMP15* were highly expressed only in MII2 and MII3 samples (Fig. 6d)^{38,39}, suggesting that these genes are good candidates for marker genes to select MII oocytes with a high quality. Finally, we performed unsupervised clustering of MII and dMII samples (Supplementary Fig. 6b), and clusters 6 and 12 were identified as highly abundant in MII2 and MII3 samples (Supplementary Table 10). *APOB* and *TNFRSF10C* were identified as MII-enriched markers, and therefore might serve as a biomarker for selecting MII oocytes with a high quality. *APOB* knockout mice have been reported to be embryonic lethality in homozygous⁴⁰. In addition, it has been reported that a positive correlation is observed between the APOB concentration in follicular fluid and the embryo quality such as fertilization rates and embryonic developmental potentials⁴¹. Furthermore, TNFRSF10C (TRAILR3) protein is decreased from a medium containing human blastocysts, suggesting

252 TNFRSF10C is utilized for embryonic development⁴². Taken together, we have not only identified
253 specific marker transcripts for each maturation state of oocytes, but also shown potential biomarkers to
254 select a high quality human oocyte for subsequent development.
255

Methods

Human oocyte. Informed consent for this prospective study was obtained from all participants. The oocyte samples used in this study were collected from 13 patients in 2019, and these oocyte samples are normally discarded as clinical waste after the completion of *in vitro* fertilization in clinical treatment. After aspirating follicles with a fine needle under the ovarian echo, the cumulus oocyte complexes (COCs) were picked up under a stereomicroscope. COCs from infertile patients were scheduled for intracytoplasmic sperm injection, and surrounding cumulus cells were removed in multipurpose handling medium (MHM; Scientific's, USA, 90163) containing 20 IU/ml Cumulus Remover (Recombinant hyaluronidase; Kitazato, Japan, 94114). After denudation, oocyte morphology was examined and an oocyte at the MII stage was used for infertility treatment. Oocytes other than the MII stage were subjected to *in vitro* maturation. The collected and denuded oocytes were incubated in the Sydney IVF fertilization medium (Cook medical, USA, K-sifm-100) at 37°C under 5% CO₂ and 5% O₂ in air for *in vitro* maturation, and oocytes that had matured to the MII stage by 16:00 on the same day of oocyte retrieval were used for the treatment of *in vitro* fertilization. The remaining oocytes that had not reached the MII stage by 16:00 were normally discarded, but further cultured for this study. A few hours after the incubation, some oocytes were matured to MII oocytes, and such matured oocytes were subjected to sampling for RNA-seq analyses. At that time, GV and MI oocytes were also sampled. For MII oocytes, zona pellucide piercing was performed on the denuded oocytes with LYKOS Clinical IVF laser system and Clinical Laser Software Legacy 5.12 (Hamilton Thorne, USA, 40025) and the first polar body was biopsied with micropipette (CooperSurgical, USA, MPBFP30). Zona was lysed with acidic-tyrode (Merck, Germany, T1788) for 20-30 seconds, and washed in Phosphate-buffered saline (PBS; Nacalai Tesque, Japan, 07269-84) containing 0.1% w/v bovine serum albumin (BSA; Merck, Germany, NM-126609-5GMCN). The zona- and polar body-free oocytes suspended in 1µl of 0.1% w/v BSA/PBS were moved into 9.5 µl of 1×Reaction buffer from SMART-seq v4 Ultra Low Input RNA Kit

(Takara Bio, Japan, 24888N), in which 10× lysis buffer (Takara Bio, Japan, 635013) and 2 unit ribonuclease inhibitor (Thermo Fisher Scientific, USA, 10777-019) were mixed. GV and MI oocytes were similarly sampled for RNA-seq analyses, except that polar body removal was not carried out. Some oocytes that remained immature at the time of the above mentioned sampling for RNA-seq analyses were left in the maturation medium overnight, and we examined the maturation state on the next day of the oocyte retrieval (9:00 am). Such oocytes were sampled as those which were delayed in their maturation by following the same sampling method as described above. The classification criteria for GV, MI and MII oocytes were explained in the corresponding result section. This study was performed within the guidelines established by the Clinical Research Ethics Review Committee of Mie University Hospital (H2018-066) and was registered in the University Hospital Medical Information Network Clinical Trials Registry in Japan (UMIN000034811). In addition, this study was registered for the research dealing with human sperm, ova, and fertilized ova in the Japan Society of Obstetrics and Gynecology.

