Exogenous thymine DNA glycosylase regulates epigenetic modifications and meiotic cell cycle progression of mouse oocytes

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ABSTRACT: In mammalian cells, 5-methylcytosine (5-meC) can be transformed into 5-hydroxymethylcytosine (5-hmC) by the methylcytosine dioxygenase TET proteins (TET1, TET2 and TET3). Thymine DNA glycosylase (TDG), a downstream enzyme of TET proteins, not only functions in base excision repair, but also acts as a key enzyme that participates in active DNA demethylation. Here we microinjected exogenous TDG-mCherry mRNAs into germinal vesicle (GV) stage mouse oocytes, and found that initially TDG-mCherry localized in the nucleus. Just before GV breakdown (GVBD), TDG-mCherry was released from the nucleus into the cytoplasm. In contrast with TDG, another active DNA demethylation-associated enzyme, activation-induced cytidine deaminase (AID) became localized in the cytoplasm of GV oocytes, but entered the nucleus of oocytes just before GVBD. However, both TDG and AID could enter the G0 stage nuclei of cumulus cells injected into the ooplasm. To analyze the effects of TDG on oocyte maturation, we over-expressed TDG-mCherry in GV oocytes, and found that the rates of both GVBD and polar body extrusion rate were significantly decreased. When the TDG over-expressed oocytes were blocked at the GV stage, the oocyte chromatin became decondensed, and the histone 3 trimethyl lysine 9 (H3K9me3) and H3K9me2 levels were decreased. We also found that TDG could reduce the 5-meC level of oocyte genomic DNA. All these results indicate that aberrant TDG expression causes epigenetic modifications and meiotic cell cycle arrest of mouse oocytes.

Key words: TDG / oocyte / meiosis / H3K9me3 / 5-meC

Introduction

The thymine DNA glycosylase (TDG) is an enzyme that can excise mispaired thymine (G:T) produced by deamination of the 5-methylcytosine (5-meC) in the genomic DNA (Wiebauer and Jiricny, 1989). In addition to thymine, TDG can also recognize and excise other substrates such as mispaired uracil (G:U) (Waters and Swann, 1998) generated by deamination of cytosine, 5-formylcytosine (5-fC) and 5-carboxylcytosine (5-caC) (He et al., 2011; Maiti and Drohat, 2011), generated during active DNA demethylation (Ito et al., 2011), and 5-hydroxymethyluracil (5-hmU) (Cortellino et al., 2011). In mammalian cells, 5-meC can be transformed into 5-hydroxymethylcytosine (5-hmC) by the methylcytosine dioxygenase TET proteins (ET1, TET2 and TET3) (Iyer et al., 2009; Ko et al., 2010; Guo et al., 2011; Iqbal et al., 2011). Both 5-meC and 5-hmC can be deaminated to thymine and 5-hmU, respectively, by the activation-induced cytidine deaminase (AICDA or AID) (Cortellino et al., 2011). In different mammalian cells, the expression and localization of the three TET proteins, AID and TDG may be different, e.g. AID can alter the cellular location of TET proteins (Arioka et al., 2012); TET3 was mainly expressed in matured oocytes and zygotes (Iqbal et al., 2011) but TDG could not be detected in oocytes (Guo et al., 2014) or zygotes (Hajkova et al., 2010). The TET3 activity in the zygote is finely regulated. The transition of 5-mC to 5-hmC in maternal genome DNA is prevented by the histone 3 lysine 9 dimethylation (H3K9me2) recruited protein developmental pluripotency-associated 3 (PGC7) (Nakamura et al., 2012). The activity of TET3 in paternal nucleus is regulated by the CRL4 complex (Yu et al., 2013) which is mainly associated with the DNA damage repair pathway. All this evidence shows that DNA demethylation is controlled...
not only by different enzyme activities and enzyme localizations, but also by histone modifications.

TDG was essential for mouse embryo development. Depletion of TDG was lethal for mouse embryos and no embryos depleted of TDG survived from embryonic day 12.5 (Cortazar et al., 2011). TDG depletion not only affected DNA demethylation of embryos, but also increased the histone 3 trimethyl lysine 9 (H3K9me3) levels of mouse embryonic fibroblasts (Cortazar et al., 2011). Evidence showed that the TDG could be recruited by DNA methyltransferase DNMT3A and DNMT3B to demethylate the 5-mCAs at the pS2 gene promoter. When fused with the rel-homology domain (RHD) of NFKB, TDG could decrease the methylation level of RHD targeted regions (Gregory et al., 2012). These results indicated that TDG could be used as a tool enzyme to modify the epigenetic code of the cell’s genome.

