Dynamics and clinical relevance of maternal mRNA clearance during the oocyte-to-embryo transition in humans

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Maternal mRNA clearance is an essential process that occurs during maternal-to-zygotic transition (MZT). However, the dynamics, functional importance, and pathological relevance of maternal mRNA decay in human preimplantation embryos have not yet been analyzed. Here we report the zygotic genome activation (ZGA)-dependent and -independent maternal mRNA clearance processes during human MZT and demonstrate that subgroups of human maternal transcripts are sequentially removed by maternal (M)- and zygotic (Z)-decay pathways before and after ZGA. Key factors regulating M-decay and Z-decay pathways in mouse have similar expression pattern during human MZT, suggesting that YAP1-TEAD4 transcription activators, TUT4/7-mediated mRNA 3'-oligouridylation, and BTG4/CCR4-NOT-induced mRNA deadenylation may also be involved in the regulation of human maternal mRNA stability. Decreased expression of these factors and abnormal accumulation of maternal transcripts are observed in the development-arrested embryos of patients who seek assisted reproduction. Defects of M-decay and Z-decay are detected with high incidence in embryos that are arrested at the zygote and 8-cell stages, respectively. In addition, M-decay is not found to be affected by maternal TUBB8 mutations, although these mutations cause meiotic cell division defects and zygotic arrest, which indicates that mRNA decay is regulated independent of meiotic spindle assembly. Considering the correlations between maternal mRNA decay defects and early developmental arrest of in vitro fertilized human embryos, M-decay and Z-decay pathway activities may contribute to the developmental potential of human preimplantation embryos.
M *atal-to-zygotic transition (MZT) is an initial step in the early development of all investigated animal species; during MZT, transcripts of maternal genes are removed by degradation and the zygotic genome is activated. The exact mechanisms by which the maternal mRNAs are degraded during MZT is a long-standing question in reproductive and developmental biology. Genetic and high-throughput sequencing studies on model systems, including Drosophila, zebrafish, and Xenopus, have indicated that the elimination of maternal transcripts is accomplished by two sequential pathways: the first pathway is entirely mediated by maternal factors accumulated in the mature oocytes and is thus termed maternal (M)-decay; the second pathway depends on de novo zygotic transcription products after fertilization and is thus termed zygotic (Z)-decay.

Significant progress has recently been made in understanding the regulation of mRNA stability in mammalian oocytes and zygotes. CNOT6L, which is a catalytic subunit of CCR4-NOT deadenylase, and its associated zinc finger protein 36-like 2 (ZFP36L2) protein were found to be essential for mRNA decay that accompanies oocyte meiotic maturation1,2. The β-cell transcription gene-4 (BTG4), which is an oocyte-specific adapter protein of CCR4-NOT, was identified as an MZT-licensing factor in mice that mediated mRNA clearance prior to ZGA3,4. These mechanisms comprise the currently known M-decay pathway in mice. In addition, terminal uridine transferase-4 and terminal uridine transferase-7 (TUT4/7)-mediated mRNA degradation not only maintained homeostasis of the maternal transcriptome during oogenesis, but also facilitated Z-decay in murine preimplantation embryos.5,6 The maternal transcriptional coactivator YAP1 and its co-transcription factor TEAD4 were found to trigger the transcription of early zygotic genes, such as TUT4/7, and possibly genes encoding other unidentified mRNA destabilizers.14,15 Further, these mechanisms comprise key components of the murine Z-decay pathway. Despite these findings in model animals of lower-level species, the dynamics of mRNA decay and mechanisms that govern stepwise maternal mRNA clearance during MZT in humans remain unclear.

Human preimplantation embryogenesis is a remarkably complicated, well-orchestrated process that relies on synchronization of oocyte maturation and zygotic genome activation (ZGA).6,17 Despite extensive research on murine as well as human oocytic and embryonic transcriptomes in recent years, many questions regarding key MZT events in humans remain unanswered. For instance, in assisted human reproduction, the extent of cytoplasmic maturation of an oocyte is considered a determining factor for its developmental potential after fertilization.19,20 However, whether the appropriate maternal mRNA degradation contributes to the cytoplasmic maturation of human oocytes and their developmental potential after in vitro fertilization (IVF) remains unclear. In addition, unlike mouse embryos, in which major ZGA is initiated at the 2-cell stage, human embryos undergo major ZGA at the 8-cell stage.21,22 The proportion of human maternal transcripts with clearance that is ZGA-dependent remains undetermined. From a broader perspective, dysregulation of the maternal mRNA clearance process may be related to various disorders of the reproductive system, such as follicle growth retardation, oocyte maturation defects, early embryo arrest, oocyte aging, and ultimately, infertility.5,9,12,23,24 Thus, investigating the stability regulation of maternal mRNAs during human MZT may facilitate the understanding of associated physiological, as well as pathological processes.

