Recent studies showed that some miRNAs (e.g., let-7, miR-134, miR-296, miR-302, miR-367, and miR-470) are involved in the regulation of stem cell function such as self-renewal, pluripotency and differentiation (9,10). MiR-145 directly regulates the reprogramming factors (OCT4, SOX2 and KLF4) and inhibits human ES cell self-renewal, represses the expression of pluripotency genes (11), while miR-203 directly represses the expression of p63, which is an essential regulator of stem cell maintenance in epithelial tissues (12). On the other hand, the "cancer stem cells (CSCs)" hypothesis has attracted lots of attention. This hypothesis suggests that cancers are maintained in a hierarchical organization of rare, slowly dividing cancer stem cells (or tumor-initiating cells), rapidly dividing amplifying cells and differentiated tumor cells. There are several similarities between CSCs and normal stem cells with respect to maintaining self-renewal and pluripotency. Therefore, miRNA may play an important role in the regulation of CSCs as well as normal stem cells. In fact, a recent study showed accumulation of tumor initiating cells after initiation of chemotherapy in breast cancer and reduced let-7 expression in these tumor initiating cells, which helped maintain the undifferentiated status and proliferative potential (13). CSCs cells are considered responsible for resistance to anticancer treatment such as chemotherapy and radiotherapy (14).

The main hypothesis of the present study was that miRNAs that regulate stem cell function are involved in resistance to chemotherapy in esophageal cancer. To test the hypothesis, we examined the expression levels of several miRNAs considered to be involved in the maintenance of stem cell function, such as let-7 family, miR-145, miR-200c, miR-21, miR-296, miR-155 miR-134 and miR-296, and analyzed their

association with the response to chemotherapy and prognosis after chemotherapy followed by surgery in esophageal cancers. Among those miRNAs, the results pointed to the involvement of miR-200c in chemoresistance in esophageal cancers.

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Material and Methods

Patients and tissue samples

All tissue samples were obtained from patients who underwent radical esophagectomy with lymph node dissection for thoracic esophageal cancers between 1999 and 2006 at The Department of Gastroenterological Surgery, Graduate School of Medicine, Osaka University. Informed consent was obtained from each patient. These patients had also received chemotherapy before the surgery (n=98). The preoperative chemotherapeutic regimen was as follows; cisplatin was administered at 70 mg/m^2, adriamycin at 35 mg/m^2 by rapid intravenous infusion on day 1; and 5-FU at 700 mg/m^2 administered by continuous intravenous infusion on day 1 through day 7. Two courses of chemotherapy were used, separated by a 4-week interval. All patients were staged pre- and post-operatively according to the criteria of the International Union Against Cancer (UICC). The median duration of follow-up was 28.8 months (range, 2.3 – 96.7 months), and 57 patients (58.2%) died during the follow-up period.

Clinical and histopathological evaluation of response to chemotherapy

Two weeks after completion of chemotherapy, all patients were re-staged by endoscopy, CT scan, and PET scan in order to evaluate the clinical response to chemotherapy. The clinical response was categorized according to the following criteria (based on the World Health Organization response criteria for measurable disease (15) and the criteria of the Japanese Society for Esophageal Diseases (16)): A complete response (CR) was defined as total regression of the disease. A CR of the

primary tumor represented disappearance of the tumor on CT scan and/or PET scan and endoscopy. A partial response (PR) was defined as more than 50% reduction in primary tumor size and lymph node metastasis, as confirmed by CT scan. Progressive disease (PD) was defined as more than 25% increase in the primary tumor or the appearance of new lesions. Cases that did not meet the criteria of PR or PD were defined as no change (NC).

After fixation in 10% buffered formalin, the surgical specimens of primary tumors were cut into 5-mm slices. All sliced tissues were embedded in paraffin, cut into 4 μm-thick sections, and then stained with hematoxylin and eosin. The extent of histopathological tumor regression was classified into five categories. The extent of viable residual carcinoma at the primary site was assessed semiquantitatively, based on the estimated percentage of viable residual carcinoma in relation to total carcinoma area. Briefly, the percentage of viable residual tumor cells within the total cancerous tissue was assessed as follows: Grade 3, no viable residual tumor cells; Grade 2, less than 2/3 residual tumor cells; Grade 1b, 1/3−2/3 residual tumor cells; Grade 1a, more than 2/3 residual tumor cells; Grade 0, no significant response to chemotherapy (16).