During mouse preimplantation embryo development, the paternal genome was globally demethylated by an active style whereas the maternal genome was globally demethylated by a passive style (Inoue and Zhang, 2011; Ma et al., 2012). However, the methylation states of regions such as imprinted regions, part repeat sequences and even some promoter regions were protected from demethylation during pre-implantation embryo development (Smith et al., 2012). These results indicated that the epigenetic modifications of gametes could be inherited by the offspring (Daxinger and Whitelaw, 2012). The gamete’s epigenetic code could be altered by environmental factors such as diabetes (Ge et al., 2013) or food supplements (Cooney et al., 2002; Ge et al., 2014). However, there is still no evidence showing that the gamete’s epigenetic modifications could be altered directly by active DNA demethylation-associated enzymes. We thus analyzed the localization of exogenous TDG in oocytes, the effects of overexpressed TDG on oocyte maturation, and the epigenetic modifications.

Materials and Methods

Oocytes collection and in vitro maturation

Oocytes were isolated from ovaries of 8- to 10-week-old female ICR strain mice. All manipulations in this study were conducted according to the guidelines of animal care and manipulation of the Institute of Zoology, Chinese Academy of Sciences. The oocytes were in vitro matured in M2 medium (Sigma-Aldrich, St. Louis, MO, USA) at 37°C, under air with 5% CO2, and a saturated humidity environment. The germinal vesicle breakdown (GVBD) rate and polar body extrusion (PBE) rate were recorded after a saturated humidity environment. The germinal vesicle breakdown was induced with milrinone was added into the M2 medium.

Primers used for amplifying the coding sequence (CDS) of TDG and AID were: forward primer with EcoRI digestion site, 5′-CCGGAGATCTCCGATGA TGCCAGAATTTCTTTAACT-3′; reverse primer with Xhol digestion site, 5′-CCGCTCGAGGCGAGGCTGCTTCTTCTCTTGCT-3′. Primers for amplifying the CDS of AID were: forward primer with Xhol digestion site, 5′-CCGCTCGAGGCGAGGCTGCTTCTTCTCTTGCT-3′; reverse primer with BamHI digestion site, 5′-CCGGAGATCTCCGATGA TGCCAGAATTTCTTTAACT-3′. The amplified TDG CDS and the pBT plasmid containing mCherry coding sequence were double digested by restriction enzymes EcoRI (all restriction enzymes were purchased from Takara, Japan) and Xhol. The amplified AID CDS and the pBT plasmid containing GFP coding sequence were double digested by restriction enzymes BamHI and Xhol. The double-digested CDSs and plasmids were purified and linked by the T4 ligase (Takara, Japan). The TDG-mCherry or AID-GFP plasmids were transformed into Top10 competent cells for amplification. Secondly, the TDG-mCherry or AID-GFP plasmids were collected from the bacteria and single digested by NdeI. The linearized plasmids were purified and the TDGmCherry mRNAs were in vitro transcribed by the mMESSAGE mMACHINE T3 kit (Invitrogen, USA). The transcribed mRNAs were purified by RNeasy Micro Kit (Qiagen, Germany). Thirdly, the purified mRNAs (~400 ng/μl for cellular localization analysis) and >1 μg/μl for over-expression) were microinjected into the cytoplasm of GV stage oocytes by using a micromanipulator. After 5 h blocking at the GV stage by 2.5 μM milrinone, the oocytes were cultured in M2 medium for in vitro maturation. To analyze the effects of TDG on the nucleus of GV oocytes, we blocked the oocytes in M2 medium with 2.5 μM milrinone for 12 h.

Cumulus cell nuclear transfer of the GV oocytes

To isolate the cumulus cells, ICR strain mice were super-ovulated by intraperitoneal injection of 10 IU hCG 48 h after 10 IU pregnant mares serum gonadotrophin injection. Fourteen hours after hCG injection, the cumulus-oocyte complexes were isolated from the mouse oviducts and treated with hyaluronidase. The separated cumulus cells were collected for nuclear transfer using a micromanipulation system. The cell membranes of cumulus cells were firstly broken with a micropipillary driven by a Piezo electric micro manipulator, and then the cumulus cell nuclei were microinjected into the cytoplasm of GV-stage oocytes (Gao et al., 2002).

