Tubastatin A inhibits HDAC and Sirtuin activity rather than being a HDAC6-specific inhibitor in mouse oocytes

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ABSTRACT

Tubastatin A (TubA) is a highly selective histone deacetylase 6 (HDAC6) inhibitor. As expected, mouse germinal vesicle oocytes fail to extrude the first polar body following TubA treatment. However, a previous study demonstrated that homozygous Hdac6 knockout (KO) mice can be viable and fertile. Therefore, we asked whether TubA is indeed a specific inhibitor of HDAC6 activity. RNA-sequencing and in silico analysis demonstrated that the TubA-treated group presented significant changes in the expression of Hdac subfamily genes such as Hdac6, 10, and 11, and Sirtuin 2, 5, 6, and 7. Additionally, gene expression related to the p53, MAPK, Wnt, and Notch signaling pathways in the TubA-treated group were increased significantly; in contrast, gene expression related to metabolism, DNA replication, and oxidative phosphorylation was decreased significantly. Furthermore, gene expression related to cell cycle, cell structure, pyrimidine metabolism, pentose phosphate pathway, mitochondrial activation, proteasome pathway, RNA polymerase, DNA replication, cyclin-dependent kinase, nucleolar activity, and MI arrest were significantly decreased, indicating that TubA-induced abnormal meiotic maturation and oocyte senescence may be due to the combined effects of HDAC and Sirtuin inhibition, and not HDAC6 inhibition alone. Thus, we believed that this system could provide a model for monitoring the effects of TubA on mouse oocytes.

INTRODUCTION

The balance between deposition and removal of histone modifications plays a critical role in the regulation of gene expression and maintenance of cellular homeostasis [1]. Epigenetic marking is known to be mediated by proteins that add, remove, or interpret the modified structures, referred to as “writers”, “erasers”, and “readers”, respectively [2]. Epigenetic changes controlled by the opposing activity of two classes of enzymes, histone acetyl transferases (HATs) and histone deacytles (HDACs), can lead to altered phenotypes and gene expression [3]. Generally, HATs are categorized into two main families, type A and type B. Although little is known about type B HATs, type A have been characterized and can be divided into three major families: (1) GNAT family (PCAF and GCN5), (2) p300/CREB proteins, and (3) MYST proteins; meanwhile, HDACs constitute one superfamily and have been categorized into four main classes (class I, II, III, and IV). Among these, class III HDACs comprise seven Sirtuins (Sirt 1-7), unlike others, defined by their deacytles activity that is dependent on nicotinamide adenine dinucleotide (NAD+) [4, 5].

Various HDAC inhibitors (HDACis) are known to selectively alter gene transcription by promoting histone acetylation [6]. Moreover, they can increase the
acetylation of many histone and nonhistone proteins, modifying their function or activity [7]. A good example of an HDACi is Tubastatin A (TubA), which drives cell cycle arrest, suggesting HDACs as therapeutic targets for cancer, chronic immune and inflammatory disorders, Alzheimer’s disease, and other tauopathies [8, 9]. TubA is known as a potent and highly selective HDAC6 inhibitor with an IC50 of 15 nM and a greater than 1000-fold selectivity for all other isoforms, except HDAC8 (57-fold) [10]. HDAC6 is a unique member of the class IIb HDACs, possessing two catalytic domains and exhibiting a predominantly cytoplasmic localization [11]. It regulates various cellular processes including microtubule-based transport, cell motility, endocytosis, cell migration, autophagy, and aggresome formation by deacetylating non-histone proteins such as α-tubulin, cortactin, and heat shock protein 90 (HSP90) [12-16]. Inhibition of HDAC6 activity by TubA supplementation in vitro has been reported to lead to the hyperacetylation of tubulin and microtubules [12]. However, the molecular basis underlying the choice between abnormal oocyte maturation and acetylation homeostasis by TubA is not well understood.

Knockout (KO) mouse models provide a valuable experimental tool for the evaluation of gene function and there are various reports on the generation of Hdac6 KO mice [13]. Although homozygous Hdac6 KO mice exhibited global tubulin hyperacetylation, they were shown to be viable and fertile and presented no major observable phenotype [14]. Recently, however, we and others reported that TubA treatment induced cell cycle arrest with a failure of spindle migration and actin cap formation in mouse oocytes [15, 16], indicating that most of the oocytes were arrested at an metaphase I (MI)- or a germinal vesicle breakdown (GVBD)-like stage. Taken together, these observations question the in vivo role of acetylated microtubules and the contribution of tubulin acetylases and deacetylases to cell function, and whether TubA inhibitory activity is indeed specific to HDAC6. To address these questions, TubA-supplemented oocytes were subjected to RNA-seq analysis to elucidate the underlying mechanism of abnormal oocyte maturation. In conclusion, RNA-sequencing and in silico pathway analysis demonstrated the potential for using mouse oocytes as an in vitro platform for the systematic validation of chemotherapeutic targets.

RESULTS

To determine whether the wave of histone acetylation/deacetylation is of functional significance for the mouse oocyte meiotic resumption, mouse oocytes were allowed to undergo meiotic maturation with or without TubA, a potent inhibitor of HDAC6. Most control oocytes progressed to germinal vesicle breakdown (GVBD) at 4 h, first metaphase (MI) at 8 h, first anaphase and telophase (AT1) at 9.5 h, and second metaphase (MII) oocytes with first polar body at 12 h. However, oocytes exposed to 20 μM TubA [15] failed to transition through the GVBD to MI or AT1 stages.