RNA isolation from formalin-fixed/paraffin-embedded and fresh frozen samples

Portions of esophageal cancer tissue samples were frozen in liquid nitrogen immediately after surgery and stored at –80°C and the remaining tissues were routinely fixed in 10% formalin and embedded in paraffin wax. Total RNA was isolated from the formalin-fixed and paraffin-embedded (FFPE) samples using the RecoverAll™ Total Nucleic Acid Isolation Kit (Ambion, Austin, TX) according to

instructions supplied by the manufacturer. Briefly, each FFPE tissue block was cut into 20-μm thick pieces, and 4 slices were placed in a centrifuge tube. To liquefy the paraffin, 100% xylene and 100% ethanol was added to each tube. After centrifugation, the precipitated samples were air dried and treated with protease in heat blocks for 3 hours at 50°C. Then, each sample was treated with isolation reagent and filtered. Each filter was treated with DNase and incubated for 30 min at room temperature. After washing the filter with washing reagents, it was treated with warmed Elution Solution and centrifuged to pass the mixture through the filter. The eluate contained the isolated RNA.

Total RNA from fresh frozen samples was isolated by using Trizol reagent (Invitrogen). Total RNA from fresh frozen samples was isolated by using Trizol reagent (Invitrogen). Briefly, 100 mg of frozen tissue sample was homogenized in 1.0 mL of Trizol reagent and incubated for 5 min at room temperature. Each sample was treated with 0.2 ml of chloroform and after incubation at room temperature for 3 min, centrifuged at 12,000 x *g* for 15 min at 4°C. Following centrifugation, the colorless supernatant was transferred to a fresh tube and 0.5 ml of isopropyl alcohol was added before incubation at room temperature for 10 min. After centrifugation at 12,000 x *g* for 10 min at 4°C, the supernatant was removed and the pellet was washed by 75% ethanol and then centrifuged at 7,500 x *g* for 5 min at 4°C. The RNA pellet was dried and dissolved in RNase-free water. The RNA concentration was quantified using NanoDrop ND-1000 Spectrophotometer (Thermo Fisher Scientific Inc.).

To evaluate the quality of the RNA extracted from FFPE samples, we compared the expression levels of miRNAs in these samples to the RNAs extracted from

fresh-frozen tissue samples. There was no significant difference in the expression of RNU48 as the internal control miRNA between the fresh-frozen and FFPE tissue samples. As reported previously (17), there was a significant correlation in miRNA expression level between fresh-frozen and FFPE tissue samples (Supplementary Figure 1). These results validated the use of FFPE samples in our study for examination of the miRNA expression.

Quantitative real-time reverse transcription-PCR

The complementary DNA (cDNA) was synthesized from 10 ng of total RNA using the TaqMan miRNA Reverse Transcription Kit and specific stem-loop reverse transcription primers (Applied Biosystems, Foster City, CA) according to protocol provided by the manufacturer. Reverse transcription conditions were as follows: 16°C for 30 minutes followed by 40°C for 30 minutes and 85°C for 5 minutes. Real-time PCR reaction was performed using TaqMan Universal PCR master mix No AmpErase UNG and TaqMan miRNA specific PCR-primers (Applied Biosystems). The 20 μl of the reaction product was incubated in a 96-well optical plate at 95°C for 10 minutes, followed by 40 cycles at 95°C for 15 seconds, and 60°C at 1 minute using ABI PRISM 7900HT (Applied Biosystems). The miRNA expression value was expressed relative to that of RNU48 and analyzed using the $2^{-\Delta\Delta Ct}$ method (18).

Cell lines and culture conditions

Five established cell lines derived from esophageal squamous cell carcinoma (TE-1, -8, -10, -13, -15) were obtained from the Riken Cell Bank (Tsukuba, Japan). All cell

lines were cultured in RPMI 1640 (Life Technologies, Gaithersburg, MD.) containing 10% fetal bovine serum (Sigma-Aldrich Co., St. Louis, MO) and 1% penicillin / streptomycin (Life Technologies Inc.), under a humidified atmosphere with 5% CO_2 at 37°C.