TUBB8 is a primate-specific β-tubulin isoform, the expression of which is confined to oocytes and the early embryo.25, TUBB8 variants are genetic determinants of human oocyte maturation arrest that cause variable and mixed phenotypes in oocyte maturation and early embryo development.26,27 However, whether the process of oocyte maturation-associated maternal mRNA degradation was also disturbed in these mutated zygotes is unclear. In this study, we define and characterize the ZGA-dependent maternal mRNA clearance process during human MZT and demonstrate that subgroups of the human maternal transcripts are sequentially removed by M-decay and Z-decay pathways before and after ZGA. We also evaluate the association of maternal mRNA degradation defects with zygotic developmental arrest due to TUBB8 mutations or unidentified reasons. These investigations aim to provide insight into the dynamics, functional importance, and pathological relevance of maternal mRNA decay during human MZT.

**Results**

**Patterns of maternal mRNA degradation in human oocytes and embryos.** To identify patterns of maternal mRNA degradation during MZT in humans, we analyzed the degradation dynamics of human maternal mRNAs in GV oocytes, zygotes, and 8-cell embryos using published RNA-seq data (GSE101571). As illustrated in Fig. 1a, ZGA occurs at the 4-8-cell stage in the human embryo. Maternal mRNAs with reliable sequence annotations and with fragments per kilobase of transcript per million reads mapped (FPKM) of ≥2 at the GV stage (7271 genes) were selected. Those with significant decreases in mRNA levels between two stages at a magnitude of more than 2-fold were considered degraded maternal mRNAs and were classified into four clusters according to their degradation patterns: Cluster I (2372 genes), degraded from the GV stage to the zygote stage, and stable after fertilization; Cluster II (2259 genes), degraded from the zygote stage to the 8-cell stage; Cluster III (1109 genes), continuously degraded from the GV stage to the 8-cell stage; and Cluster IV (1531 genes), stable during MZT (Fig. 1a, b). To assess whether all Cluster IV transcripts remained stable beyond the 8-cell stage or if a subset of transcripts were degraded after this timepoint, we also analyzed transcript levels at the morula stage (Fig. 1c). This analysis indicated that only 176 of the 1531 Cluster IV transcripts were, in fact, degraded between the 8-cell and morula stage. The majority of Cluster IV transcripts remained stable between the 8-cell and morula stage. In human embryos, zygotic transcription activity is first detected at the 4-cell or 8-cell stage; thus, maternal mRNAs in Clusters II and III were considered candidates for ZGA-dependent decay, or Z-decay; further, Cluster I was considered a candidate for a maternally encoded mRNA decay pathway that acts before ZGA and is defined as M-decay.

We then asked whether maternal mRNA degradation in human embryos after fertilization depends on ZGA. Both in vitro controls and α-amanitin-treated human zygotes successfully developed to the 8-cell stage (Fig. 1a). These 8-cell embryos were collected for RNA-seq analysis. Significantly, transcripts of Clusters II and III were stabilized in α-amanitin-treated embryos (Fig. 1d). However, α-amanitin treatment only blocked the degradation of nearly half of the transcripts of Clusters II and III in mice. Thus, a ZGA-dependent mRNA decay pathway was found to operate during human MZT, in which it played a more important role than in mouse MZT.

In mice, long 3′-UTRs and high translational activity of Z-decay mRNAs conferred resistance to CCR4-NOT-mediated deadenylation during MZT, which showed that the length of the 3′-UTR is also a factor that determines mRNA stability.29,30 We also observed in the human transcriptome that: (1) M-decay transcripts possessed shorter 3′-UTRs compared to Z-decay transcripts (Fig. 1e); (2) When multiple cytoplasmic polyadenylation element (CPE) and polyadenylation signal (PAS) were present in the 3′-UTR of mRNAs, they contributed to mRNA translation in an additive manner during oocyte maturation in...
mRNA were classed into four clusters according to the level of expression during MZT. We made comparisons of these four groups of mRNAs in human 2-cell embryos and mouse 8-cell embryos (Fig. 2a). Maternal transcriptomes were overlapping (Fig. 2a), indicating that the homology between human and mouse maternal transcriptomes is high. To demonstrate the differences between human and mouse during the MZT, we collected oocytes (GV and ZGA) and preimplantation embryos at the 8-cell and morula stages from volunteers (25–35 years old) for RNA extraction and quantitative RT-PCR (RT-qPCR). We detected levels of transcripts that were shown to be targets of BTG4 and CNOT6L in mice. Similarly, we found that, in humans, the numbers of CPEs and PASs were present in the 3′-UTRs of Z-decay mRNAs at 2 folds compared to those in the 3′-UTRs of M-decay mRNAs (Fig. 1f).

Comparisons of human and mouse transcriptomes during the MZT. To demonstrate the differences between human and mouse maternal transcriptomes, we directly compared the transcripts in human and mouse GV oocytes. This revealed that only half of the transcripts were overlapping (Fig. 2a), indicating that the homology between human and mouse maternal transcriptomes is low. Even fewer zygotically activated genes were shared by mouse 2-cell embryos and human 8-cell embryos (Fig. 2a). Maternal mRNAs were classed into four clusters according to the level change during MZT. We made comparisons of these four groups between mouse and human since they may utilize the same mechanisms for maternal mRNA decay. Despite these four clusters being defined by similar criteria, the genes in each cluster were significantly different in human and mouse (Fig. 2b). Examples in Fig. 2c, d shows that some human M-decay transcripts were degraded by Z-decay pathways in mouse, and vice versa. Therefore, subsets of human mRNA might be regulated differently from mouse during the MZT.