Nuclear staining. Nuclear staining was performed using the surplus oocytes at each stage as follows. Oocytes were incubated in the fixation medium, 4% w/v paraformaldehyde (Nacalai tesque, Japan, 26126-25), for 30 min at room temperature, followed by three washes with 0.2% w/v BSA/PBS. After permeabilization with 0.2% v/v Triton X-100/PBS for 30 min at room temperature, they were washed with 0.2% w/v BSA/PBS. Then, nuclei were stained with 1 µg/µL DAPI (Nacalai tesque, Japan, 11034-56) in 0.2% w/v BSA/PBS solution for 10 min, followed by washes with 0.2% w/v BSA/PBS. The fluorescence signals were observed using a confocal microscope (Carl Zeiss, Germany, LSM800), equipped with a laser module (405/488/561/640 nm) and GaAsP detector. Z-slice thickness was determined by using the optimal interval function in the ZEN software (Carl Zeiss).

Library preparation for RNA-seq. A single oocyte at each maturation stage was subjected to cDNA synthesis and amplification using SMART-Seq® v4 Ultra® Low Input RNA Kit (Takara Bio, Japan, Z4889N) according to the vendor's instruction with some modifications. Briefly, collected surplus oocytes were subjected to micromanipulation to remove their sibling polar bodies, and then zona pellucida was enzymatically removed as described above. After removing the polar body and the zona pellucida, oocytes were washed with 0.1% w/v BSA/PBS, and then transferred to a 0.2 ml tube containing 9.5 µl of reaction buffer to make the final volume of 10.5 µl. After cell lysis in the buffer, 1 µl of the solution was removed and, instead, 1 µl of the diluted ERCC RNA Spike-In Mix was added (diluted for 350 pg of total RNA according to the vendor's instruction; Thermo Fisher Scientific, 4456740). The lysed RNA solution with the spike-in RNA was subjected to reverse transcription. The produced cDNA was amplified by PCR with 16 cycles. The amplified cDNA was purified using AMPure XP beads (Beckman Coulter, USA, A63882). The purified cDNA was measured on the Bioanalyzer (Agilent, USA) using High Sensitivity DNA Kit (Agilent, 5067-4626) and the normalized volume of DNA was subjected to library preparation using Nextera XT DNA Library Preparation Kit (Illumina, USA, FC-131-1024) by following the vendor's instruction. The obtained library was quality-checked by Bioanalyzer and quantified using Qubit (Thermo Fisher Scientific). The experiments have been approved by the Clinical Research Ethics Review Committee of Kindai University at the Department of Biology-Oriented Science and Technology (H30-1-006) and followed the university's guidelines to store the sequencing data.

Sequencing data filtering. Paired end sequencing (50 bp + 25 bp) was carried out using the Illumina NextSeq system. Fastq files from Illumina sequencing were filtered for low quality reads by sliding window trimming (window size 10, <QV20), and low quality bases were trimmed from the ends of the reads (<QV20) using Trimmomatic⁴³. Reads of less than 20 bases and unpaired reads were removed.

Furthermore, adaptor, polyA, polyT and polyG sequences were removed using trim_galore and cutadapt⁴⁴. The sequencing reads were then mapped to the human genome (hg19) using STAR⁴⁵. Reads on annotated genes were counted using featureCounts⁴⁶.

RNA-seq analysis. RNA-seq reads were visualized using Integrative Genome Viewer⁴⁷. FPKM values were calculated from mapped reads by normalizing to total counts and transcript. Differentially expressed genes (DEGs, $P < 0.05$) were then identified using DESeq2⁴⁸. An unsupervised hierarchical clustering of the read count values was performed using hclust in TCC (unweighted pair group method with arithmetic mean: UPGMA). Gene ontology terms showing over-representation of genes that were up or down-regulated were detected using DAVID tools ($P < 0.05$)^{49,50}. Furthermore, the gene list of DEGs was compared to the categorized gene lists found in a paper²³. Each gene list was further subjected to an Ingenuity Pathway Analysis (IPA; Qiagen, Redwood City, USA, <https://www.qiagenbioinformatics.com/products/ingenuity-pathway-analysis>). Using IPA, enriched canonical pathways, upstream transcriptional regulators, gene regulatory networks, and diseases and biological functions in the identified DEGs were investigated. Expression levels of DEGs were displayed in heatmaps with z-scores obtained from FPKM values of genes in the DEG lists by using heatmapper⁵¹. The DEG lists consist of genes that satisfy the condition $P \text{ value} < 0.05$ and $\log_2\text{FC} > 2$ or $\log_2\text{FC} < -2$.

