High DNA Methyltransferase 3B Expression Mediates 5-Aza-Deoxycytidine Hypersensitivity in Testicular Germ Cell Tumors

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Abstract

Testicular germ cell tumors (TGCT) are the most common solid tumors of 15- to 35-year-old men. TGCT patients are frequently cured with cytotoxic cisplatin-based therapy. However, TGCT patients refractory to cisplatin-based chemotherapy have a poor prognosis, as do those having a late relapse. Pluripotent embryonal carcinomas (EC) are the malignant counterparts to embryonic stem cells and are considered the stem cells of TGCTs. Here, we show that human EC cells are highly sensitive to 5-aza-deoxycytidine (5-aza-CdR) compared with somatic solid tumor cells. Decreased proliferation and survival with low nanomolar concentrations of 5-aza-CdR is associated with ATM activation, H2AX phosphorylation, increased expression of p21, and the induction of genes known to be methylated in TGCTs (MGMT, RASSF1A, and HOXA9). Notably, 5-aza-CdR hypersensitivity is associated with markedly abundant expression of the pluripotency-associated DNA methyltransferase 3B (DNMT3B) compared with somatic tumor cells. Knockdown of DNMT3B in EC cells results in substantial resistance to 5-aza-CdR, strongly indicating that 5-aza-CdR sensitivity is mechanistically linked to high levels of DNMT3B. Intriguingly, cisplatin-resistant EC cells retain an exquisite sensitivity to low-dose 5-aza-CdR treatment, and pretreatment of 5-aza-CdR resensitizes these cells to cisplatin-mediated toxicity. This resensitization is also partially dependent on high DNMT3B levels. These novel findings indicate that high expression of DNMT3B, a likely byproduct of their pluripotency and germ cell origin, sensitizes TGCT-derived EC cells to low-dose 5-aza-CdR treatment. [Cancer Res 2009;69(24):9360–6]

Introduction

Testicular germ cell tumors (TGCT), the most common solid tumors of adolescent and young men, are thought to derive from transformation of primordial germ cells (PGC) or early gonocytes (1, 2). TGCTs are classified as seminomas and nonseminomas (1). Within nonseminomas are undifferentiated, pluripotent cells, known as embryonal carcinoma (EC). ECs are proposed to represent the stem cells of TGCTs and to be the malignant counterparts to embryonic stem (ES) cells (1, 2). EC cells can differentiate in vivo as mature teratomas toward extrabryonic tissues and embryonic tissues (1, 2).

Patients with TGCTs, even those with advanced metastatic disease, are often successfully treated with cisplatin-based chemotherapeutic regimens (3, 4). However, 15% to 20% of patients are refractory to this treatment and succumb to progressive disease (5). Some TGCT patients, who initially respond to treatment, can exhibit a late relapse and have a poor prognosis (4, 5). Testicular cancer survivors have increased incidence of infertility, cardiovascular disease, and secondary malignancies (6).

Reasons for the high curability of TGCTs have been elusive. Mouse models of testicular cancer do exist, but they do no recapitulate key features of this human malignancy (1). Mechanisms of inherent or acquired cisplatin resistance in other tumors have not yet provided insights into the exquisite cisplatin sensitivity of TGCTs (4). That patients with advanced stage TGCTs can be cured implies that the stem cells of TGCTs are effectively targeted with cisplatin-based chemotherapy (1, 4).

To date, DNA methylation inhibitors have been more active in leukemia than in solid tumor cells (7). There is currently little information available on the effects of DNA methylation inhibitors against TGCT cells. In the current study, we establish that TGCT cells are hypersensitive to the DNA methylation inhibitor 5-aza-deoxycytidine (5-aza-CdR). This response is associated with remarkably high levels of DNA methyltransferase 3B (DNMT3B). Notably, high DNMT3B expression is validated as functionally important for 5-aza-CdR-mediated hypersensitivity in both cisplatin-sensitive and cisplatin-resistant TGCT cells. Indeed, 5-aza-CdR can resensitize cisplatin-resistant cells to cisplatin-mediated toxicity. Together, these findings indicate that high basal DNMT3B expression in pluripotent EC cells can account for 5-aza-CdR hypersensitivity in cisplatin-sensitive and cisplatin-resistant TGCTs.