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Establishment of cisplatin-resistant cell lines

A cisplatin-resistant cell line (TE8-R) was developed by a stepwise increment of cisplatin concentration as follows. The initial concentration of cisplatin was 2 μM and after 3 days, the cells were passaged into a cisplatin-free medium. Upon reaching
215 confluence, the cells were treated with higher concentration of cisplatin (1.5-2.0 fold). The dose of cisplatin was gradually escalated every few passages, up to a concentration of 35 μM over a period of 2 months. The cisplatin-resistant subline (TE8-R) was 5.8-fold more resistant to cisplatin than the parent cell line (TE8-P). The IC_{50} values for TE8-P and TE8-R were 8.33 and 48.19 μM, respectively.

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Anti-miRNA transfection

Cells were cultured to 60-80% confluence and transfected with 5 nM of anti-miR-200c or negative control oligonucleotides (Applied Biosystems) using siPORT™ NeoFX™ Transfection Agent (Ambion) according to the protocol provided
225 by the supplier. After transfection, the cells were cultured for 72h and intermediate samples were collected at 12, 24 and 48 h and analyzed by immunoblotting, RT-PCR, MTT assay and flow cytometry.

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MTT assay

The 3- (4, 5- dimethylthiazol-2-yl)- 2,5- diphenyltetrazolium bromide (MTT) assay was used to assess IC_{50} (the 50% inhibition concentration) value for cisplatin. Cells were seeded into 96-well plates at 5×10^3 per well and incubated overnight under usual culture condition, and then exposed to cisplatin at various concentrations ranging from 0 to 400 μM (0, 3.125, 6.25, 12.5, 25, 50, 100, 200, and 400 μM) for 6 hours. After treatment, the medium was changed to cisplatin-free medium. Following 48-h incubation, 10 μL of MTT solution was added to each well and the plates were incubated for another 3 h at 37°C, and formazan crystals were dissolved with 100 μL of 0.04 N HCl-isopropanol. The absorbance of individual wells was read at 550 nm test wavelength and 655 nm reference wavelength using a microplate reader (Bio-Rad Laboratories, Hercules, CA). The IC_{50} value for cisplatin was calculated from the dose-response curve.

Apoptosis assay

Annexin V-FITC and propidium iodide (PI) staining were used to determine the percentage of cells undergoing apoptosis and necrosis among cells treated with 30 μM of cisplatin for 4 hours. Apoptosis assay was conducted using the protocol supplied by the manufacturer (BioVision Inc., Mountain View, CA). Briefly, cells were trypsinized gently and resuspended with 500 μL of 1× binding buffer and then treated with 5 μl of Annexin V-FITC and 5 μl of propidium iodide. After incubation for 5 minutes on ice, each sample was analyzed immediately using the FACScalibur flow cytometer (Becton Dickinson, Mountain View, CA). Approximately 15,000 cells were

detected for each sample. Cytogram analysis was performed using the Cell Quest software and unstained cells were classified as "live"; cells stained for Annexin V only were "early apoptotic"; cells stained for both Annexin V and propidium iodide were "late apoptotic"; and cells stained for propidium iodide only were "dead" cells.

MiRNA target prediction

The analysis of miR-200c predicted targets was determined using the algorithms called Target scan (http://www.targetscan.org/) and miRBase Targets database (http://microrna.sanger.ac.uk).

Immunoblotting

Adherent cells were washed with ice-cold phosphate-buffered saline (PBS) and lysed in RIPA buffer (Thermo Fisher Scientific Inc.) or Sample buffer (Wako Pure Chemical Industries, Osaka, Japan) on ice. Lysates were spun and the supernatant was collected. Equal amounts of cell extracts (15μg) were fractionated by SDS-PAGE gel (Bio-Rad Laboratories Inc.) and transferred onto membranes (ImmobilonP, Millipore, Bedford, MA). After blockade by incubation with milk, the membranes were incubated overnight at 4°C with the primary antibodies, and with secondary antibodies for 1 h at room temperature. The following antibodies were used in this study; anti-actin (dilution, 1:1000 Sigma-Aldrich Co.), anti-PTEN (dilution, 1:200), anti-Apaf1 (dilution, 1:200), anti-TFAP2α (dilution, 1:200) and anti-SOCS6 (dilution, 1:200, all from Santa Cruz Biotechnology, Santa Cruz, CA), anti-LATS2 (dilution, 1:1000) and anti-A20 (dilution, 1:200, both from Abcam, Cambridge, UK), anti-Akt (dilution,