RNA-seq analysis of mouse oocytes

Control GV- and MII-stage oocytes, as well as TubA-supplemented oocytes (10 and 20 μM) were subjected to RNA-seq to elucidate the underlying mechanism of abnormal oocyte maturation. As shown in Fig. 1A, a total of 49,430,590, 78,472,444, 78,239,478, and 53,687,286 reads were obtained from four RNA-seq libraries from GV, MII, TubA-10 (10 μM treatment) and TubA-20 (20 μM treatment), respectively. To reveal the molecular events underlying transcriptomic profiles, sequence reads were mapped to a reference transcriptome of control GV- or MII-stage oocytes. From the above reads, 15,593, 15,687, 15,815, and 14,667 genes from GV, MII, TubA-10, and TubA-20, respectively, were mapped to a unigene. To determine differences in gene expression between TubA-treated and control GV or MII oocytes, gene expression data from control GV oocytes were compared to data from control MII (MII/GV) or TubA-treated oocytes (TubA/GV). MII/GV and TubA/GV pairs had 13,756 commonly expressed genes, while 718, 723, and 294 differentially expressed genes (DEGs) were unique to GV, MII, and TubA, respectively (Fig. 1B). After normalization (Fig. 1C), 2,034 and 693 DEGs in MII/GV and TubA-10 pairs were significantly up- and downregulated (R=0.899; \log_{10}FPKM, FC>2), respectively, whereas 2,065 and 313 DEGs were significantly up- and downregulated, respectively, in TubA20/GV pairs (Fig. 1D). All the DEGs with FC>2 and FDR<0.1 in one comparison were selected for canonical pathway analysis using IPA software. P-values were calculated based on the number of DEGs and total mapped genes, as described previously [17].

Gene network and IPA analysis

An RNA-seq-based transcriptome analysis was used to investigate key genes involved in abnormal meiosis of mouse oocyte maturation. The most enriched gene ontology (GO) terms for biological process, cellular component, and molecular function using GOpplot analysis are shown in Fig. 1E-G, respectively. The common GO terms from MTII/GV and TubA/GV pairs are translation and cellular response to DNA damage stimulus for biological process (Fig. 1E), structural constituent of ribosome, iron-sulfur cluster binding, proton-transporting ATP-synthase activity, and NADH
Figure 1. Gene expression is altered in TubA treated oocytes. (A) Sequence reads and number of unique genes in GV, MII, and the TubA (10 and 20 μM)-treated group. (B) Venn diagram showing the overlap between DEGs in GV, MII, TubA-10-, and TubA-20-treated oocytes. (C) Violin plots of differential gene expression profiles identified by SCPattern analysis. The y-axis indicates normalized expression values, FPKM. The x-axis indicates oocyte stage or TubA dosage of each sample. (D) Scatterplot comparing the transcriptomes of GV, MII, with or without TubA-10 and/or TubA-20. Blue dots indicate significantly decreased genes, while red dots indicate more highly expressed genes. (E-G) GO circle plot analysis of RNA-seq in MII/GV and TubA20/GV pairs. The outer circle shows a scatter plot for each term of the log2FC of the assigned genes in each enriched gene ontology (GO) term: biological process (E), molecular function (F), cellular component (G). Red circles display upregulation and blue circles display downregulation by default. The inner ring is a bar plot where the height of the bar indicates the significance of the GO terms (log_{10} adjusted p value), and color corresponds to the z-score: green, decreased; red, increased; and white, unchanged. (H, I) Canonical signalling pathways enriched in MT/GV and TubA-20/GV differentially expressed genes (ingenuity pathway analysis [IPA]). The ratio of differentially expressed genes in the pathways is shown for pathways with Benjamini-Hochberg-corrected p values <0.01. Activated or repressed pathways in (I) are shown as red and blue dots, respectively. Specific enriched pathways are highlighted. (J) Genome browser view depicting differential expression in fragment count for the Hdac6 gene. At the gene level, overall, there is a large difference between control-GV (or –MII) and TubA-20, as shown in read coverage profiles. Red color indicates abundant transcripts of Hdac6 mRNA.

Figure 2. Major canonical pathways influenced TubA in control MII/GV and TubA/GV pairs. (A) Time schedule of control GV to MII oocyte transition. (B, C) Ingenuity pathway analysis (IPA) and functional categorization of the top selected genes by RNA-Seq of control MII/GV (B) and TubA/GV (C) pairs. The secondary Y-axis shows the -log (p-value) of the probability for genes in a data set to associate with identified pathways by chance. A threshold p value of 0.05 is presented as a yellow dotted line. The ratio of the number of genes from the data set that map to a given pathway divided by the total number of genes that map to the canonical pathway is shown as a solid line.
dehydrogenase activity for cellular component (Fig. 1F). However, no common category was found for molecular function (Fig. 1G). Next, we examined the dysregulation of canonical signaling pathways using Ingenuity Pathway Analysis (IPA) and plotted the ratio of dysregulated genes in specific pathways against their p value for overrepresentation (Fig. 1H and I). Of note, MII/GV pairs showed a negative feedback loop in EIF2 signaling and the NRF2-mediated oxidative stress response pathway (Fig. II, blue color).

An important quality parameter of RNA-seq mapping is the percentage of mapped reads, which is an indicator of the overall RNA-seq accuracy. When reads were mapped against the transcriptome of the Hdac6 gene, the TubA-20 treated group showed marginally lower multi-mapping read percentages compared to control GV or MII oocytes (Fig. 1J), indicating that TubA is a critical inhibitor of HDAC6 and that our approach was shown to work well with normalized RNA-seq data.

After RNA-seq quantification of the normalized data, we examined DEG profiles between control (GV or MII) and TubA-treated groups. The number of up- and downregulated DEGs in the top 10 enriched pathways are shown in Supplementary Table 1 and 2. Of note, metabolic-related pathways (pentose phosphate, pyrimidine metabolism, oxidative phosphorylation, and ribosome pathways) in the TubA-treated group were strongly downregulated. However, the top 10 upregulated KEGG pathways in the TubA-treated group were MAPK signaling, progesterone-mediated oocyte maturation, p53 signaling, insulin signaling, and cell cycle (Supplementary Table 1). As shown in Fig. 2B and C, IPA canonical pathway analysis showed that EIF2 signaling, mitochondrial dysfunction, oxidative phosphorylation, regulation of EIF4 and p70S6K signaling, Sirtuin signaling, colanic acid building block biosynthesis, GDP-mannose biosynthesis, mTOR signaling, role of PKR in interferon, induction and antiviral response, tRNA charging, and ERK signaling were simultaneously affected in both the MII/GV and TubA/GV pairs. However, a significant proportion of the genes in TubA-treated oocytes were enriched in cell cycle-related pathways (cyclins and cell cycle regulation, role of BRCA1 in DNA damage response, CHK proteins in cell cycle checkpoint control, cell cycle regulation by BTG family proteins, cell cycle G1/S checkpoint regulation, G2/M DNA damage checkpoint regulation, and MAPK/ERK signaling). The observed dysregulation of these genes would be expected to prolong cell cycle checkpoint signaling leading to a further delay or block in entry into meiosis.

