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Original Article

MiR-212 suppressed the metastases of non-small cell lung cancer by reducing ZEB2 expression

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Abstract: MicroRNAs (miRNAs) play an essential role in the tumor tumorigenesis. Recently, miR-212 was reported as a tumor suppressor in non-small cell lung cancer (NSCLC). However, the underlying mechanisms were still unclear. Here, we reported that miR-212 levels were down-regulated and ZEB2 levels were up-regulated in NSCLC tissues compared to the paired adjacent non-tumor tissues. Moreover, the levels of miR-212 and ZEB2 were inversely correlated in NSCLC specimens. Bioinformatics analyses showed that miR-212 targeted the 3'-UTR of ZEB2 mRNA to inhibit its translation, which was confirmed by luciferase reporter assay. Moreover, over expression of miR-212 inhibited ZEB2 mediated cell invasiveness, while depletion of miR-212 augmented it. Together, our data indicated that miR-212 suppression in NSCLC could promote ZEB2 mediated cancer metastasis.

Keywords: Non-small cell lung cancer, ZEB2, miR-212, progression

Introduction

Lung cancer is the leading cause of all cancer related mortality worldwide, and non-small cell lung cancer (NSCLC) accounts for approximately 85% of lung cancer cases [1, 2]. The leading death cause of NSCLC is metastasis, but the underlying mechanisms are still unclear [3]. Despite great advances in chemotherapy and surgical techniques, the 5-year survival rate of NSCLC patients is still around 15% [4]. Thus, it is necessary to elucidate novel mechanism and to facilitate the treatment of NSCLC.

MicroRNA (miRNA) is a class of non-coding small RNA that comprised of about 18-23 nucleotides [5]. MiRNAs regulate the gene expression post-transcriptionally, through its base-pairing with the 3'-untranslated region (3'-UTR) of the mRNA of the target gene [6]. MiRNAs are involved in a variety of biological processes, including cell proliferation, differentiation, chemotherapy resistance, and metastasis [7, 8]. Aberrant alteration of miRNAs has been suggested to play critical roles in the tumor development and progression. For example, Yang et al. suggested that miR-506 was down-regulated in clear cell renal cell carcinoma and inhibited cancer cell growth and metastasis via targeting FLOT1 expression [9]. Kawakita et al. showed miR-21 promoted oral cancer invasion via the Wnt/β-catenin pathway by targeting DKK2 [10]. Mao et al. reported that miR-449a enhanced radiosensitivity through modulating pRb/E2F1 in prostate cancer cells [11].

In the present study, we investigated the role of miR-212 in the metastasis of NSCLC. Our findings showed that the expression level of miR-212 was decreased and ZEB2 was increased in NSCLC tissues. The levels of miR-212 and ZEB2 were inversely correlated. Furthermore, we identified that ZEB2 was a target of miR-212 in NSCLC cells. In addition, our data showed that miR-212 over-expression suppressed ZEB2 mediated cell metastasis, while depletion of miR-212 augmented it. Together, our data suggested that miR-212 acted as a tumor suppressor in NSCLC partially by targeting ZEB2.

Materials and methods

Patient tissue specimens

A total of 26 resected specimens from NSCLC patients were collected for this study. NSCLC specimens were compared with the paired non-tumor tissue (NT) from the same patient. All specimens had been histologically and clinically diagnosed at the Department of Thoracic Surgery, Shanghai Tenth's People Hospital of Tongji University Chongming Branch, from 2013 to 2014. For the use of these clinical materials for research purposes, prior patient's consents and approval from the Institutional Research Ethics Committee were obtained.

Cell lines

Human NSCLC cell lines A549 were purchased from American Type Culture Collection (ATCC) and was cultured in RPMI1640 medium (Invitrogen) supplemented with 10% fetal bovine serum (FBS) in a humidified chamber with 5% CO_2 at 37°C.

Plasmid transfection

MiR-212-expressing and antisense (anti) plasmids were prepared with general method. Transfection was performed with Lipofectamine 2000 reagent (Invitrogen), according to the instructions of the manufacturer. The cells that were transfected with plasmid expressing a scrambled sequence were used as control (Ctl). Transfected cells expressing miR-212 or anti-miR-212, or Ctl were purified by flow cytometry based on green fluorescent protein (GFP).