Classification of maternal mRNA degradation in human embryos. To verify the RNA-seq data regarding mRNA dynamics during MZT in human embryos, we collected oocytes (GV and MII) and preimplantation embryos at the 8-cell and morula stages from volunteers (25–35 years old) for RNA extraction and quantitative RT-PCR (RT-qPCR). We detected levels of transcripts that were shown to be targets of BTG4 and CNOT6L in mice and were eliminated during oocyte meiotic maturation, i.e.,
M-decay. Single cell RT-qPCR results showed that these transcripts were also degraded during the GV-to-MII transition in human oocytes (Fig. 3a). Furthermore, the levels of these transcripts were comparable in MII oocytes and in zygotes, suggesting that degradation of these transcripts was largely completed by the MII stage and there is no significant degradation during the MII-to-zygote transition (Fig. 3a). Some transcripts that were eliminated by Z-decay were stable before fertilization and were degraded at the 8-cell stage (Fig. 3b). As in mice, the delayed removal of mRNAs encoding BTG4 and the catalytic subunits (CNOT7 and CNOT6L) of CCR4-NOT deadenylase, as well as PAN2 RNA deadenylase, was also observed in the human embryos: RT-qPCR and RNA-seq results showed that these transcripts were relatively stable until the 4–8-cell stage (Fig. 3c and d). BTG4 and CNOT7 proteins were undetectable in GV oocytes before meiotic maturation, but accumulated in maturing oocytes and in zygotes, as detected by immunofluorescence (Fig. 3e). Then these proteins decreased to basal levels at the 8-cell stage. This observation suggests that BTG4 and CCR4-NOT may play roles in human maternal mRNA decay, whereas they themselves were degraded until other maternal mRNAs were eliminated.

We also examined the potential ZGA factors that were involved in the Z-decay pathway in human embryos. The transcription factor TEAD4 was zygotically expressed in mice and was required for Z-decay in preimplantation embryos. Similarly, TEAD4 transcription in human embryos was activated at as early as the 4-cell stage, and its mRNA levels increased 4-fold from the 8-cell stage to the morula stage, as determined by RNA-seq results (Fig. 3f). In contrast, the mRNA levels of TUBB8 were relatively stable during MZT (Fig. 2f). Immunofluorescence results showed that YAP protein evenly distributed in the human GV oocytes and zygotes, but accumulated in the nuclei of 8-cell embryos (Fig. 3g). The 3′-terminal uridylyl transferase 4 and 7 (TUT4/7)-dependent mRNA 3′-oligouridylation in mice participated in mRNA decay and sculpted the maternal transcriptome. TUT7, which is the downstream factor of TEAD4, was also expressed in human oocytes at higher levels compared to TUT4, but maternal TUT7 transcripts were removed during oocyte matura
tion and fertilization. Nevertheless, transient expression of zygotic TUT4/7 was detected from the 8-cell stage to the morula stage, with TUT7 levels being higher than those of TUT4 (Fig. 3f). The TUT7 expression levels peaked at the 8-cell stage and then rapidly decreased at the morula stage. The expression window of the human zygotic TUT4/7 gene overlapped with the time frame of Z-decay.

The TUBB8 mutation did not affect M-decay in humans. In a clinical context, normal embryos should develop at the 8-cell stage 3 days after IVF; however, there were embryos that were fertilized, as evidenced by the formation of pronuclei, that remained arrested at the 1-cell stage. Zygotic arrest of some embryos was due to gene mutations, such as TUBB8 mutations that affect cell division.

To investigate whether mRNA degradation was blocked by TUBB8 mutation-induced cell cycle arrest, we microinjected mRNA encoding mutated TUBB8 (TUBB8V255M), which is a dominant negative mutant, into mouse oocytes (Fig. 4a). Consistent with the results of previous studies, oocytes that overexpress TUBB8V255M had a GVBD rate similar to that of control oocytes that overexpress wild-type TUBB8, however, the PB1 emission rate of oocytes that overexpress TUBB8V255M was significantly lower than that of the control group (Supplementary
with those in 3PN zygotes, suggesting that they are normally not affected by nocodazole-induced meiotic spindle assembly abnormalities of the oocytes. If M-decay of maternal transcripts, arrested 1-cell embryos from two TUBB8V255M-mutated patients had high correlations, and those in 3PN zygotes, suggesting that they are normally not affected by nocodazole-induced meiotic spindle disruption.

Collectively, these results indicated that the arrest of meiotic cell division did not affect M-decay of maternal transcripts. If mRNA degradation defects were found in some arrested human embryos, these defects were not considered secondary consequences of spindle assembly abnormalities of the oocytes.