**TubA effect is not solely mediated by HDAC6-specific inhibition in mouse oocytes**

TubA-treated oocytes underwent GBVD on schedule, but the meiosis I to meiosis II transition was impaired (Fig. 3A). Given that TubA is known as a specific inhibitor of HDAC6 [6], we examined whether inhibition or blocking the GV to GVBD or MI (or ATI) transition observed with TubA treatment resulted from disruption of Hdac6-specific gene expression. As shown in Fig. 3B and C, the expression of Hdac6 in control MII-stage oocytes was marginally lower compared to control GV-stage oocytes, whereas Hdac6 mRNA levels in TubA-treated groups were significantly decreased compared to control GV- or MII-stage oocytes. Similar results were obtained for Hdac10 and 11 mRNA expressions, although the expression of these genes during the GV to MII transition in the control group was marginally higher than the TubA-treated group. Although Hdac9 mRNA expression during the GV to MII transition showed a small decrease in the control group, expression in the TubA-treated group increased slightly or was not altered compared to the control during the GV to MII transition. Therefore, our RNA-seq analysis indicates that significant changes in the expression of these mRNAs, as well as the observed abnormal oocyte maturation, were closely related to TubA treatment.

Of the pathways analyzed, the Sirtuin signaling pathway exhibited the highest p value (p = 2×10^{-17}), and is predicted to be activated in the TubA-treated samples (Fig. 2C). Consequently, we examined Sirtuin family-related gene expression and found that TubA was not selective for HDAC6 alone, instead inhibiting both Class II (Hdac6 and 9) and III HDACs (Sirt2, 5, 6, and 7) (Fig. 3B-D). Previous data suggested that a specific Sirt2 inhibitor blocked the progression of GVBD during *in vitro* oocyte maturation [18] and that Sirt2 knockdown resulted in abnormal spindle and chromosome alignment during meiosis [19]. Furthermore, Sirt6 or 7 deficiencies lead to replication stress, an important source of endogenous DNA damage [20, 21]. Therefore, TubA-induced spindle or chromosome organization failure may be due to reduced Sirt2, 5, 6, and 7 mRNA expressions.

**Effects of TubA on the expression of oocyte maturation-related genes**

To examine cyclin-dependent kinase gene expression (Cdk1, Cdk2, Cdk4, and Cdk6), we visualized the enrichment of each mRNA expression at the candidate gene loci using Integrative Genomics Viewer (IGV). As shown in Fig. 4A, expression of these mRNAs in TubA-
treated oocytes was significantly reduced compared to the control GV- or MII-stage oocytes. Even though mRNA expression levels of Cdk1, Cdk2, Cdk4, Cdk6, and Cdc25b in the TubA-treated groups showed a significant decrease, CDK1 and Cyclin B protein content in the TubA-treated groups increased continuously in a time-dependent manner (Fig. 4B) compared to the control. Previously, it was reported that protein synthesis rates were significantly reduced during oocyte maturation [22], indicating that the failure of developing oocytes in transition from the GV to MI or MI to MII stage following TubA treatment may be correlated with impaired CDK and Cyclin B accumulation; this may disrupt the nuclear shape and chromatin organization and subsequently lead to DNA damage accumulation and senescence. Furthermore, 19 genes involved in degradation through the 26S proteasome pathway (Psmb5, Psmf1, Psmb4, Psmd14, Psmb7, Psme5, Psmd13, Psmb6, Psma6, Psme4, Psmb1, Psme2, Psme3, Psme2, Psme1, Psma3, Psmd4, Psmd6, and Psmd8), showed significantly decreased expression levels in the TubA/GV compared to the MII/GV pair (Supplementary Table 2). This may also explain why protein levels increased despite the downregulation of mRNA expression after TubA supplementation.

We next determined the effects of TubA on the expression of factors involved in the GVBD to MI transition (or MI to AT1 transition) arrest. As shown in

Figure 3. Heatmap and IGV of DEGs in control GV, TubA (10 and 20 µM)-treated, and MII oocytes. (A) A representative oocyte of control-GV, TubA-treated (10 and 20 µM), and control-MII-stage oocytes. Of note, control mouse GV oocytes can undergo meiotic maturation in vitro, whereas 20 µM of TubA supplementation inhibited progression through the MI (or AT1) to MII transition in mouse oocytes. (C, D) Visualization of mRNA expression enrichment of the Class IIb Hdac and Class III sirtuin loci using IGV.
Fig. 4C and D, the expression of MI arrest-related genes (Cops3, Cops5, Tkt, Zap70, and Obox5) and cytokinesis-related gene (Arp2, Arp3, Aurora C, and Cdc14b) in TubA-treated oocytes was significantly reduced compared to the control. Considering a relative increase in these transcript levels was observed during the GV to MII transition in control oocytes, a significant reduction in the levels of these transcripts in the TubA-treated group may result in the failure of spindle or chromosome organization during oocyte maturation. Additionally, there was a significant decrease in RNA polymerase (Popr3h, Polr2e, Polr3k, Polr1e, Polr2l, Polr1d, Polr2k, Polr2l, Znrd1, Polr3a, Polr1c, and Polr3c)- and DNA replication (Rpa2, Pold4, Rfc3, Rfc4, Sshp1, Rfc2, Pold2, Rnaseh2a, Fen1, Rnaseh2c, and Mcm5)-related genes in the TubA/GV pair.
Furthermore, gene expression related to cell structure (Tubb1a, Tubb2b, and Tubb6), mitochondrial activation (Atp5D, Atp5E, Atp5h, Atp5k, Atp5j, Atp5O, Atp6v0d1, Ndufs6, Ndufs5, Ndufs8, Ndufs3, and Ndufs2), pyrimidine metabolism (Dyynk, Uppl, Znrd1, Tk2, Tyns, Nt5c3, Uck2, Entpd1, Nudt2, Nme6, Umps, Nme3, and Nme1), and the pentose phosphate pathway (Aldoa, Pgm2, Pgls, G6pdx, Fbp1, Pfkp, Dera, Tki, Fbp2, and Gpi1) also exhibited a significant decrease (Supplementary Table 2). Together, these observations suggest that TubA-treated oocytes may arrest at the MI stage due to reduced metabolism induced by the TubA treatment.