Antibodies and immunostaining

The antibodies used in our experiments were: α-tubulin antibody conjugated with fluorescein isothiocyanate (FITC) (1:200, Sigma-Aldrich); H3K9me3 antibody (1:500, Merck Millipore, Billerica, MA, USA); H3K9me2 antibody (1:200, Merck Millipore) and antibody for 5-mec (1:100, Abcam, Cambridge, UK). For labeling the normal proteins with antibodies, cells were firstly fixed with 4% (w/v) paraformaldehyde in phosphate-buffered saline (PBS) for 40 min. Next, the cells were permeabilized with 0.1% (v/v) Triton X-100 in PBS for 20 min and blocked in PBS containing 1% BSA (w/v) for 1 h. The blocked cells were incubated with first antibodies at 4°C overnight. After washing three times, the cells were incubated with the second antibody at room temperature for 2 h and washed again. After staining the DNA with Hoechst 33342 and mounting on slides, the samples were observed with the laser confocal fluorescence microscope (LSM 710, Zeiss, Germany). For labeling the normal proteins with antibodies, cells were firstly fixed with 4% (w/v) paraformaldehyde in phosphate-buffered saline (PBS) for 40 min. The cells were permeabilized with 0.1% (v/v) Triton X-100 in PBS for 20 min and blocked in PBS containing 1% BSA (w/v) for 1 h. The blocked cells were incubated with first antibodies at 4°C overnight. After washing three times, the cells were incubated with the second antibody at room temperature for 2 h and washed again. After staining the DNA with Hoechst 33342 and mounting on slides, the samples were observed with the laser confocal fluorescence microscope (LSM 710, Zeiss, Germany). For labeling the normal proteins with antibodies, cells were firstly fixed with 4% (w/v) paraformaldehyde in phosphate-buffered saline (PBS) for 40 min. The cells were permeabilized with 0.1% (v/v) Triton X-100 in PBS for 20 min and blocked in PBS containing 1% BSA (w/v) for 1 h. The blocked cells were incubated with first antibodies at 4°C overnight. After washing three times, the cells were incubated with the second antibody at room temperature for 2 h and washed again.

Time-lapse live cell observation

The ZEISS live cell station was used for observation of the TDG-mCherry or AID-GFP localization in mouse oocytes during meiosis resumption. The culture parameters were the same as for oocyte in vitro maturation. The fluorescence signals were recorded every 10 min for ~5–6 h. The total or max intensity of mCherry or GFP signals were calculated by the live cell station system.

Statistical analysis

Differences between experimental groups and control groups were tested with Fisher’s exact test.
Results

The localization of exogenous TDG and AID in oocytes around GVBD

Time-lapse live cell observation revealed that the exogenous TDG localized at the nucleus of GV oocytes but just before GVBD, the TDG dispersed from the nucleus into the cytoplasm (Fig. 1A).

Because AID could form a complex with TDG in P19 embryonic carcinoma cells (Cortellino et al., 2011), to analyze the localization of AID in oocytes, we also constructed an AID-GFP plasmid and microinjected the AID-GFP mRNAs into the oocytes. We found that, unlike TDG, exogenous AID mainly localized to the cytoplasm of GV-stage oocytes. Just before GVBD, AID partially entered the nucleus but after GVBD AID dispersed into the cytoplasm like the exogenous TDG (Fig. 1B).

In addition, we also found that both TDG-mCherry and AID-GFP total signals displayed no significant change during meiosis resumption (Fig. 1C and D). Because the GV stage is the diplotene stage of prophase of meiosis I, to analyze if TDG and AID localized to the nucleus at the G0 stage, we injected membrane-ruptured cumulus cells (which are arrested at the G0 stage) into the cytoplasm of oocytes after TDG-mCherry and AID-GFP mRNA microinjection. We found that both the TDG-mCherry and AID-GFP became localized to the cumulus cell nucleus (Fig. 2).