M-decay was frequently impaired in development-arrested human zygotes. To verify whether the defects of maternal mRNA degradation were causes of human preimplantation embryo arrest, we profiled the transcriptome of arrested 1-cell embryos that were derived from seven mutation-identified (unid) patients at day 3 after IVF. Since it is ethically difficult to collect normal human zygotes for this specific experimental purpose and since the TUBB8 mutation did not affect M-decay of known maternal transcripts, arrested 1-cell embryos from two TUBB8-mutated patients (V255M and G308S) were used as controls for single-cell RNA-seq.

The gene expression levels were assessed by FPKM. A principal component analysis revealed that two samples from TUBB8-mutated patients had high correlations, and five out of seven samples from unid-patients also had high correlations (Fig. 6a). A heatmap also showed high correlations among five samples from unid-patients (average $r = 0.900$; Supplementary Fig. 1c, f and Supplementary Table 1), whereas these five samples were significantly different from the two samples of TUBB8-mutated patients (Fig. 6a, Supplementary Fig. 1c and Supplementary Table 1).
Table 1). These observations indicate that the five embryos from unid-patients may have been arrested for the same reason, but not due to meiotic division defects like the $TUBB8$-mutated oocytes.

It was found that 3712 and 2493 transcripts were upregulated and downregulated more than 2-fold in the five unid-patient embryos with high transcriptome correlations (Fig. 6b, c). More transcripts were upregulated than they were downregulated in these embryos when we increased the thresholds of the analyses (Fig. 6d, e). The #6 unid-patient also displayed remarkable correlations, they were used for further analyses. Nearly 50% (1490 in 3712) of the transcripts that were upregulated in the embryos of this patient when we increased the thresholds of the analyses were upregulated than they were downregulated in the embryos of this patient compared to control embryos (Supplementary Fig. 1d). However, relatively small numbers of transcripts in the #7 unid-patient were upregulated or downregulated (Supplementary Fig. 1e), and the numbers of upregulated and downregulated genes were not very different (646 versus 494). Overall, mRNA clearance was impaired in 6 of 7 unid-patients.

Since the transcriptomes of the #1–5 embryos had high correlations, they were used for further analyses. Nearly 50% (1490 in 3712) of the transcripts that were upregulated in the unid-patient embryos should have been degraded in normal embryos during the GV-to-zygote transition (i.e., Cluster I and III of Fig. 1b) (Fig. 6f). Among mRNAs degraded in normal embryos during the GV-to-zygote transition, only 1431 of 3179 were degraded in unid-patient embryos (Fig. 6g); nearly 50% (1490/3179) were stabilized in unid-patient embryos. In contrast, only <10% (285/3179) were downregulated in unid-patient embryos. We further performed gene ontology (GO) analyses on the transcripts that are upregulated in the arrested embryos with fold changes of >2. Transcripts that are related to translation-related functions (red bars) and mRNA stability (green bars) were enriched (Fig. 6h), and they may have caused over-translation of the accumulated maternal mRNAs and led to cell division defects.

**M-decay defects potentially cause embryo arrest in humans.** To further determine whether M-decay defects associated with early embryo arrest in humans, we collected arrested zygotes from 4 $TUBB8$-mutated patients and 15 unid-patients and verified the levels of known M-decay transcripts by RT-qPCR (Fig. 7a, b). The mRNA levels of indicated transcripts were consistently low in the samples from $TUBB8$-mutated patients (Fig. 7a, b). In zygotes from 8 unid-patients (#1–8), at least 4 out of 6 detected transcripts showed significant accumulation compared to that in the maternal $TUBB8$-mutated zygotes, suggesting that M-decay was defective (Fig. 7a and Supplementary Table 2). In contrast, in the other 7 unid-patients (#9–15), these M-decay transcripts were not synergistically upregulated, suggesting that these embryos were arrested due to reasons other than M-decay defects (Fig. 7b and Supplementary Table 2).

Recent studies have indicated that the oocyte-expressed MZT licensing factor BTG4 mediates maternal mRNA degradation in mouse oocytes and zygotes by recruiting the CCR4-NOT complex to transcripts that undergo active translation. Murine CNOT6L, which is a CCR4-NOT catalytic subunit, is required for meiosis-coupled maternal mRNA decay. RT-qPCR results indicate that the CNOT6L, CNOT7, and BTG4 expression was significantly lower in human embryos that have M-decay defects compared to that in $TUBB8$-mutated embryos, whereas the decrease in CNOT6L, CNOT7, and BTG4 levels was less remarkable in the arrested embryos of unid-patients without M-decay defects (Fig. 7c, d and Supplementary Table 2). These results suggest that similar to the mechanisms of the mouse MZT,
BTG4 and CCR4-NOT may also participate in the M-decay pathway of human embryos.