For verification of the RNA-seq data, the expression levels of these RNAs were quantified using quantitative real time reverse transcription PCR (RT-qPCR). For these experiments, a separate group of TubA-treated or

Figure 5. Ribosome biosynthesis in GV, MII, and TubA-treated oocytes. (A) GSEA plots showing preferential downregulation of the KEGG ribosome in matched pairs of TubA20 treated (TubA/GV pairs) versus untreated control (MII/GV pairs). (B) Expression of ribosome biosynthesis-related functional genes in GV-, TubA-20 treated, and MII-stage oocytes. Y-axis shows the FPKM values of genes inferred from the transcriptome data. Right indicates a heatmap showing DEGs of Rps family genes in GV, MII, and TubA-treated oocytes. (C) IPA analysis of MII/GV and TubA/GV pairs. Shapes and lines are color-coded based on predicted associations and functions. Green and red indicate up- and downregulated gene expression.
control GV- or MII-stage oocytes was prepared under the same experimental conditions used for the RNA-seq analysis. For validation, we examined the expression of MI arrest-related genes (Cops3, Cops4, Tkt, Zsp70, and Obox1) and cytokinesis-related genes (Cdc14b, Arp2, Arp3, and Aurora C). As shown in Fig. 4E, we found that the expression of the selected mRNAs detected by RT-qPCR agree with the RNA-seq data.

**Effects of TubA on nucleolar activity**

The nuclear structures responsible for ribosome biogenesis are nucleoli. To examine the roles of TubA in ribosome biogenesis, we performed an analysis of gene set enrichment using KEGG pathway mapping. The "ribosome" pathway was the top differentially expressed pathway with both the control (MII/GV) and TubA/GV pairs (Fig. 5A), with genes encoding ribosomal subunits such as Rps5, 6, 10, 13, 15, 17, 18, 19, and 20 presenting significant reductions (Fig. 5B, left). Of the 73 transcripts that encode cytoplasmic ribosomal proteins from both the large and small ribosomal subunits, most transcripts in the control MII-stage oocytes showed a significant decrease compared to control GV oocytes. Notably, TubA-treated groups showed a greater reduction in mRNAs encoding these ribosomal proteins, with a few exceptions (Right, Fig. 5B). As shown in Supplementary Table 2, Polr1e, Polr1d, and Polr1c mRNA expression in the TubA/GV pair was significantly downregulated compared to the MII/GV pair. Furthermore, Rpl3, Rpl5, Rpl11, and Rpl23 gene expressions as well as p53, Mdm2, E2fs, and c-myc in TubA/GV pair are significantly altered, compared to MII/GV pair (Fig.5B and Fig.6). Considering that initiation of RNA synthesis is associated with a characteristic remodeling of the nucleolar architecture, TubA may disrupt remodeling of the nucleolar architecture during oocyte maturation. As a result, perturbation of ribosome biogenesis in developing oocytes by TubA treatment may cause nucleolar stress, leading to cell cycle arrest in a p53-dependent manner.

Previous studies reported that SIRT7 was the only sirtuin localized to nucleoli [23, 24] and that SIRT7 depletion led to hyperacetylation of H3K18 in the promoters of the Rps20, Rps7, Rps14, Nme1, and Cops2 genes [25]. In this study, we found that Cops3, Cops5, and Sirt7 gene expression was significantly downregulated (Fig. 3D and Fig. 4D). Considering that Sirt7 may be a positive regulator of rDNA transcription via its association with RNA Polymerase I [26], we propose that reduced rDNA transcription in TubA-stimulated oocytes may be associated with Sirt7 and linked to replicative senescence, which may be responsible for inducing oocyte aging (Fig. 5C).

**Effects of TubA on the regulation of NAT10, a nucleolar protein**

In control oocytes, NAT10 protein expression patterns was changed from the nucleolus in GV oocytes to the cytoplasm in MI oocytes, and the membrane and first polar body of MII-stage oocytes (Fig. 6A). Nine hours after TubA supplementation, however, NAT10 protein staining was strongly localized to the peri-nucleus including the cytoplasm. After 12 h, NAT10 protein staining became limited to the peri-chromosomes. Of note, Ac-p53 (K120) staining patterns in both the control and TubA-treated groups were very closely correlated with those of NAT10. In addition, Nat10 mRNA expression in the TubA-treated groups decreased slightly, whereas Mdm2 mRNA expression was similar to control MII-stage oocytes (Fig. 6B and C). Collectively, immunostaining patterns in the TubA-treated groups demonstrate that nuclear NAT10 and Ac-p53 (K120) were correlated with the poorest outcome of meiotic exit compared to cytoplasmic and membrane-associated NAT10 and Ac-p53 (K120), indicating that the subcellular distribution may be important for the progression of meiotic oocyte maturation.

In conclusion, RNA-seq and in silico pathway analysis demonstrated the potential of using mouse oocytes as an in vitro platform for the systematic validation of chemotherapeutic targets. In this study, we propose a key underlying mechanism to explain the failure of meiosis in TubA-treated oocytes (Fig. 6D). These observations strongly suggest that TubA is not a specific inhibitor of HDAC6 in mouse oocytes. Rather, we believe that the effects of TubA disrupt spindle migration and asymmetric division in mouse oocytes, and then induce oocyte senescence and aging, which eventually results in female infertility.

**DISCUSSION**

*In vitro* oocyte maturation in the presence of 20 μM TubA (p<0.05) is arrested at the MI or ATI stage, and shows abnormal spindle and chromosome alignment [15]. Here, we performed single oocyte RNA-Seq and analyzed the in silico data to address several unanswered questions regarding TubA effects and to determine the epigenetic effects of TubA on oocyte maturation and viability. Additionally, we used the R package GOplot, KEGG, and IPA analysis to identify the underlying mechanism of TubA-induced abnormal oocyte maturation.