Quantitative real-time PCR

Total RNA and miRNAs were extracted from tissue or cells with miRNeasy mini kit or RNeasy kit (Qiagen), respectively. cDNA was randomly primed from 2 μg of total RNA using High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems). Reverse transcription quantitative PCR (RT-qPCR) was subsequently performed in triplicate with QuantiTect SYBR Green PCR Kit (Qiagen). All primers were purchased from Qiagen. Data were collected and analyzed using the $2^{-\Delta\Delta Ct}$ method for quantification of the relative mRNA expression levels. Values of genes were first normalized against GAPDH and then compared to the experimental controls.

Luciferase reporter activity assay

Luciferase reporters were successfully constructed using molecular cloning technology. Target sequence for ZEB2 miRNA 3'-UTR clone was purchased from Creative Biogene (Shirley). MiR-212-modified A549 cells were seeded in 24-well plates for 24 h, after which they were transfected with 1 μg of Luciferase-reporter plasmids per well. Luciferase activities were measured using the dual-luciferase-reporter gene assay kit (Promega), according to the manufacturer's instructions.

Scratch wound healing assay

Cells were seeded in 24-well plates at a density of 10^4 cells/well in complete RPMI1640 and cultured to confluence. The cell monolayer was serum starved overnight in RPMI1640 prior to initiating the experiment. Confluent cell monolayer was then scraped with a yellow pipette tip to generate scratch wounds and washed twice with media to remove cell debris. Cells were incubated at 37°C for 24 h. Time lapse images were captured after 24 h. Images were captured from five randomly selected fields in each sample, and the wound areas are calculated by NIH ImageJ software (Bethesda).

Transwell cell invasion assay

Cells (10^4) were plated into the top side of polycarbonate transwell filter coated with Matrigel in the upper chamber of the BioCoatTM Invasion Chambers (Becton-Dickinson Biosciences). After incubation for 48 h, the non-invading cells remaining on the upper surface of the membrane was removed, and the invaded cells through the membrane were fixed and stained with 0.1% crystal violet, imaged and counted under a microscope (Olympus).

Western blotting

Treated cells were washed twice with PBS and proteins were extracted with SDS lysis buffer (Beyotime), and separated by 10% SDS-PAGE gel. Protein samples were transferred to PVDF membrane (Millipore) and were probed with primary antibodies against ZEB2 or GAPDH (Abcam). Membranes were incubated at 4°C overnight, followed by incubation with AP-conjugated secondary antibodies and detected by ECL.

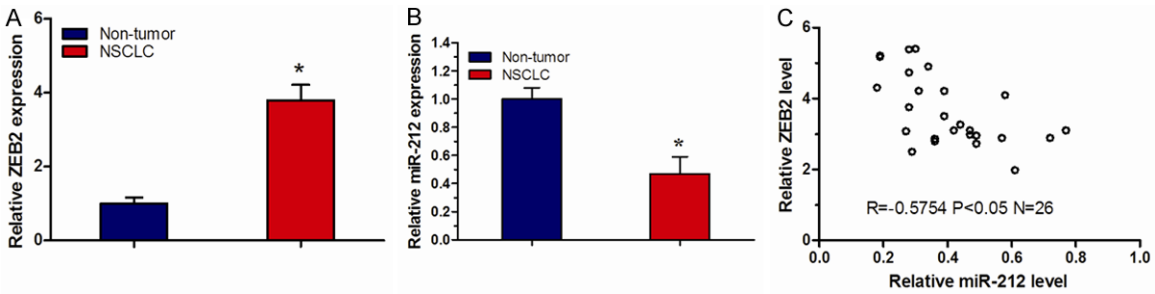

Figure 1. Increased ZEB2 and decreased miR-212 were detected in NSCLC. RT-qPCR on ZEB2 and miR-212 were performed on paired NSCLC and adjacent non-tumor tissues (NT) from 26 patients. (A, B) NSCLC contained increased ZEB2 levels (A) and decreased miR-212 levels (B) compared to adjacent non-tumor tissues. (C) Correlation tests were performed between ZEB2 and miR-212. *P<0.05.