Human embryos with ZGA defects also have defects to remove maternal transcripts through Z-decay. ZGA have been shown to occur at the 8-cell stage in human embryos, but the association between Z-decay and the developmental potential of early embryos has never been assessed in human. In a clinical context, normal embryos should develop into blastocysts 5 days after IVF. However, some embryos reached the 8-cell stage but fail to form blastocysts. Thus, we compared the transcriptomes of normal and 8-cell stage-arrested human embryos. Morphologically normal embryos at the 1-cell and 8-cell stages were collected. The 8-cell arrested embryos at day 5 after IVF were separately collected from six patients who experienced repeated developmental failure of preimplantation embryos after IVF (Fig. 8a, b). Individual normal and arrested embryos were subjected to single-embryo RNA-seq analyses, and the gene expression levels were assessed by FPKM.
All three normal embryo samples at the 1-cell or 8-cell stages had high correlations following principal component analysis, whereas 4 (#1, 4–6) of 6 arrested 8-cell embryos had high correlations (Fig. 8c). A heatmap also showed that these four samples had high correlations (average $r = 0.748$) and significantly differed from three normal 8-cell embryos (Supplementary Fig. 2a and Supplementary Table 3). In the following experiment, RNA-seq results of these four samples were further compared to those of normal embryos. There were 2968 and 5297 transcripts that were downregulated and upregulated more than 2 folds in arrested embryos when compared to normal 8-cell embryos (Fig. 8d). When the threshold of the fold changes was increased to 5, there were more genes that were upregulated than those that were downregulated (Fig. 8e and Supplementary Fig. S2b). In the
arrested embryos #2 and #3, however, the numbers of upregulated and downregulated genes were comparable (Supplementary Fig. 2c, d). Thus, mRNAs were accumulated in the arrested embryos #1, #4, #5, and #6.

A gene set enrichment analysis of the 2926 downregulated transcripts in the arrested embryos revealed that 1373 of these (~50%) belonged to early zygotically expressed genes of normal embryos (Fig. 8f). Thus, ZGA was at least partially impaired in these embryos. In addition, among the 4074 Z-decay transcripts that were detected in normal embryos, 775 transcripts were stabilized in the arrested embryos (Fig. 8g). Among these transcripts, 223 belonged to the previously identified ZGA-dependent Z-decay transcripts (Fig. 8g). A heatmap showed that the changed transcriptomes of four arrested embryos were consistently and significantly different from those of normal embryos (Fig. 8h). Further, a GO analysis revealed that the genes that failed to be expressed in arrested embryos were primarily associated with genome transcription and mRNA splicing (Fig. 8i). In contrast, the maternal transcripts that were accumulated in the arrested embryos were associated with the cell cycle, maternal behavior, and protein ubiquitination (Fig. 8j). These results were consistent with the phenotype of the prolonged 8-cell stage in these embryos.

Z-decay defects were detected in the early development arrested human embryos. We next investigated whether the Z-decay defects are frequently associated with the 8-cell arrest of human embryos. Embryos that were arrested as 8-cell embryos were separately collected at day 5 after IVF from 14 patients who experienced repeated preimplantation developmental failure. In a high proportion of these embryos, mRNA expression levels of the key ZGA factor MYC (12/14) and Z-decay factors (10/14) for TEAD4 and 9/14 for TUT7, except for TUT4, were significantly lower than normal (Fig. 9a, b and Supplementary Table 4). Although these 8-cell arrested embryos had developed 2 days longer than the control embryos before RNA extraction, the maternal transcripts that were known to be degraded through the Z-decay pathway remained at higher levels in these embryos than in the normal 8-cell embryos. These include factors that are associated with the cell cycle (CENP), protein degradation (SKP1), maternal mRNA degradation (CNOT7), and histone H3 methylation (SUZ39H2) (Fig. 9c, d and Supplementary Table 4)

These results indicate an association between the Z-decay of maternal mRNAs and preimplantation developmental competence of human embryos.

Inhibition of YAP–TEAD4 activity impaired TUT4/7 expression and Z-decay in mouse and human embryos. In the following experiments, we aimed to repress the YAP–TEAD4 activity in early human embryos using verteporfin, a small molecule that prevents the YAP–TEAD4 interaction, and then determine if the zygotic expression of TUT4/7 and removal of Z-decay transcripts were impaired. First, we confirmed the effects of verteporfin treatment in mouse. When GV oocytes were cultured in medium containing 1 µM verteporfin, meiotic maturation were normal as the control group (Supplementary Fig. 3a, b), suggesting that the molecule is not toxic at this concentration. However, zygotes cultured at the presence of verteporfin had lower developmental rates than the control zygotes, with significant arrest at the 2–4-cell stages (Supplementary Fig. 3c, d). In verteporfin-treated 2-cell embryos, expression of known YAP–TEAD4 target genes, including TUT4/7, was repressed (Supplementary Fig. 3e). Meanwhile, the known mouse Z-decay transcripts accumulated in these embryos (Supplementary Fig. 3f). These phenotypes were similar to those observed in maternal Yap1 knockout or TEAD4-inhibited embryos, suggesting that verteporfin effectively inhibited YAP–TEAD4 activity in cultured embryos.