Materials and Methods

Cell culture and drug treatments. All cell lines were cultured in DMEM media with 10% fetal bovine serum supplemented with glutamine and antibiotics with the exception of MCF7 cells that were cultured in F12-DMEM. The derivation of the NT2/D1-resistant NT2/D1-R1 cell line was previously described (8, 9). With the exception of results in Fig. 4D, cells were treated with the indicated dosages of 5-aza-CdR or cisplatin for 3 d and drug was replenished each day. For the experiment in Fig. 4D, 3 × 10^5 cells were plated per well in 24-well plates. The next day, cells were treated with the indicated doses of 5-aza-CdR daily for 3 d and then treated with the indicated doses of cisplatin for 3 d. Cells were then fixed and stained with Giemsa stain. Cisplatin (Bristol Laboratories) treatments were performed at the concentrations and time points indicated. To assess cell proliferation and survival, Cell-Titer Glo (Promega) assays were performed.

Real-time PCR and immunoblot analyses. Reverse transcription was performed on I μg RNA using the Tagman RT kit (Applied Biosystems). Twenty nanograms of the resulting cDNA was used with SYBR green (Applied Biosystems) for quantitative real-time PCR assays using the ddCT...
method normalized to glyceraldehyde-3-phosphate dehydrogenase and the ABI Prism Sequence Detection System 7700. Primers are provided in Supplementary Table S1. For Western analyses, cells were lysed in a radiomune precipitation buffer and separated by SDS-PAGE, as previously described (8, 9). Antibodies to DNMT3B (H-230; sc-20704, Santa Cruz, and Ab2851, Abcam), actin (C-11; sc01615, Santa Cruz), 1981-ATM (Epitomics), and 139-H2AX (Cell Signaling) were used.

Lentiviral production. Silencing shRNAs targeting human DNMT3B were purchased (Open Biosystems). A pLKO.1-shRNA lentiviral construct was also purchased (pLKO.1, Sigma) and used as a control. Lentiviral stocks were generated from 293T cells, and psPAX2 and pMD2G packaging and envelop vectors using standard protocols. Viral stocks were independently added to NT2/D1 and NT2/D1-R1 cells plated at 0.2 × 10⁶ cells per well per six-well plates. The lentiviral stock was cultured with each of these cells for 24 h. Cells were then harvested and placed onto 10-cm plates in selection media containing 1.0 μg/mL puromycin. Selection continued for 48 h, after which no viable cells remained in the mock-transduced control plates. The puromycin-resistant cell pools were passaged for at least 10 d before use in these experiments.

Figure 1. EC cell lines are sensitive to low-dose 5-aza-CdR. Indicated doses of 5-aza-CdR were added fresh each day for 3 d to exponentially growing cultures. Viable cell growth and survival were measured. Data were normalized to no drug treatment. EC cell lines are NT2/D1, NT2/D1-R1, 833K, 833K-CP, and Tera-1. Data are the average of three biological replicates. Each line was assayed in at least two independent experiments with similar results. Points, mean; bars, SD; *, P < 0.002 for U2OS, MCF7, and HCT116 versus NT2/D1 cells.

Figure 2. EC cell lines express DNMT3B much more highly than do somatic tumor cell lines. Western analyses for DNMT3B expression in various EC and somatic cancer cell lines. EC cells are NT2/D1, NT2/D1-R1, 833K, 833K-CP, Tera-1, 577M, and 2102EP. H23, HÖP62, U1752, A549, H522, and H157 are lung cancer cell lines. U87 and U251 are glioblastoma cell lines. A549, H522, and H157 are lung cancer cell lines. HCT116 and PANC1 is a pancreatic cancer cell line. DNMT3B antibody H-230 was used in A, whereas DNMT3B antibody ab2851 was used in B to D.

Statistics. Where a value for statistical significance is indicated, a two sample two-tailed t test assuming unequal variances was performed.