TubA supplementation in culture medium resulted in the combined inhibition of Hdacs (6, 9, 10, and 11) and Sirtuins (Sirt2, 5, 6, and 7) in cultured oocytes. TubA is a highly selective HDAC6 inhibitor and is reported to
inhibit the enzymatic activity of HDAC6 in cells. In this and a previous study, the protein and mRNA levels of 
\(\text{Hdac6}\) in TubA-treated mouse oocytes were significantly reduced. This posed the question as to how a specific enzymatic inhibitor could result in mRNA degradation and reduction in protein expression. There are some possible mechanisms to explain how specific inhibition of an enzyme could result in mRNA degradation and subsequent reduction in protein expression. First, HDAC6 may directly target the 
\(\text{Hdac6}\) gene in a negative feedback mechanism, as described previously [27]. A second possible reason may be that the observed TubA effects were not specific to HDAC6. In this study, we found that both \(\text{Hdacs}\) (6,
9, 10, and 11) and Sir tuins (2, 5, 6, and 7) were
dysregulated in the TubA-treated group compared to the
control. Therefore, we believe that inhibition of both
HDACs and sirtuins by TubA may disrupt spindle
migration and asymmetric division in mouse oocytes.
Furthermore, previous studies have demonstrated that
histones are globally deacetylated during meiosis at the
MI and MII stages by histone deacetylase activity in
mammalian oocytes [28-31]. Taken together, our
analysis suggest that TubA induced inadequate histone
deacetylation causes chromatin perturbation, spindle
defects, and chromosome misalignment [32].

Both of Sirt2 and Hdac6 are tubulin deacetylases and
that are thought to function as essential coenzymes [33,
34], and it has been shown that knockdown of either
Sirt2 or Hdac6 by siRNA disturbed the mouse meiotic
apparatus [35]. In this study, the mRNA levels of both
Sirt2 and Hdac6 were significantly downregulated in
TubA-exposed oocytes (Fig. 3). Our results are
consistent with previous data showing that a specific
Sirt2 inhibitor blocked the progression of GVBD during
oocyte maturation in vitro [18] and that Sirt2
knockdown resulted in abnormal spindle and
chromosome alignment during meiosis [19]. However,
remains to be determined how TubA treatment
interrupts spindle assembly and chromosome movement
during oocyte meiosis. Previous studies indicated that
histone H4K16 and α-tubulin-K40 may be the potential
targets of Sirt2 in modulating chromatin conformation
and microtubule stability [33, 36]. Therefore, we
conclude that TubA supplementation induced the
reduction of Sirt2 mRNA expression, which in turn led to
α-tubulin hyperacetylation and consequently contributed to the failure of spindle or chromosome
organization or to aneuploidy.

We also observed a significant downregulation of Sirt6
and 7 mRNA levels in TubA-treated oocytes, two genes
that play a pivotal role in controlling meiotic
progression and transcriptional regulation of ribosomal
DNA, respectively [32, 37, 38]. Sirt1-7 genes have all
been knocked out in mice [21, 39-48]. Among these,
Sirt6 KO mice died within one month of birth with
accelerated aging, while Sirt7 KO mice also died at a
late stage of embryonic development. In somatic cells,
SIRT7 depletion resulted in an increased mutation rate,
sensitivity to different DNA damage agents, and
abnormal rates of apoptosis. Sirt6 or 7 deficiencies,
similar to that observed in Sirt2 KO cells [13, 49],
resulted in replication stress, which is an important
source of endogenous DNA damage [20, 21, 45, 50]. In
addition, knockdown of Sirt7 led to reduced RNA Pol I
levels, but RNA Pol I mRNA levels was not changed.
This suggests that Sirt7 plays a crucial role in
connecting the function of chromatin remodeling
complexes to RNA Pol I machinery during transcription
[51, 52]. Furthermore, decreased levels of Sirt7 during
senescence led to increased acetylated forms of NPM1,
known to promote cellular senescence [53]. Thus, these
observations suggest that the expression of both of Sirt6
and 7 is critical for the meiotic maturation of mouse
oocytes.

In this study, mRNA expression of most of cyclin-
dependent kinases such as Cdk1, Cdk2, Cdk4, Cdk6 and
Cdc25b exhibited significant decreases in the TubA-
treated group, whereas Cenbl mRNA expression was
increased slightly, or was similar, compared to control
GV- or MII-stage oocytes. Previous studies
demonstrated that Cdk2, Cdk3, Cdk4 or Cdk6 KO mice
were healthy when Cdk1 can compensate for their loss
by forming active complexes with Cyclin A, B, D, and
E, indicating that these CDKs are not essential for
completion of the mitotic cell cycle [54]. Other studies
demonstrated that Cdk2 KO in mice leads to postnatal
loss of all oocytes, indicating that CDK2 is an important
factor for oocyte survival, and likely also oocyte
meiosis [55, 56]. However, Cdk1 KO mice, unlike
Cdk2, suffered early embryonic death, suggesting that
Cdk1 is the only CDK essential for driving the mitotic
cell cycle in mammals [57, 58]. Furthermore, Cdk1 KO,
but not Cdk2, resulted in female infertility due to a
failure in the resumption of meiosis in oocytes [54].
Taken together, our observations and these results
suggest that lower Cdk5 mRNA levels induced by TubA
may block the resumption of oocyte meiosis.