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1:200), anti-pAkt (dilution, 1:200), anti-PPP2CA (dilution, 1:500) and anti-PPP2R1B (dilution, 1:500, all from Cell Signaling Technology, Beverly, MA). Immune complexes were detected using Detection Kit (GE Healthcare, Buckinghamshire, UK).

Statistical analysis

All data are expressed as mean±SD. The relationship between miRNA expressions and each clinicopathological variable was analyzed by χ^2 test, Fisher's exact test or Mann-Whiteny U test. Time to recurrence was defined as the time interval between the date of surgery and the date of diagnosis of first recurrence or last date of follow up if recurrence was not observed. Overall survival time was censored at the date of the last follow-up if death did not occur. For survival analysis, Kaplan-Meier method was used to assess survival time distribution according to miRNA expression level and the log-rank test was used to examine the differences between groups. A P value of <0.05 denoted the presence of statistically significant difference between groups. All statistical analyses were performed with JMP ver.8.0 software (SAS Institute Inc., Cary, NC).

Results

Overexpression of miR-200c was associated with poor response to preoperative chemotherapy and poor prognosis in patients with esophageal cancers

Several miRNAs are known to regulate stem cell function. In this study, we used qRT-PCR to quantitate the expression of nine miRNAs: let-7a, let-7g, miR-21, miR-134, miR-145, miR-155, miR-200c, miR-203 and miR-296 in esophageal cancer tissues harvested during surgery after a course of preoperative chemotherapy. The expression of miR-200c, but not any other miRNAs, correlated inversely and significantly with the response to chemotherapy (Table 1, Supplementary Table 1). Furthermore, overexpression of miR-200c, underexpression of miR-145 and overexpression of miR-21, correlated significantly with shorter overall survival of patients who received preoperative chemotherapy (Figure 1, Supplementary Figure 2). On the other hand, the expressions of other miRNAs, including let-7a, let-7g, miR-134, miR-155, miR-203 and miR-296, did not correlate with survival. These findings suggest the involvement of miR-200c overexpression in chemoresistance and poor prognosis.

Table 2 summarizes the relationship between miR-200c expression and other clinicopathological parameters of patients who received preoperative chemotherapy followed by surgery. The expression of miR-200c correlated significantly with tumor depth ($p<0.001$), lymphatic invasion ($p=0.022$) and vessel invasion ($p=0.002$). In addition, we measured miR-200c expression level in paired tumor and non-tumor surgical tissues from 15 patients. The expression level of miR-200c was higher in the

tumor tissues than in non-tumor tissues in most patients (Figure 2a), and the mean expression level of miR-200c in tumor tissues was significantly higher than in non-tumor tissues (Figure 2b).

Inhibition of miR-200c expression increases chemosensitivity to cisplatin and apoptosis in esophageal cancer cells

In the next series of studies, we established the relationship between miR-200c expression and chemoresistance using esophageal squamous cell carcinoma cell lines. We first established the cisplatin-resistant cell line (TE8R) because the expression level of miR-200c was lower in TE8 than other esophageal cancer cell lines (Figure 3a). The IC_{50} of TE8R was about 5.8-fold higher than that of parent cells (TE8P) (Supplementary Figure 3). Real-time RT-PCR confirmed a significantly higher miR-200c expression in TE8R than in their parent cells (about 1.7-fold change, Figure 3b).

To investigate the role of miR-200c in the proliferative activity and chemosensitivity in esophageal cancer, we transfected TE13 cells, which overexpressed miR-200c (Figure 2c), with a specific inhibitor of miR-200c (anti-miR-200c) (Supplementary Figure 4). In the proliferation assay, inhibition of miR-200c expression did not have a significant impact on the proliferative activity (Figure 3a). Furthermore, the MTT assay showed that inhibition of miR-200c expression with anti-miR-200c reduced the IC_{50} value for cisplatin (17.84 μM), compared with the negative controls (163.40 μM, Figure 3b), suggesting the role of miR-200c in sensitivity to cisplatin in esophageal cancer cells.