Results

EC sensitivity to low-dose 5-aza-CdR. We sought to identify whether different EC cell lines were sensitive to DNA methylation inhibition. Five different EC cell lines, including two that were cisplatin resistant, NT2/D1-R1 and 833K-CP, compared with parental NT2/D1 and 833K lines, were highly sensitive to inhibition of cell growth and viability with the DNA methylation inhibitor 5-aza-CdR with IC₅₀ values in the 5 to 25 nmol/L range (Fig. 1). These doses are substantially lower than those typically reported for diverse solid somatic tumors that have IC₅₀ values in the 500 nmol/L to 10 μmol/L range (7, 10). This is in agreement with our somatic tumor cell data as human breast (MCF7), osteosarcoma (U2OS), and colon (HCT116) cancer cells were relatively resistant to 5-aza-CdR treatment at doses as high as 5 μmol/L (Fig. 1). Interestingly, the line most sensitive to 5-aza-CdR was the cisplatin-resistant line, 833K-CP (also called 833K64-CP10).

EC cells overexpress DNMT3B compared with somatic cancer cells. Recent microarray studies indicated that ES and EC cells as well as clinical EC and nonseminomas highly express mRNA for the DNA methyltransferase, DNMT3B, compared with normal and somatic tumors (11–13). However, this differential expression has not been confirmed or shown at the protein level. We found a striking difference in DNMT3B protein expression in the EC cell lines NT2/D1, NT2/D1-R1, 833K, 833K-CP, Tera-1, 577M, and 2102EP compared with U2OS, MCF7, and lung, pancreatic and glioblastoma cancer cell lines (Fig. 2). HCT116 cells also did not express appreciable DNMT3B compared with EC cells (data not shown). Notably, the high expression of DNMT3B in EC cells could be detected with two distinct DNMT3B antibodies (Fig. 2). Densitometry measurements revealed at least a 30-fold increase in DNMT3B expression in the EC cells compared with somatic tumor cells. Thus, the hypersensitivity of TGCTs to low-dose 5-aza-CdR is tightly associated with high expression of DNMT3B in EC cells. It should be noted that there is not an exact correlation between the EC cell lines with regard to the level of DNMT3B and sensitivity to 5-aza-CdR. This is most evident in the NT2/D1-R1 line that is less sensitive to 10 nmol/L 5-aza-CdR compared with other EC cells, yet is one of the highest expressers of DNMT3B. Thus, there seems to be other cell line–specific factors,
Five different lentiviral shRNAs for DNMT3B hypersensitivity.

...and the induction of genes known to be methylated in TGCTs. Low doses of 5-aza-CdR can elicit both a DNA damage response in response to DNA damage (22). Together, the data suggest that be commonly methylated in tumor cells but is commonly induced HOXA9 in NT2/D1-R1 cells. In contrast, expression of BRCA1 was dose 5-aza-CdR induced the expression of MGMT, RASSF1A, and RASSF1A, HOXA9, and BRCA1 (19

dosage, two hallmarks of the DNA damage response these mechanisms at the low doses affecting cell proliferation (18). We asked whether 5-aza-CdR engaged

Figure 3. Low-dose 5-aza-CdR activates the ATM pathway and induces the expression of methylated genes in EC cells. A, indicated doses of 5-aza were added fresh each day for 3 d to NT2/D1-R1 cells. Expression profiles of P(S198) ATM and P(S139)H2AX were assessed. The experiment was repeated with similar results. B, NT2/D1-R1 cells were treated as in A above and expression of the indicated genes assessed by quantitative PCR assays. Columns, mean of biological triplicate determinations; bars, SD; *, P < 0.01; #, P < 0.05.

other than DNMT3B, which affect the relative sensitivity of the EC cells to 5-aza-CdR.

Low-dose 5-aza-CdR induces H2AX and ATM phosphorylation and tumor suppressor gene expression. Two mechanisms have been proposed to account for the antitumor effects of 5-aza-CdR, namely, activation of the DNA damage response pathway and re-expression of tumor suppressor genes through DNA de-methylation (14–18). We asked whether 5-aza-CdR engaged these mechanisms at the low doses affecting cell proliferation and survival of EC. As shown in Fig. 3A, low-dose 5-aza-CdR treatment of NT2/D1-R1 cells increased the levels of activated ATM and phosphorylated H2AX, two hallmarks of the DNA damage response previously shown to be induced with high-dose 5-aza-CdR in somatic tumor cells (7, 17, 18).