Very recently, Balmus et al [59] and Liu et al [60]
reported that NAT10 acetylates p53 at K120 and
stabilizes it by counteracting MDM2 action, and
that chemical or genetic targeting of NAT10 decreased
genomic instability. In this study, the expression
patterns of the NAT10 and Ac-p53 (K120) proteins
were changed from the nucleolus to the cytoplasm
and/or cell membrane during control oocyte maturation,
whereas expression was limited to the nucleus with
TubA treatment. Furthermore, Nat10 mRNA levels
were significantly decreased with 20 μM TubA
treatment compared to control GV or MII oocytes.
A previous report suggested that NAT10 translocates to
the cytoplasm or membrane to facilitate α-tubulin
acetylation and enhance microtubule stability or activate
rRNA transcription by binding and acetylating UBF
[61]. Even though a Nat10 biallelic KO was lethal in
mice, a more recent study showed that monoallelic
Nat10 KO mice were subfertile [59]. Taken together,
our observations suggest that the NAT10 protein
expression pattern is closely linked to Ac-p53, and that
the levels and localization of the NAT10 protein is
critical for oocyte maturation in the mouse.
In conclusion, knockdown screening techniques using chemical inhibitors are extremely valuable tools. However, this system may also have its limitations such as differences in drug penetration rates into oocytes, oocyte state during culture, and the fraction of unaffected oocytes. More studies are required to prove batch effects caused by knockdown screen techniques to improve the performance of cluster analysis.

MATERIALS AND METHODS

Unless otherwise stated, chemical and media were obtained from Sigma-Aldrich (St. Louis, MO, USA).

Mice

Mice were housed in wire cages at 22±1°C under a 12 h light-dark cycle with 70% humidity. All studies were approved by the Animal Care and Use committee of Konkuk University (IACUC approval number: Konkuk university 16122) and were done in accordance with Konkuk University guidelines. B6D2F1 (C57BL/6 x DBA) female mice aged from 6 to 8 weeks were used for collecting oocytes as previously described [15].

Oocyte collection and TubA treatment

Female mice were injected with 5 IU pregnant mare serum gonadotropin (PMSG) and were sacrificed 48 h after PMSG injection. Germinal-vesicle (GV) oocytes were collected using a mouth pipette. Cumulus cells were dispersed with hyaluronidase (300 IU/mL) for 3-5 min in M16 medium. Denuded oocytes were rinsed thoroughly 5 times in M16 medium and then randomly cultured in M16 medium with or without 10 or 20 μM TubA, after covering with sterile mineral oil, under 5% CO2 at 37°C for 12 h. TubA (Catalogue no. 27108) was obtained from BPS Bioscience (San Diego, USA) and dissolved in DMSO. In this study, TubA was used at a final concentration of 10 or 20 μM, as previously described [15]. In vitro-matured MII oocytes were collected if they had a polar body extrusion, and subjected to further analyses.

RNA library preparation and sequencing

This experiment was performed by TheragenEtex (www.theragenetex.com) according to their protocol. Libraries from pooled (n=100) oocytes (GV, MII, and 10 or 20 μM TubA-treated) were constructed by TheragenEtex. Briefly, total RNA was extracted from GV, MII, and TubA-treated oocytes using the PicoPure® RNA Isolation Kit (Arcturus, Carlsbad, CA, USA) following the manufacturer’s instructions. Prior to cDNA library construction, 2 μg of total RNA was treated with DNase I, and magnetic beads with Oligo (dT) were used to enrich poly (A) mRNA. The efficacy of each step of library construction was ascertained using a 2100 Bioanalyzer (Agilent Technologies, CA, USA). The library was sequenced using a HiSeq 2500 sequencer (Illumina, San Diego, CA, USA). Cluster generation was performed, followed by 2×100 cycle sequencing reads separated by a paired-end turnaround. Image analysis was performed using the HiSeq control software version 1.8.4.

Quantification of gene expression and analysis of differentially expressed genes

This experiment was analyzed by LAS (http://www.lascience.co.kr) according to their protocol. To quantify the mapped reads on the reference genome, cufflinks with the strand-specific library option, --library-type=fr-firststrand and other default options were used. Gene annotation of the mm10 reference genome from UCSC genome (https://genome.ucsc.edu) in GTF format was used as a gene model and the expression values were calculated in the Fragments Per Kilobase of transcript per Million fragments mapped (FPKM) unit. Unsupervised clustering by in-house R scripts of the normalized expression values from the few hundred selected differentially expressed genes (DEGs) was used to compare the expression profiles among the samples. The scatter plots for the gene expression values, the volcano plots for the expression-fold changes, and p-values between the two selected samples also were drawn by in-house R scripts.

Functional category analysis

To elucidate the biological functional role of the differential gene expression between the compared biological conditions, we tested gene set overlapping between the analyzed differentially expressed and functionally categorized genes, including biological processes, using Gene Ontology (GO), Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways, and Ingenuity Pathway Analysis (IPA). Network analysis was performed using the IPA web-based application (QIAGEN, Ingenuity Systems). The integrated genome viewer (IGV) was used for genomic annotation as IGV supports various genomic annotation formats [62] and visual representation of annotations follows many of the conventions introduced by the UCSC Genome Browser. Graphs were visualized using GraphPad Prism v6.0 (GraphPad Software, Inc., CA, USA). Venn diagrams were constructed using the web-based program at http://bioinfopp.cnb.csic.es/tools/venny/. RNA sequencing data were deposited in GEO with the accession number GSE115008.
Immunostaining and confocal imaging

Immunostaining and confocal imaging were prepared as described earlier [15]. Briefly, oocytes were fixed in 4% paraformaldehyde for 30 min and permeabilized for 20 min with PBS containing 0.5% Triton X-100. Permeabilized oocytes were blocked for 2 h at room temperature in 1% bovine serum albumin (BSA) or 10% normal goat serum, then incubated at 4°C overnight with the primary antibodies [anti-CDK1-FITC (ab203852, Abcam), anti-Cyclin B1-Alexa Fluor® 647 (ab215945, Abcam), Anti-NAT10 (ab194297, Abcam), and anti-acetyl-p53 at K120 (ab78316, Abcam)]. All stained samples were mounted using Vectashield mounting medium containing 4′,6-diamidino-2-phenylindole (DAPI) (H-1200, Vector Laboratories, Burlingame, CA, USA). Images were acquired using a confocal laser scanning microscope (LSM800, Zeiss, Germany), and the Fiji open-source platform was used to process and measure image fluorescence intensity, according to a previous report [63].