Next, we cultured human zygotes with 3PN in medium containing verteporfin. The 3PN zygotes are usually caused by polyspermic IVF and are ethically approved to be used for research purpose. Approximately 40% 3PN zygotes developed to the 8-cell stage, with or without verteporfin treatment (Fig. 10a). These 8-cell embryos were collected for RT-qPCR analyses. The results showed that TUT4/7 expression was repressed (Fig. 10b, c), whereas representative Z-decay transcripts accumulated (Fig. 10d, e) in the verteporfin-treated embryos. Therefore, YAP–TEAD4 is likely to have a conserved function to trigger zygotic TUT4/7 expression as well as Z-decay transcript removal in both mouse and human early embryos.

Discussion

Studies in model systems have shown that both maternal and zygotic transcript degradation pathways are functional in the early mouse embryo during MZT. When M-decay was impaired in mice, the embryos were arrested at the 1–2-cell stages, whereas Z-decay is required for mouse embryo development beyond the 4-cell stage. However, whether mRNA decay (including M-decay and Z-decay) also plays a key functional role in human embryo development has not been investigated until this study. Thus, maternal mRNA decay defects have never been associated with early developmental arrest of human embryos after IVF. The current data mainly provided correlative rather than causal evidence that the factors facilitating mouse maternal mRNA decay may also be involved in the regulation of maternal mRNA stability during human MZT. Meanwhile, in another study we have identified infertile women carrying BTG4...
mutations. The zygotes from these women were arrested at the 1-cell stage and exhibited defects in maternal mRNA degradation. The phenotypes were similar to those we have observed in Btg4 knockout mice\(^1\). The identification of Btg4 mutations in infertile women supports our hypothesis that Btg4/CCCR4-NOT-induced mRNA deadenylation is involved in the regulation of maternal mRNA stability during human MZT. Also consistent with our working model, TUT4/7 expression and Z-decay of maternal transcripts was impaired in human 8-cell embryos derived from 3PN zygotes, when Yap–Tead4 activity was inhibited. These results provide evidence that Yap and TUT4/7 are likely regulating Z-decay of maternal mRNA during human MZT.

In this study, oocyte and embryo transcriptomes of human and mouse origin, as well as human embryo transcriptomes generated by different groups were compared. The absolute FPKMs can vary among different datasets due to differences in input RNA quantity, the efficiency of reverse transcription, and detection sensitivity. Furthermore, human samples obtained from the clinic often vary significantly across many factors, including patient age, genetic background, living environment, diet, and other factors. These factors cannot be strictly controlled as they are in experiments using a mouse model. Therefore, it is common to observe fewer overlaps of transcriptomic datasets published by different groups. The actual overlapped genes in most analyses of this study should be more than it appeared.

Fig. 7 Levels of maternal transcripts in development-arrested human zygotes. a and b RT-qPCR results showing the mRNA levels of selected M-decay transcripts in arrested zygotes derived from TUBB8-mutated and unid-patients 3 days after IVF. c and d RT-qPCR results showing the mRNA levels of Btg4, CNOT7, and CNOT6L in arrested zygotes that were derived from TUBB8-mutated and unid-patients. Data are presented as mean values ± SEM. P value by one-way ANOVA. n = 3 independent experiments.
It is likely that, in addition to mRNA degradation, the maternal proteins are also removed for embryo development. The results of our GO analyses suggest that the zygotes failed to undergo normal M-decay of maternal mRNAs, which indicates that the transcripts related to protein translation and mRNA stability were enriched and may be the cause of over-translation of the accumulated maternal mRNAs eventually leading to cell division defects. Similarly, due to ZGA defects, many of the maternal mRNAs that were associated with the meiotic cell cycle and that should have been degraded by the Z-decay pathway were...
accumulated, leading to embryonic developmental retardation at the 8-cell stage. It was unclear whether timely mRNA degradation occurs in oocytes that are arrested in meiosis I or II due to spindle assembly defects or whether M-decay depended on progression to meiosis II or even meiosis exit after fertilization. In previous studies, we have performed experiments in mouse oocytes to address this unanswered question. We artificially arrested the maturing oocytes in meiosis I by treatment with nocodazole, which is a widely used microtubule disruptor. We then detected the degradation of selective mRNAs that should have been removed by M-decay in these oocytes using RT-qPCR. The results showed that, while the degradation of these mRNAs was impaired by M-decay-associated genetic defects, they were not affected by nocodazole treatment. In this study, we further provided in vivo evidence that M-decay in humans is not impaired by meiosis defects caused by TUBB8 mutations. This is evidence that the delayed mRNA decay observed in some arrested zygotes is primarily due to a lack of M-decay factors, rather than secondary consequences of cell cycle arrest. In the Z-decay in Drosophila and zebrafish embryos, microRNAs play an important role. Smaug, which is a master MZT
regulator in Drosophila, is required for zygotic synthesis of the miR-309 family of microRNAs, which targets several hundred maternal transcripts for degradation during MZT. Zebras fish miR-430 is expressed at the onset of zygotic transcription and facilitates the deadenylation and clearance of maternal mRNAs during early embryogenesis. However, it has also been reported that microRNA function is globally suppressed in mouse oocytes during early embryogenesis. However, it has also been reported that microRNA function is globally suppressed in mouse oocytes during early embryogenesis. Thus, mRNAs of certain early zygotic genes may be the major zygotic transcription products that regulate Z-decay in mammals.