Statistical and Reproducibility. Significant GO terms were identified using the modified Fisher Exact P-values according to the DAVID functional annotation tool. In IPA, P values were obtained according to the statistical methods applied to each analysis explained in the vendor's instruction. A value of $P < 0.05$ was considered significant. Number of replicates is indicated in the figure legends.

Reporting summary. Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data availability

The dataset generated during this study are available in the Supplementary Tables and in the Gene Expression Omnibus (GEO) public repository under accession GSE166533. All the other data are available from the corresponding author upon reasonable request.

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Author contributions

K.Mi. conceived the project. H.T. and K.Mi. designed experiments. H.T., M.Y., M.F., and K.Mi. performed experiments. M.Y. and K.Mi. analyzed data. K.Ma. provided an analytic tool. T.M., M.N., E. K., T.I., and K.Mi. supervised research. K.Mi, mainly wrote the manuscript. H.T., and M.Y. helped writing the manuscript.

Competing interests

The authors declare no competing financial interests.

Additional information

Supplemental information is available for this paper.

Fig. 1. Transcriptomic changes of human oocytes during *in vitro* maturation. **a** A schematic diagram of human oocyte retrieval for single cell RNA-seq analyses. Ten oocytes were collected

from 7 patients. **b** Representative images of human oocytes at different maturation stages. The bottom panels show higher magnification images of the regions indicated in the top panels by dashed boxes. DNA was stained by DAPI. Scale bars represent 20 μm for whole oocyte images and 10 μm for enlarged images. **c** PCA of the gene expression profiles (3-4 biological replicates per each maturation stage). Blue dots: GV samples, Orange dots: MI samples, and Green dots: MII samples. **d** Hierarchical clustering dendrogram generated using the gene expression profiles of human oocytes at different maturation stages. Spearman correlation was used. **e, f** Heatmaps depicting the gene expression levels of DEGs identified in the indicated comparisons. Color key with z-score is shown. **(e)** shows upregulated genes in MI (GV vs MI), MII (MI vs MII), and MII (GV vs MII) samples, while **(f)** indicates downregulated genes in the same comparisons. **g** Heatmaps depicting the gene expression levels of Tubulin-related and myosin-related genes. GV; oocytes at the germinal vesicle stage, MI; maturing oocytes at the metaphase I stage, MII; oocytes at the metaphase II stage.

Fig. 2. Gene regulatory networks that are associated with dynamic transcriptome changes during human oocyte maturation. **a** Canonical pathways predicted by IPA using the DEG list of GV vs MI or GV vs MII. Significant terms are indicated in red colors: darker the color more significant the term is ($P < 0.05$, Fisher's exact test). Closely related terms are connected to each other. A green arrow indicates Sirtuin signaling pathway as explained in the relevant text. **b** Downstream targets of AKT were found in the DEG list by IPA. Gene regulatory networks of AKT in human oocytes were predicted. **c** Downstream

528 targets of HIF1A were found in the DEG list by IPA. Blue arrows mean 'Leads to inhibition', orange
529 arrows mean 'Leads to activation', a yellow arrow means 'Inconsistent with state of downstream
530 molecules', black arrows mean 'Effect not predicted'. Molecules colored green, red and blue show
531 'downregulated', 'upregulated', and 'predicted inhibition', respectively.

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535 **Fig. 3. Human oocytes that are delayed in their maturation show different transcriptomes from**

those matured in an earlier timing. a PCA of the gene expression profiles (3-4 biological replicates per each category). The top left image in the rectangle includes samples collected on the day for oocyte retrieval (Fig. 1a) (GV, MI and MII). Nine delayed oocytes were collected from 7 patients. Blue dots: GV samples, light blue dots: MI samples, dark orange dots: MII samples, light orange dots: dGV samples, dark green dots: dMI samples, and light green dots: dMII samples. **b, c** Heatmaps depicting the gene expression levels of DEGs identified in the indicated comparisons. **(b)** shows upregulated genes in dGV (GV vs dGV), dMI (MI vs dMI), and dMII (MII vs dMII) samples, while **(c)** indicates downregulated genes in the same comparisons. Color key with z-score is shown. dGV; oocytes that were at the germinal vesicle stage the day after oocyte retrieval, dMI; oocytes that were at the metaphase I stage the day after oocyte retrieval, dMII; oocytes that were at the metaphase II stage the day after oocyte retrieval.