Quantitative real time reverse transcription PCR (RT-qPCR)

Total RNAs were extracted from GV, MII, and TubA-treated oocytes using the Dynabeads mRNA Direct Kit (Thermo Fisher Scientific, Rockford, IL, USA) according to the manufacturer’s instructions, respectively. RT-qPCR was performed using a ViiA 7 Real-time PCR system (Applied Biosystems, OR, USA). Target gene expression levels were normalized to Gapdh mRNA expression, which was unaffected by TubA treatment. The RT-qPCR primer sets are listed in Table 3. RT-qPCR was performed independently in triplicate for each of the different samples, and the data are presented as the mean values of the gene expression levels measured in the TubA treated samples versus the controls, according to a previous report [15].

Data analysis

All analysis for real time RT-qPCR and RNA-seq are presented as the mean±S.E.M. [15]. Each experiment was performed at least three times and subjected to statistical analysis. One-way analysis of variance (ANOVA) was first performed to determine differences among groups (*: p<0.05; **: p<0.01). Fisher’s post hoc test was then performed to determine significant differences between pairs. Values of p<0.05 and p<0.01 were considered significant. Statistical tests were performed using Stat View software version 5.0 (SAS Institute Inc., Cary, NC, USA).

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CONFLICTS OF INTEREST

The authors have no conflicts of interest to declare.

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**SUPPLEMENTARY MATERIAL**

Supplementary Table 1. Top 10 KEGG pathway analysis up-regulated in TubA/GV pair, compared to MII/GV pair.

| Pathway                                      | Count | Genes                                      |
|----------------------------------------------|-------|--------------------------------------------|
| Ubiquitin mediated proteolysis              | 21    | Ube3b, Ddb1, Ube2g2, Ube4b, Skp2, Cdc34, Ube3c, Herc1, Cdc26, Birc3, Plas4, Slah1b, Slah1a, Trim32, Uba3, Ddb2, Mdm2, Itch, Rchy1, Ube2s, Cul1 |
| Cell cycle                                   | 20    | Trp53, Cdc7, E2f4, E2f5, Dbf4, Skp2, Ywhab, Pkmyt1, Chek1, Rb1, Pttg1, Cdc26, Ccnb1 (cyclin B1), Rad21, Eps300, Cend3, Mdm2, Ccna2, Cul1, Stag1 |
| Insulin signaling pathway                    | 20    | Map2k1, Prkb2, Mnk2, PrkabB1, Raf1, Pde3b, Rhoq, Foxo1, Socs4, Ppp1cb, Akt1, Prkar2b, Ppp1r3d, Nras, Prkar2a, Sorbs1, Rheb, Prkaca, Shc1, Crk |
| Prostate cancer                              | 15    | Trp53, Fgfr2, Map2k1, Pdgfa, Raf1, Nfkbia, Lef1, Foxo1, Nfkb1, Rb1, Pten, Akt1, Nras, Ep300, Mdm2 |
| Pathways in cancer                           | 34    | Fgfr2, Pdgfa, Fgfr9, Foxo1, Nfkbia, Nfkb1, Cdh1, Ttp, Pten, Akt1, Rac1, Rala, Trp53, Dvl2, Ctbp2, Map2k1, Ralbp1, Runxl1, Skp2, Raf1, Lef1, Rb1, Ccnb1, Birc3, Dapk2, Nras, Ccnc6, Rassf5, Ep300, Hif1a, Itga6, Pias4, Mdm2, Crk |
| p53 signaling pathway                        | 12    | Ccnb1, Trp53, Ccnb3, Siah1b, Siah1a, Shisa5, Ddb2, Mdm2, Chek1, Rrm2b, Rchy1, Pten |
| Renal cell carcinoma                         | 12    | Akt1, Nras, Ep300, Hif1a, Map2k1, Pdgfa, Pak4, Rac1, Rap1a, Raf1, Fcln, Crk |
| Tight junction                               | 18    | Ppp2r1a, Gnai3, Gnai2, Cldn4, Gnai1, Mpp5, Cask, Ctnnb1, Pten, Tjap1, Lgl2, Csnk2a2, Akt1, Nras, Csnk2a1, Ash1, Pard6g, Milt4 |
| Progesterone-mediated oocyte maturation      | 13    | Gnai3, Gnai2, Map2k1, Gna1, Raf1, Pkmyt1, Pde3b, Cdc26, Ccnb1, Akt1, Rps6ka6, Prkaca, Ccna2 |
| MAPK signaling pathway                       | 28    | Trp53, Fgfr2, Map2k1, Pdgfa, Fgfr9, Taok3, Ptprr, Mnk2, Ppm1a, Raf1, Nfkb1, Tab2, Daxx, Atf2, Akt1, Nras, Rps6ka6, Dusp1, Dusp14, Pla2g12a, Rac1, Rap1a, Prkaca, Crk, Dusp7, Rasa1, Rasa2, Ppp5c |
Supplementary Table 2. Top 10 KEGG pathway analysis down-regulated in TubA/GV pair compared to MII/GV pair.