On the contrary, the involvement of de novo proteins translated from early zygotic transcripts in Z-decay is not clearly described in all model systems. Our studies suggest that early zygotic expression of T read4 and Tut4/7 may be required for Z-decay in both mice and humans. In 4-cell embryos derived from oocyte-specific Yap1 knockout mice, maternal transcripts were accumulated, particularly those that were destined to be removed by the Z-decay pathway. Similarly, in the arrested human embryos, the decreased expression of these factors (BTG4, CNOT7, T read4, Tut4/7) was closely associated with maternal mRNA degradation defects, which suggests that the maternal mRNA clearing pathway is highly conserved in vertebrate species.

Transcriptome analyses indicated that the Z-decay process is largely completed by the 8-cell stage in human embryos. Different from its involvement in mice, zygotic transcription plays a more important role in the Z-decay of human maternal transcripts, probably due to a longer duration from ZGA to the completion of Z-decay in humans compared to that in mice.
has been noted in clinically assisted reproduction practices that many poor-quality embryos or embryos derived from aged oocytes were arrested at the 8–16-cell stage. Therefore, the maternal and zygotic components of the Z-decay pathway may be key factors that determine the quality and developmental potential of human embryos.

Methods

Human oocyte and early embryo collection. All of the oocytes and embryo were obtained with signed informed consent by the donor couples. The ovaries were stimulated using GnRH analogs combined with recombinant follicle stimulating hormone (FSH). Oocytes were obtained through follicle puncture at 36 h after hCG administration. The donated oocytes were randomly picked. The cumulus cells around each oocyte were removed using hyaluronidase treatment. MIL oocytes were acquired 36 h from in vitro maturation of the immature (GV/MII) oocyte.

To collect early embryos, in vitro fertilized eggs were cultured until the 8-cell stage using a G1 (Vitrolife) human embryos culture medium. The G2 (Vitrolife) medium was used to culture the 8-cell embryos to the blastocyst stage. When normal embryos developed to the 8-cell stage at day 3 after fertilization, the embryos that were arrested at the 1-cell stage were collected. 8-cell embryos of the control groups were from abnormal zygotes (3PN fertilization) and collected at day 3 after fertilization. The arrested 8-cell embryos that have no signs of degeneration were collected at day 5 after fertilization. None of the donated oocytes were fertilized for the purposes of this study.

In some experiments, oocytes were acquired from 6 donors; 39 arrested zygotes (3PN included) were acquired from 30 donors; and 23 arrested 8-cell embryos were acquired from 30 donors. The experiments performed in this study were approved and guided by the ethical committee of Guangdong Second Provincial General Hospital (Research license YY-2018-009-01) and the Reproductive & Genetic Hospital of CITIC-XIANGYA (Research license LL-SC-2019-030).

Animals. All the used mouse strains were of a C57/S6 background. Wild type C57/S6 mice were obtained from the Zhejiang Academy of Medical Science, China. The experimental protocols that involved mice were approved by the Zhejiang University Institutional Animal Care and Research Committee (Approval # ZJU20170014), and mouse care and use was performed in accordance with the relevant guidelines and regulations.

Mouse oocyte culture. Female mice (21–23 days old) were injected with 5 IU of PMSG and were humanely euthanized after 44 h. Oocytes at the GV stage were harvested in M2 medium (M7167; Sigma-Aldrich) and cultured in mini-drops of M16 medium (M7292; Sigma-Aldrich) that were covered with mineral oil (M5310; Sigma-Aldrich) at 37 °C in a 5% CO2 atmosphere.

Microinjection of mouse oocyte. All injections were performed using an Eppendorf transferman NK2 micromanipulator. GV oocytes were incubated in M2 medium before to inject spontaneous GVBD and microinjected at 5–10 pl samples per zygote. The concentration of all microinjected RNAs was then adjusted to 1000 ng/µl. After microinjection, oocytes were washed and cultured in M16 medium at 37 °C with 5% CO2.

Immunofluorescence. Oocytes and embryos were fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS) for 30 min and permeabilized in PBS containing 0.3% Triton X-100 for 30 min. After being blocked with 1% bovine serum albumin in PBS, the oocytes were incubated with primary antibodies for 1 h and sequentially labeled with Alexa Fluor Cy3-conjugated or 488-conjugated secondary antibodies and 4,6-diamidino-2-phenylindole (DAPI) for 30 min. A confocal microscope was used to image oocytes.

In vitro transcription and preparation of mRNAs for microinjection. To prepare mRNAs for microinjection, expression vectors were linearized and subjected to phenol/chloroform extraction and ethanol precipitation. Linearized DNAs were in vitro transcribed using the SP6 message mMACHe Kit (Life, AM1340). Transcribed mRNAs were then added to poly (A) tails (~200–250 bp) using the mMACHe Kit (Life, AM1350), recovered by lithium chloride precipitation, cleaned by ethanol, and lastly resuspended in nuclease-free water.