TDG over-expression caused chromatin decondensation

To analyze the effects of exogenous TDG on the nucleus of GV-stage oocytes, we blocked the oocytes at the GV stage for 12 h using milrinone after TDG-mCherry mRNA microinjection. As a result, we found that the number of the condensed chromatin dots was reduced as the TDG-mCherry signals increased (Figs 3 and 4). When the TDG-mCherry

Figure 1 Cellular localization of mCherry coupled thymine DNA glycosylase (TDG-mCherry) (A) and green fluorescent protein (GFP) coupled activation-induced cytidine deaminase (AID-GFP) (B) during oocyte meiosis resumption. (C) The changing patterns of the total TDG-mCherry fluorescence signals (red line, sum of mCherry signals/100 000) and the maximum fluorescence intensity of TDG-mCherry. (D) The changing patterns of the total AID-GFP fluorescence signals (green line, sum of mCherry signals/100 000) and the maximum fluorescence intensity of AID-GFP. GV, germinal vesicle; GVBD, GV breakdown; MI, metaphase of the first meiosis. Blue, Hoechst 33342; Red, TDG-mCherry; Green, AID-GFP; Scale bar, 20 μm.
Figure 2  Both thymine DNA glycosylase (TDG) and activation-induced cytidine deaminase (AID) localized to the nucleus of a cumulus cell injected into an oocyte. (A) Co-expression of TDG-mCherry and AID-green fluorescent protein (GFP) in an oocyte. (B) Oocyte co-expressing TDG-mCherry and AID-GFP injected with a membrane-ruptured cumulus cell. Arrow indicates the nucleus of the cumulus cell. Scale bar, 20 μm.

Figure 3 Histone 3 trimethyl lysine 9 (H3K9me3) immunostaining of normal and thymine DNA glycosylase (TDG) over-expressed oocytes. SN, Hoechst-positive ring surrounded nucleolus; NSN, Hoechst-positive ring non-surrounded nucleolus; GV, germinal vesicle. Scale bar, 5 μm.
**Figure 4** Histone 3 dimethyl lysine 9 (H3K9me2) immunostaining in normal and thymine DNA glycosylase (TDG) over-expressed oocytes. Scale bar, 5 μm.

**Figure 5** Thymine DNA glycosylase (TDG) decreased the 5-methylcytosine (5-meC) level in germinal vesicle stage oocytes. The 5-meC was labeled by Cy5. Scale bar, 20 μm.
was strongly expressed, we also found that the formation of the nucleolus surrounded Hoechst-positive ring was prevented (Figs 3 and 4). These results showed that over-expression of exogenous TDG induced chromatin remodeling in GV-stage oocytes.

**Over-expression of TDG decreased the H3K9me3 and H3K9me2 levels in GV stage oocytes**

As the chromatin of GV-stage oocytes was remodeled by TDG over-expression, to analyze whether the histone modifications were altered by TDG, we immunostained the trimethylated lysine 9 of histone 3 (H3K9me3) and H3K9me2 in TDG over-expressed oocytes. We found that both the levels of H3K9me3 and H3K9me2 were reduced at the GV stage when TDG was strongly expressed (Figs 3 and 4), suggesting that TDG could change the histone modifications in GV oocytes.

**The over-expression of TDG decreased the 5-meC level in GV stage oocytes**

As TDG plays important roles in active DNA demethylation, we analyzed the 5-meC level in TDG over-expressed oocytes. To remove the errors by manipulations, we placed the control oocytes and TDG over-expressed oocytes together firstly, and immunostained the DNA 5-meC. We found that the 5-meC signal in TDG over-expressed oocytes was significantly weaker compared with that of oocytes without the TDG-mCherry signal (Fig. 5), indicating that the DNA methylation of GV-stage oocytes could be removed by TDG over-expression.

**Over-expression of TDG blocked maturation of oocytes**

To analyze the effects of exogenous TDG on oocyte maturation, we over-expressed TDG-mCherry at the GV stage and cultured these oocytes in M2 medium. Compared with the normal oocytes group (N = 69), the polar body extrusion (PBE) rate of TDG over-expressed oocytes (N = 116) was significantly decreased (Fig. 6, normal oocytes 85.5% and TDG over-expressed oocytes 30.2%, P < 0.01). Correspondingly, compared with normal oocytes, the TDG over-expressed oocytes were blocked at the GV stage (normal oocytes 1.4% and TDG over-expressed oocytes 12.9%, P < 0.01) or pre-metaphase I to anaphase I stage (normal oocytes 13.0% and TDG over-expressed oocytes 56.9%, P < 0.01).