| Pathway                                | Members                                                                 |
|-----------------------------------------|-------------------------------------------------------------------------|
| Ribosome                                | Rpl18, Rpl36a, Rpl19, Rpl14, Gm6251, Rpl2211, Rps3, Rpl39l, Fau, Rpl10, Rpl12, Rps27a, Rpl36al, Rpl35a, Rps4x, Rps18, Rps19, Rps17, Rps15, Rps13, Rps10, Ubb, Kpna2, Gm12191, Rpl27a, Rpl35, Rpl36, Rpl38, Rpl39, Rps25, Rps26, Mrp113, Rpl30, Rps29, Rpl6, Rpl31, Rpl8, Rpl3, Gm15772, Rpl5, Rpl110a, Rpl7a, Rps20, Rpl4, Rps21, Rps23, Rps24, Rpsa, Rpl26, Rps9, Rpl27, Rpl24, Rpl23A, Rps6, Rps5, Rpl28, Rps7, Rpl29, Rpl118a, Rpl23, Rpl13a, Rpl21 |
| Parkinson's disease                     | Atp5d, Atp5e, Uqerc1, Cyc1, Uchl1, Uqercf1, Uqercq, Cox5b, Ndufs7, Ndufs6, Ndufs5, Ndufs8, Atp5o, Ndufs3, Atp5h, Ndufs2, Rps27a, Atp5j, Ndufb10, Cyc, Cycs, Ndufc2, Ube2j1, Gm5801, Ndufc1, Uqcrh, Ubb, Uqcrb, Ndufb3, Ndufb4, Ndufb5, Ndufb7, Ndufa11, Uqcrh, Uqcrb, Ndufb3, Ndufb4, Ndufb5, Ndufb7, Cox7b, Cox7c, Atp5g2, Cox7a2l, Atp5g1, Atp5g3, Ndufa2, Ndufa8, Ndufa9, Ndufa7, Atp5f1, Vdac2, Vdac3, Park7, Ndufv3, Sdhb, Ndufv1, Sdh, Ndufv2, Cox6a2, Atp5a1 |
| Oxidative phosphorylation               | Atp5d, Atp5e, Clta, Uqerc1, Cltb, Cyc1, Uqercfs1, Uqercq, Cox5b, Ndufs7, Ndufs6, Ndufs5, Ndufs8, Atp5o, Atp6v0d1, Ndufs3, Cox17, Atp5h, Ndufs2, Atp5k, Atp5j, Ndufb10, Ndufc2, Cox4i1, Ndufc1, Ndufa11, Uqcrh, Uqcrb, Ndufb3, Ndufb4, Ndufb5, Ndufb7, Cox7b, Cox7c, Atp5g2, Cox7a2l, Atp5g1, Atp5g3, Atp6v0c, Atp5j2, Ndufa2, Ndufa8, Ndufa9, Ndufa7, Atp5f1, Lhpp, Ndufv3, Sdhb, Ndufv1, Atp6v1e1, Sdh, Ndufv2, Cox6a2, Atp5a1 |
| Huntington's disease                    | Atp5d, Atp5e, Clta, Uqerc1, Cltb, Cyc1, Uqercfs1, Uqercq, Cox5b, Ndufs7, Ndufs6, Ndufs5, Ndufs8, Atp5o, Atp6v0d1, Ndufs3, Cox17, Atp5h, Ndufs2, Atp5k, Atp5j, Ndufb10, Cyc, Cycs, Ndufc2, Cox4i1, Ndufc1, Dctn2, Uqcrh, Uqcrb, Ndufb3, Ndufb4, Ndufb5, Polr2e, Ndufb7, Polr2l, Polr2k, Cox7b, Polr2l, Cox7c, Atp5g2, Cox7a2l, Atp5g1, Atp5g3, Ndufa2, Creb3, Ndufa8, Ndufa9, Ndufa7, Atp5f1, Sod1, Vdac2, Vdac3, Ndufv3, Sdhb, Ndufv1, Sdh, Ndufv2, Cox6a2, Atp5a1 |
| Alzheimer's disease                     | Atp5d, Atp5e, Uqerc1, Cyc1, Uqercfs1, Uqcrd, Cox5b, Ndufs7, Ndufs6, Ndufs5, Ndufs8, Atp5o, Ndufs3, Atp5h, Ndufs2, Atp5j, Ndufb10, Cyc, Cycs, Ndufc2, Cox4i1, Ndufc1, Dctn2, Uqcrh, Uqcrb, Ndufb3, Hsd17b10, Ndufb4, Ndufb5, Ndufb7, Aph1c, Cox7b, Cox7c, Atp5g2, Cox7a2l, Atp5g1, Atp5g3, Ppp3cc, Ndufa2, Ndufa8, Ndufa9, Ndufa7, Atp5f1, Bad, Ndufv3, Sdhb, Ndufv1, Sdh, Ndufv2, Cox6a2, Calm3, Atp5a1, Calm2 |
| Proteasome ubiquitin mediated proteolysis| Psmb5, Psmb1, Psmb4, Psmd14, Psmb7, Psmc5, Psmd13, Psmb6, Psma6, Psmc4, Psmc1, Psme2, Psmc3, Psmc2, Psme1, Psma3, Psmc4, Psmc6, Psmc8 |
| Pyrimidine metabolism                   | Polr2e, Polr2l, Polr2k, Polr2i, Dtymk, Upp1, Znrd1, Tk2, Tym, Nt5m, Nt5c3, Uck2, Entpd1, Nudt2, Polr3h, Polr3k, Polr1e, Polr1d, Polr1c, Polr3a, Polr3e, Nme6, Pold4, Umps, Nme3, Nnm1, Pold2 |
| Pathway                          | Genes                                                                 |
|---------------------------------|----------------------------------------------------------------------|
| RNA polymerase                  | Polr3h, Polr2e, Polr3k, Polr1e, Polr2l, Polr1d, Polr2k, Polr2i, Znrd1, Polr3a, Polr1e, Polr2k, Polr1c, Polr3c |
| Pentose phosphate pathway       | Aldoa, Pgm2, Pgl5, G6pdx, Fbp1, Pfkp, Dera, Tkt, Fbp2, Gpi1         |
| DNA replication                 | Rpa2, Pold4, Rfc3, Rfc4, Ssbp1, Rfc2, Pold2, Rnaseh2a, Fen1, Rnaseh2c, Mcm5 |

**Supplementary Table 3. List of primers used in this study.**

| Genes | Forward           | Reverse                          |
|-------|-------------------|----------------------------------|
| **Cdc14b** | GTGAAGAAGAGCCGCAG | GCTGTAGAGAATGGCAAAAAC           |
| **Arp3**   | CGCCATGGTATAGTTGAAGA | AGTGGAGGTTCAGTCAAAAG          |
| **Aurora c** | CGTACAGCCACGATAATAC | CCTGTGAATACCTTCTTCTTCT          |
| **Cops3**  | TGTGGAAGAAACAGCCC | GTCAGTTGGTTGGTATTCATC           |
| **Cops5**  | AAACCTGGGACTAAGGATCA | TCACCATTTCAGTAGAGCC          |
| **Tkt**    | ATGGCATACACAGGCAATA | TTGTAATTTCCAGCAAAGGC           |
| **Obox1**  | GGCACATATCGTGGTGTTA | ATATCTGGAGGTTCTCCCG          |
| **Gapdh**  | AGGTGCGGTGTGAAAGGATTTG | TTAGACCATGTTGAGGTTGCA         |