To address whether low-dose 5-aza-CdR induced the expression of genes known to be methylated in TGCTs, expression of MGMT, RASSF1A, HOXA9, and BRCA1 (19–21) was assessed (Fig. 3B). Low-dose 5-aza-CdR induced the expression of MGMT, RASSF1A, and HOXA9 in NT2/D1-R1 cells. In contrast, expression of BRCA1 was not induced by low-dose 5-aza-CdR. Low-dose 5-aza-CdR was also able to induce the expression of p21. The p21 gene is not known to be commonly methylated in tumor cells but is commonly induced in response to DNA damage (22). Together, the data suggest that low doses of 5-aza-CdR can elicit both a DNA damage response and the induction of genes known to be methylated in TGCTs.

Knockdown of DNMT3B in EC cells reverses 5-aza-CdR hypersensitivity. Five different lentiviral shRNAs for DNMT3B were independently used to knockdown DNMT3B. Six potential alternatively spliced isoforms of DNMT3B exist; the most biologically relevant isoforms are variants 1, 2, 3, and 6 (14). Quantitative reverse transcription-PCR assays using isoform-specific primers revealed that these shRNAs (relative to controls) reduced expression of these DNMT3B isoforms (Fig. 4A). Furthermore, none of the DNMT3B-specific shRNAs affected levels of DNMT1 or DNMT3A (Fig. 4A). DNMT3B-targeting shRNAs also reduced DNMT3B protein in both NT2/D1 and NT2/D1-R1 cells (Fig. 4B). Because NT2/D1 cells stably expressing sh84 and NT2/D1-R1 cells stably expressing sh84, sh85, or sh86 had the most efficient knockdown of DNMT3B (Fig. 4A and B), these cells were tested for 5-aza-CdR sensitivity. Cells expressing DNMT3B-targeting shRNAs exhibited dramatic reduction of 5-aza-CdR sensitivity compared with control cells (Fig. 4C). However, knockdown of DNMT3B by itself had no apparent effect on the growth of NT2/D1 or NT2/D1-R1 cells (data not shown). These results strongly support a functional link between sensitivity of EC cells to 5-aza-CdR and high DNMT3B expression.

Chronic low-dose scheduling has been suggested to be the optimal protocol for 5-aza-CdR in the treatment of leukemia (15). Therefore, the effect of DNMT3B expression on the sensitivity of NT2/D1-R1 cells to chronic 5-aza-CdR treatment was assessed. NT2/D1-R1 cells and control shRNA cells showed an even greater sensitivity to a prolonged 10-day treatment with 5-aza-CdR compared with the 3-day treatment (Fig. 4D). These cells were sensitive to 5-aza-CdR at doses as low as 2.5 nmol/L. In contrast, NT2/D1-R1 cells treated with DNMT3B-targeting shRNA showed a dramatic resistance to 5-aza-CdR (Fig. 4D).

Restoring cisplatin sensitivity to resistant cells with 5-aza-CdR. We previously reported that cisplatin causes a global p53-dominant transcriptional response in EC cells (23). Through microarray and other studies, we found that the p53 response is repressed in NT2/D1-R1 cells despite having abundant wild-type p53 expression (data not shown; refs. 8, 9, 23). Figure 5A shows that pretreatment of NT2/D1-R1 cells with low-dose (10 nmol/L) 5-aza-CdR for 3 days at least partially restored cisplatin induction of the p53 target genes GDF15 and BTG2 in NT2/D1-R1 cells. Notably, 5-aza-CdR treatment alone substantially induced the expression of GDF15, BTG2, and the p53 target gene, FDXR, in these cells. In contrast, 5-aza-CdR treatment had little or no detected effect on cisplatin induction of FDXR in NT2/D1-R1 cells. This dose of 5-aza-CdR inhibits proliferation of NT2/D1-R1 cells by only ~10% versus controls after 3 days (Fig. 1). Viable cells were counted and replated after 5-aza-CdR treatment and allowed to recover for 24 hours before cisplatin treatment. Notably, pretreatment with low-dose 5-aza-CdR restored cisplatin growth suppression and toxicity to two separate cisplatin-resistant TGCT cell lines, NT2/D1-R1 and 833K-CP (Fig. 5B and C). 833K-CP cells were pretreated with 2.5 nmol/L 5-aza-CdR, a dose that results in a 10% growth inhibition (Fig. 1). Pretreatment with 5-aza-CdR did not alter the cisplatin sensitivity of parental NT2/D1 cells, but did partially increase the cisplatin sensitivity of 833K cells (data not shown). These data indicated that 5-aza-CdR can restore cisplatin cytotoxic response to cisplatin-resistant EC cells.