By immunostaining of α-tubulin, we found that over-expression of TDG affected the spindle shape and chromosome organization. As the TDG-mCherry signals increased, the chromosomes became scattered within the spindle and the spindle formed multiple poles (Fig. 7).

**Discussion**

During mammalian germ line development and cell cycle progression, epigenetic modifications can be erased and re-established by intrinsic or extrinsic factors. When the epigenetic code of parent gametes was changed by extrinsic environmental factors, it could be inherited by the offspring, which was termed transgenerational epigenetic inheritance (Daxinger and Whitelaw, 2012). It remains unclear, whether the epigenetic code of the gamete could be modified artificially to change the epigenetic code of the offspring in animal models and how this might be achieved. TDG is an essential enzyme in the active DNA demethylation pathway (Kangaspeska et al., 2008; Metivier et al., 2008; Cortazar et al., 2011). When fused with sequence-specific DNA binding proteins, TDG could demethylate a specific region of the genome (Gregory et al., 2012). Here we analyzed the effects of exogenous TDG on mouse oocytes.

Firstly, we found that TDG localized to the nucleus of GV-stage oocytes but AID mainly localized to the cytoplasm. When we injected the G0-stage nucleus of a cumulus cell into the oocyte, both TDG and AID entered the cumulus cell nucleus. When AID is over-expressed in cell lines such as DLD1 and 293FT, the localization of TET proteins will be partially altered, re-distributing from the nucleus into the cytoplasm (Arioka et al., 2012). These results indicated that in different cell cycle stages, the cell may utilize different ways to demethylate the 5-meCs on DNA, and there are some unknown mechanisms controlling the nuclear localization of active DNA demethylation-associated proteins in the cell.

Secondly, we over-expressed TDG in GV-stage oocytes and found that TDG affected the oocyte epigenetic modifications. Not only DNA methylation and histone modification, but also the chromatin structure could be altered by TDG. These results indicated that TDG could be a potential tool for modifying or correcting the gamete’s epigenetic code.

Thirdly, we found the over-expression of TDG was harmful to oocyte maturation. Meiotic cell cycle progression and polar body emission were evidently reduced after exogenous TDG expression. The methylation of histone 3 lysine 9 is the marker of pericentric major satellite repeats (Lehnertz et al., 2003), which are the main component of DNA centromeres (Joseph et al., 1989; Guenatri et al., 2004). We propose that the abnormal spindle induced by TDG over-expression may be mediated by the decrease in H3K9 methylation. When the DNA topoisomerase 2 (TOP2) was inhibited, the oocyte chromosomes could also be decondensed and the PBE rate was decreased (Li et al., 2013). Unlike the exogenous TDG over-expression, inhibition of TOP2 did not affect the
H3K9 methylation (Li et al., 2013), indicating the target genome regions of TOP2 and TDG may be different. The pericentric heterochromatin is mainly marked by the H3K9me3 (Muramatsu et al., 2013), and the maternal nuclear DNA is protected from 5-mC to 5-hmC transition by the H3K9me2 mediated recruitment of PGC7 (Nakamura et al., 2012). The evidence indicates that the decrease in H3K9 methylation level induced by exogenous TDG expression may affect both the pericentric chromatin structure and the DNA modifications, which may result in the spindle assembly defects in oocytes.

To alter the epigenetic code but without changing the developmental potential of oocytes, it will be important to construct proteins which could bind to the specific region and not be harmful to oocyte development. Although TDG alone could alter the oocyte’s epigenetic code, the negative effects of TDG on chromatin structure blocked oocyte maturation, suggesting that further adjustments are necessary to improve the system. During early embryo development, maternal and paternal DNA will be globally demethylated after fertilization, and began to be remethylated at the late morula stage. During the global DNA

Figure 7  Thymine DNA glycosylase (TDG) over-expression induced abnormal spindle and chromosome organization. Scale bar, 20 μm.
Thymine DNA glycosylase and oocyte epigenetics

193
demethylation, the methylation state of imprinted genome regions, part of repeat sequences and some gamete specific region will be maintained (Ma et al., 2014). Evidence showed that the TDG mRNA levels were very low in oocytes and early embryos and only increased at the blastocyst stage (Guo et al., 2014). By applying the exogenous TDG to modify the DNA methylation and histone modifications, we can understand the effect aberrant epigenetic modifications on oocyte and early embryo development.

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Authors’ roles

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Conflict of interest
None declared.

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