We also examined the effect of miR-200c knockdown on apoptotic cell death. For this purpose, we used flow cytometry to determine the percentages of Annexin-V-positive cells by among anti-200c-transfected cells and control cells treated with cisplatin. Knockdown of miR-200c expression significantly increased the proportion of apoptotic cells after cisplatin treatment, compared with the negative control (11.30±2.76% vs 6.82±1.26% at 24 h, P=0.026, Figure 3c, d).

Overexpression of miR-200c-induced chemoresistance is mediated through Akt pathway

To explore the downstream mechanism through which miR-200c expression modulates chemosensitivity of esophageal cancer cells, we searched for potential targets of miR-200c by using the Target scan (19) and miRBase Targets database (20). Several putative miR-200c targets, including Apaf-1, PTEN, TFAP2α and LATS2, were chosen as potential direct targets of miR-200c. We considered that these proteins are candidate targets of miR-200c when their inhibition resulted in less apoptosis. First, we applied western blotting to examine whether exogenous repression of miR-200c expression affected the expression of these proteins. Knockdown of miR-200c expression had no apparent effect on the expression of Apaf-1, TFAP2, and LATS2. Low expression of miR-200c also had no effect on PTEN expression (Figure 4a), but resulted in decreased expression of Akt, which is downstream of PTEN and known to be involved in chemoresistance (Figure 4b). To explore the mechanism through which down-regulation of miR-200c reduces the expression of Akt, we searched again for potential direct target(s) of miR-200c that is (are) involved in the

regulation of Akt signaling, using the target scan and miRBase Targets database. Thus, in these experiments, we also examined the expression of several potential miR-200c targets such as PPP2CA, PPP2R1B, SOCS6 and A20, all of which are known to negatively regulate Akt signaling (21-23). Knockdown of miR-200c expression resulted in increased expression of PPP2R1B, a subunit of protein phosphatase 2A (PP2A), which is known to inhibit the phosphorylation of Akt (21), at 48h after treatment (Figure 4b). However, knockdown of miR-200c had no apparent effect on the expression of PPP2CA, SOCS6 and A20 (Figure 4b). Considered together, these results indicate the involvement of miR-200c expression in chemoresistance by directly targeting PPP2R1B following upregulation of Akt signaling.

Discussion

In clinical setting, development of resistance to chemotherapy is one of the major challenges in treatment of patients with cancer. In this study, we focused on miRNAs that are known to maintain stem cell function as regulators of sensitivity to anti-cancer therapies. We reported three new findings in this study. First, in esophageal cancer, high expression of miR-200c was closely associated with poor response to preoperative chemotherapy. Second, high expression of miR-200c correlated with poor prognosis. Third, the effects of miR-200c on chemosensitivity were mediated through the Akt pathway.

The results showed that overexpression of miR-200c correlated with poor response to cisplatin-based chemotherapy in patients with esophageal cancer. Several miRNAs are known to be associated with chemotherapeutic efficacy. For example, overexpression of miR-214 is reported to induce cisplatin-resistance by targeting PTEN in ovarian cancer (24), and high expression of miR-199a is also reported to be involved in chemoresistance in cervical carcinoma (25). On the other hand, low expression levels of let-7i, miR-181a, miR-630, miR-7 and miR-345 are associated with chemoresistance in patients with ovarian cancer (26), lung cancer (27) and breast cancer (28). To our knowledge, the relationship between miR-200c expression and chemoresistance in human cancers has not been analyzed previously. In esophageal cancers, one recent study described the relationship between miRNA expression and chemoresistance (29). The study suggested that miR-296 mediated drug resistance in part through multidrug resistance 1 (MDR1) and apoptosis although the study was

limited by the small sample size. In the present study, we showed that miR-200c expression is significantly associated with the response to chemotherapy in a large sample of patients with esophageal cancer.