Single cell RNA-Seq library preparation. To remove the zona pellucida, the embryos were exposed to acidic Tyrode’s solution (pH 2.5, Sigma, Cat#T7788) for 3–5 s and then washed thoroughly in PBS containing 0.5% bovine serum albumin (BSA) (Sigma, Cat#A933). Single cells were placed into individual tubes that contained 4 µl of lysis buffer (1.96 µl of nuclease-free water, 1 µl of 10 mM DNTP mix (NEB, Cat#N0447), 0.1 µl of 40 U/ml RNase-inhibitor (NEB, Cat#M0314)), 0.04 µl of 10% Triton X-100 (Sigma, Cat#T8787), and 1 µl of 10 mM modified oligo-dT primer (5’-AACCCAGACTGACT30VN-3’). After 3 min of cell lysis at 72 °C, Smart-seq2 reverse transcription reactions were performed. After the first-strand reaction, the cDNA was amplified using a limited number of cycles (~13 cycles). Sequencing libraries were constructed from 500 pg of amplified cDNA using the TruePrep DNA Library Prep Kit V2 for Illumina (Vazyme, TD920) according to the manufacturer’s instructions. Barcoded libraries were pooled and sequenced on the Illumina HiSeq X Ten platform in the 150 bp paired-end mode.

RNA seq data analysis. All the raw reads were first preprocessed using Trimomatic (v0.35) to remove sequencing adapters, trim low-quality bases from both read ends (with the parameters LEADING:3 TRAILING:3 SLIDINGWINDOW:4:15), and remove reads <36 bp in length. The clean reads were then mapped to the human reference genome of GRCh38 (without masking repeats) using STAR aligner (v2.5.2b). Ensemble genes were calculated using HTSeq (v0.6.1p1). The expression levels of each gene were quantified using normalized FPKM. Two-tailed Student’s t-test was used to determine statistical significance of differences between samples. PCA clustering for different embryos was performed using the R pcompc function. Summaries of the RNA-seq data generated in this study are shown in Supplementary Tables 5 and 6.

RNA isolation and real-time RT-PCR. Oocytes or embryos were collected and lysed in 2 µl of lysis buffer (0.2% lysis buffer (0.2% Triton X-100) and 4 U/RNase inhibitor) followed by reverse transcription with primer transcript II reverse transcriptase (Takara), according to the manufacturer’s instructions. A real-time RT-PCR analysis was performed using the Power SYBR Green PCR Master Mix (Applied Biosystems, Life technologies) and an Applied Biosystems 7500 Real-Time PCR System. The respective cycle threshold (Ct) values were obtained, and relative mRNA levels were calculated by normalization to the endogenous mRNA levels (internal control) using Microsoft EXCEL®. The gene expression levels were calculated by 2^ΔCt (2^ΔCt (gene–GAPDH)). The relative transcript levels of the samples were compared to those of controls, and fold changes were determined. For each experiment, qPCR was performed in triplicate. Primer sequences are listed in Supplementary Table 7.

Maternal transcript clustering. The data was extracted from previously published dataset (GV data from GSE107746 and others from GSE36552; The datasets of a-amantatin treatment are from GSE105171). Maternal mRNAs with reliable sequence annotations and FPKM of ≥2 at the GV stage were retained for further analysis. Expression levels of each gene were added to one and then transformed by log2 in the following analysis. Cluster I–IV consisted of genes that satisfy the following formulas:

Cluster I: Expression (GV) > Expression (zygote) + 1; Expression (zygote) ≤ Expression (8-cell) + 1.
Cluster II: Expression (GV) ≤ Expression (zygote) + 1; Expression (GV) > Expression (zygote) – 1; Expression (zygote)–Expression (8-cell) + 1.
Cluster III: Expression (GV) > Expression (zygote) + 1; Expression (zygote)–Expression (8-cell) + 1.
Cluster IV: Expression (GV) > Expression (zygote) + 1; Expression (GV) > Expression (zygote) – 1; Expression (zygote) ≤ Expression (8-cell) – 1; Expression (zygote)–Expression (8-cell) – 1.

3’-UTR analysis. The 3’-UTR sequences of humans (grch37) were extracted from the UCSC Table Browser. The conserved sequences 5’-GAGAGCUUUAAAGUCUUUAA-3’ and 5’-AAUAAAUAUAAUAAUAA-3’ were used to identify CPEs and PASs, respectively. The lengths of 3’-UTRs and numbers of CPEs and PASs in 3’-UTRs were calculated using an in-house Python script.

Statistical analysis. Results were presented as mean ± SEM. Most experiments included at least three samples and were repeated at least three times. The results for the two experimental groups were compared using two-tailed unpaired Student’s t-tests and one way ANOVA. Values were considered statistically significant at P < 0.05, P < 0.01, and P < 0.001.

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

Data availability. RNA-seq data have been deposited in the NCBI Gene Expression Omnibus database under accession code PRJNA635859. Source data are provided with this paper.

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Additional information
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