Fig. 4. Gene regulatory networks are altered in the human oocytes that are delayed in their maturation. **a** Canonical pathways predicted by IPA using the DEG list of MII vs dMII. Significant terms are indicated in red colors: darker the color more significant the term is ($P < 0.05$, Fisher's exact test). Closely related terms are connected to each other. A green arrow indicates Sirtuin signaling pathway as explained in the relevant text. **b** Downstream targets of SMARCA4 were found in the DEG list by IPA. Gene regulatory networks of SMARCA4 in human oocytes were predicted. SMARCA4 affects molecules indicated at the end of arrows, blue arrows mean 'Leads to inhibition', black arrows mean 'Effect not predicted'. Molecules colored green, red and blue show 'downregulated', 'upregulated', and 'predicted inhibition', respectively.

Fig. 5. Gene regulatory networks that are altered in different patients. a, b Heatmaps depicting the gene expression levels of DEGs identified in the indicated comparisons. **(a)** shows upregulated genes in MII2 (MII1 vs MII2), MII3 (MII2 vs MII3), and MII1 (MII3 vs MII1) samples, while **(b)** indicates

565 downregulated genes in the same comparisons. Color key with z-score is shown. **c** Canonical pathways
566 predicted by IPA using the DEG list of MII1 vs MII2 or MII1 vs MII3. Significant terms are indicated in
567 red colors: darker the color more significant the term is ($P < 0.05$, Fisher's exact test). Closely related
568 terms are connected to each other. Green arrows indicate commonly misregulated pathways.

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Fig. 6. Marker genes for human GV and MII oocytes and potential biomarkers for MII oocytes with high quality. a A heatmap of gene expression levels after clustering based on expression patterns.

575 Twenty clusters were generated and clusters 5, 9, 12, and 16 are indicated. Color key indicates
576 $\log_2FC(\text{FoldChange})$. **b** Venn diagrams showing the numbers of total and overlapping genes among
577 genes that belong to cluster 5, upregulated genes in the GV vs MI comparison, and upregulated genes in
578 the GV vs MII comparison. **c** Venn diagrams showing the numbers of total and overlapping genes among
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580 upregulated genes in the MI vs MII comparison. **d** A heatmap depicting the gene expression levels of
581 candidate genes for human MII oocytes with high quality. Gene symbols are listed. Color key with
582 z-score is shown.

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Figures

Figure 1

Transcriptomic changes of human oocytes during in vitro maturation. a A schematic diagram of human oocyte retrieval for single cell RNA-seq analyses. Ten oocytes were collected from 7 patients. b Representative images of human oocytes at different maturation stages. The bottom panels show higher magnification images of the regions indicated in the top panels by dashed boxes. DNA was stained by

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Human oocytes that are delayed in their maturation show different transcriptomes from those matured in an earlier timing. a PCA of the gene expression profiles (3-4 biological replicates per each category). The top left image in the rectangle includes samples collected on the day for oocyte retrieval (Fig. 1a) (GV, MI and MII). Nine delayed oocytes were collected from 7 patients. Blue dots: GV samples, light blue dots: MI samples, dark orange dots: MII samples, light orange dots: dGV samples, dark green dots: dMI samples, and light green dots: dMII samples. b, c Heatmaps depicting the gene expression levels of DEGs identified in the indicated comparisons. (b) shows upregulated genes in dGV (GV vs dGV), dMI (MI vs dMI), and dMII (MII vs dMII) samples, while (c) indicates downregulated genes in the same comparisons. Color key with z-score is shown. dGV; oocytes that were at the germinal vesicle stage the day after oocyte retrieval, dMI; oocytes that were at the metaphase I 545 stage the day after oocyte retrieval, dMII; oocytes that were at the metaphase II stage the day after oocyte retrieval.

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Marker genes for human GV and MII oocytes and potential biomarkers for MII oocytes with high quality. a A heatmap of gene expression levels after clustering based on expression patterns. Twenty clusters were generated and clusters 5, 9, 12, and 16 are indicated. Color key indicates $\log_2FC$ (FoldChange). b Venn diagrams showing the numbers of total and overlapping genes among genes that belong to cluster 5, upregulated genes in the GV vs MI comparison, and upregulated genes in the GV vs MII comparison. c Venn diagrams showing the numbers of total and overlapping genes among genes that belong to

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