The results also showed that high expression of miR-200c was closely associated with poor prognosis of patients with esophageal cancer. Several studies examined the prognostic value of miR-200c expression in human cancers. The expression of miR-200c was found to be up-regulated in of epithelial ovarian (30) and hepatocellular (31) cancer tissues, compared with the relative non-cancerous tissue. Our results are in agreement with those of previous studies, which showed significant correlation between high expression of miR-200 and poor prognosis of patients with ovarian (32) and colorectal cancers (33). Other studies, however, reported downregulation of miR-200c in bladder cancer (34) and renal clear cell carcinoma (35). Thus, the impact of miR-200c expression on the progression and prognosis of patients with malignancies seems to vary according to human cancer type, histopathological type (36) and stage of cancer (37).

The present study also showed that the effect of miR-200c on the development of chemoresistance is mediated through the Akt pathway. Akt, a serine/threonine kinase, plays a pivotal role in oncogenesis, and its altered expression is observed in various human cancers. Akt is also involved in resistance to chemotherapeutic agents and to radiotherapy in various cancers (38,39). In our previous study, we reported an increase in phospho-Akt expression after chemotherapy and that high expression correlated with poor prognosis in patients with esophageal squamous cell carcinoma (40). Recently, several studies examined the relationship between miRNA expression

and Akt-mediated chemoresistance. In breast cancer, the oncosuppressor miR-205, which is downregulated in cancer tissue, directly targeted the HER3 receptor and inhibited the activation of the downstream mediator Akt (41). In pancreatic cancer, overexpression of miR-21 downregulates the expression of PTEN and consecutively upregulates phospho-Akt, resulting in reduced apoptosis of gemcitabine-treated cells (42). In ovarian cancer, miR-214 is reported to enhance cell survival and cisplatin-resistance primarily through targeting the PTEN/Akt pathway (24). The present study showed for the first time that resistance of esophageal cancer to chemotherapy is induced in the presence of high expression of miR-200c through its action on the Akt pathway, and this effect is likely mediated through downstream target of miR-200c, PPP2R1B, rather than via PTEN.

The PPP2R1B gene encodes the beta isoform of the A subunit of the protein phosphatase 2A (PP2A). PP2A is one of the major cellular serine/threonine phosphatases and is a well recognized regulator in the control of Akt activity (21). Previous studies showed that increased PP2A activity leads to inhibition of tumor invasiveness and enhancement of chemosensitivity through dephosphorylation of Akt in various cancers (43-45), suggesting that PP2A plays a tumor suppressive role through downregulation of Akt signaling. Similarly, reduced expression of PPP2R1B is reported in human lung cancers, colorectal cancers (46), and ovarian cancers (47). Recently, Wong et al (48) showed that overexpression of miR-222 enhanced Akt signaling through directly targeting PP2A, and this change enhanced the metastatic potential of hepatocellular carcinoma. Here, we identified PPP2R1B as a potential

target of miR-200c, and we propose that PPP2R1B is a likely intermediate that interplays between miR-200c and Akt pathway in esophageal cancer.

In present study, we focused on nine miRNAs that are known to be involved in the regulation of stem cell function according (9-13), and among those miRNAs, we found miR-200c expression to correlate with chemoresistance and poor prognosis in esophageal cancer. However, it is possible that miRNAs other than those that are involved in stem cell function are also associated with chemoresistance. In fact, we analyzed the miRNA expression profile of a cisplatin-resistant esophageal cancer cell line by miRNA microarray, and compared such profile with that of parental cell line. The results showed that the expression of other miRNAs that are unlikely to regulate stem cell function was significantly altered in chemoresistant cell line, compared with parental cell line (data not shown), although the expression of several miRNAs that are involved in stem cell function were significantly up- or down-regulated in chemoresistent cell line, compared with parental cell line (miR-200c was 1.7 fold upregulated, miR-296 was 0.37 fold downregulated; miR-145 was 0.52 fold downregulated). Further studies are needed to investigate whether miRNAs other than those that are involved in stem cell function are also associated with chemoresistance.

In summary, we examined in the present study the role of various miRNAs, which regulate stem cell function, in resistance to chemotherapy in esophageal cancer and identified miR-200c as the miRNA responsible for chemoresistance in esophageal cancer. Moreover, the results showed that the miR-200c-induced resistance is mediated through the Akt pathway. Further studies are needed to confirm the clinical role of miR-200c in the response to chemotherapy in patients with esophageal cancer.

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