Relationship between DNMT3B levels and cisplatin sensitivity. Because DNMT3B knockdown reversed 5-aza-CdR hypersensitivity in cisplatin-sensitive and cisplatin-resistant EC cells, it was of interest to investigate whether cisplatin sensitivity itself depended on DNMT3B expression. As shown in Fig. 6, DNMT3B knockdown had no appreciable effect on cisplatin sensitivity of the cisplatin-resistant EC line NT2/D1-R1. This is in agreement with the finding...
that both cisplatin-sensitive (NT2/D1 and 833K) and cisplatin-resistant (NT2/D1-R1 and 833K-CP) EC cells abundantly express DNMT3B (Fig. 2). However, the ability of 5-aza-CdR to sensitize NT2/D1-R1 cells to cisplatin was substantially and significantly repressed by DNMT3B depletion. In summary, these data indicate that 5-aza-CdR hypersensitivity of EC cells is dependent on DNMT3B expression regardless of whether the cells are sensitive or resistant to cisplatin. Yet, cisplatin sensitivity per se is not affected by DNMT3B expression.

**Discussion**

Few studies have addressed 5-aza cytidine analogue treatment effects in TGCT cells. In the current study, we show that TGCT cells are hypersensitive to the DNA methylation inhibitor, 5-aza-CdR. This response is tightly associated with high levels of DNMT3B protein, which was validated as an important target of 5-aza-CdR-mediated hypersensitivity in cisplatin-sensitive as well as cisplatin-resistant TGCTs. Based on these findings, we propose that TGCT cells may be distinctly sensitive to DNA methylation inhibitors due to high basal levels of DNMT3B that are likely a consequence of the PGC origins of EC and their similarities to ES cells, which also are known to express high levels of DNMT3B (11, 12). Indeed, several genomic studies highlighted DNMT3B as a marker of pluripotency (11, 12).

The 5-aza cytidine–related compounds become incorporated into DNA and have been shown to mediate covalent adduct formation with DNMTs (16). The most widely studied and accepted mechanism for the antitumor effects of 5-aza-CdR is related to DNA demethylation and re-expression of specific tumor suppressor genes (reviewed in ref. 14). However, other studies indicate that DNA methylation–independent effects of 5-aza-CdR are critical, especially for effects mediated through the ATM/ATR/p53-dependent DNA damage checkpoint (7, 16–18, 24, 25). The degree each mechanism is used may be cell context–dependent. The studies cited above use doses of 5-aza considerably higher than the doses used here in the treatment of EC cells. We provide evidence that in EC cells, low-dose 5-aza-CdR is able to activate the ATM pathway as well as induce the expression of genes known to be methylated in TGCTs (Fig. 3).

![Figure 4.](image-url) DNMT3B knockdown results in resistance to 5-aza-CdR in EC cells. A, real-time PCR assays of DNMT3B isoforms in control NT2/D1 and in NT2/D1-R1 cells and cells transduced with DNMT3B shRNA lentiviruses. Results 10 d after selection are indicated. Similar results were also obtained in two independent experiments 1 and 2 mo postselection, indicating that knockdown was maintained (data not shown). B, Western analyses of NT2/D1 and NT2/D1-R1 cells independently transduced with DNMT3B shRNA (sh84, sh85, sh86, sh88, sh93, sh97, and sh98) using an anti-DNMT3B antibody, H-230. Ctrl, control lentivirus. C, dose response after 3 d of 5-aza-CdR treatment of lentiviral control NT2/D1 as well as NT2/D1-R1 cells (ctrl) and cells independently transduced with DNMT3B sh84, 85, and 86. Points, mean of biological triplicates and representative of at least two independent experiments; bars, SD; *, P < 0.002 for control cells versus the respective shRNAs for that cell line; #, P < 0.02 for NT2/D1-R1 control versus NT2/D1-R1 cells transduced with shRNAs at the 5 nmol/L 5-aza-CdR dose. D, NT2/D1-R1, NT2/D1-R1 control shRNA (sh-ctrl), and NT2/D1-R1 DNMT3B shRNA84 and shRNA86 cells were independently plated in individual wells of 24-well plates (3 × 10³ cells per well) and treated with the indicated dosages of 5-aza-CdR for 10 d before staining with Geimsa. Fresh 5-aza CdR was added every day for the first 3 d and every other day thereafter. This experiment was repeated with similar results (data not shown).
At first glance, it may seem paradoxical that EC cells are hypersensitive to a DNA methylation inhibitor, but are resistant to that inhibitor when one of its targets, DNMT3B, is depleted (Fig. 4). This result argues that the actions of 5-aza-CdR in EC cells cannot be explained fully by inhibition of DNMT3B activity and suggests that high basal levels of DNMT3B are needed to efficiently elicit an acute cytotoxic response to 5-aza-CdR.

This finding is not without precedence. Juttermann and colleagues (16) showed that ES cells and embryos with DNMT1 knockdown were significantly less sensitive to 5-aza-CdR-mediated toxicity. Oka and colleagues (26), using DNMT3B/DNMT3A null ES cells, also showed that DNMT3B/DNMT3A knockdown results in decreased sensitivity to 5-aza-CdR. HCT116 cells that contain a hypomorphic, truncated allele of DNMT1 were shown to have less...
H2AX phosphorylation and ATM activation in response to 5-aza-CdR, compared with wild-type HCT116 cells (17). However, cells with the hypomorphic DNMT1 allele were sensitized to 5-aza-CdR cytotoxicity, presumably due to a failure to effectively arrest in G2-M (17). In contrast, our preliminary data have thus far not detected large differences in 5-aza-CdR–mediated ATM and H2AX activation in DNMT3B knockout cells, compared with wild-type EC cells (data not shown). As DNMTs are auxiliary components of the DNA replication and repair machinery (17, 27), it is possible that depleting DNMT3B in the EC context alters downstream responses to 5-aza-CdR–mediated DNA damage that perturbs the balance between cell cycle checkpoint arrest, DNA repair, and apoptosis. In contrast to 5-aza-CdR sensitivity, cisplatin sensitivity is independent of DNMT3B levels, as both cisplatin-sensitive and cisplatin-resistant cells express abundant DNMT3B (Fig. 2) and knockdown of DNMT3B fails to alter the cisplatin response (Fig. 6). Thus the cytotoxic effects of DNMT3B appear not to be engaged by general DNA-damaging agents. Rather, it is the combination of 5-aza-CdR and high DNMT3B levels that seems critical for the hypersensitivity of EC cells to 5-aza-CdR.

The methylation status of germ cell tumors has only recently been investigated on a genome-wide level, and findings suggest that TGCTs have distinct methylation signatures compared with other solid tumors (19, 28). Seminomas are prominent hypomethylated compared with nonseminomas and somatic tumors (19, 28). This signature is likely related to the PGC origins of TGCTs. Male PGCs can undergo complete erasure and re-establishment of DNA methylation during development that is associated with dynamic changes in the expression of the de novo DNA methyltransferase, DNMT3B (29).

5-Aza-CdR is Food and Drug Administration approved for the treatment of myelodysplastic syndrome and shows promise for the treatment of certain leukemias. Recent data suggest that 5-aza-CdR is most efficacious when given chronically and at low dosages (15). It has been suggested that less compelling results with 5-aza-CdR in solid tumor trials may have been due in part to the high doses administered and to the monitoring of early rather than later clinical treatment responses (15). Based on these arguments, a call to revisit 5-aza-CdR in solid tumors as a single or combined agent has been made (15). In agreement with our data, it has been shown that 5-aza-CdR can sensitize somatic tumor cells to cisplatin, but at dosages considerably higher than that shown here for EC cells (30–33).

A number of experimental antitumor drugs were propelled to be successfully tested for treating other solid cancers based primarily on initial studies conducted in testicular cancer (3). We propose that high expression of DNMT3B in EC cells is a consequence of their pluripotent and germ cell origin, and results in hypersensitivity to DNA methylation inhibitors. The finding that cisplatin-resistant EC cells retain a high degree of sensitivity to low-dose 5-aza-CdR and that pretreatment of 5-aza-CdR restores cisplatin cytotoxicity to resistant EC cells is notable because it can be clinically exploited.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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References


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