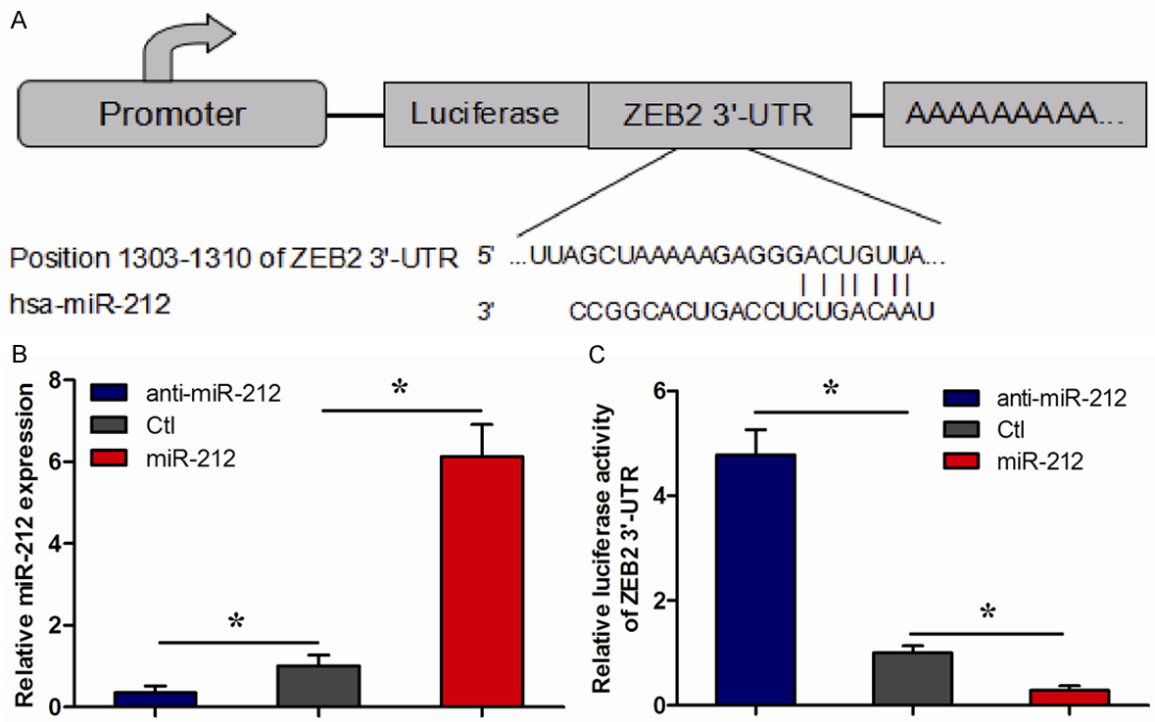

Figure 2. ZEB2 was a target of miR-212 in NSCLC cells. A. Bioinformatics analyses of binding of miR-212 to the 3'-UTR of ZEB2 mRNA. B. In order to examine whether miR-212 may regulate ZEB2 in NSCLC cells, we either overexpressed miR-212 or inhibited miR-212 in A549 cells by transfection of the cells with a miR-212 expressing plasmid (miR-212) or with a plasmid carrying miR-212 antisense (anti-miR-212). The transfected efficient was confirmed by RT-qPCR. C. miR-212-modified A549 cells were then transfected with 1 μg of ZEB2-3'-UTR luciferase reporter plasmid. The luciferase activities were quantified in these cells, indicating that miR-212 targets 3'-UTR of ZEB2 mRNA to inhibit its translation. *P<0.05.

Statistical analysis

All statistical analyses were carried out using the SPSS 18.0 statistical software package. Data are presented as mean ± SD. Statistical differences were determined by ANOVA or Student t test. The correlation between miR-212 and ZEB2 expression was analyzed using Spearman's correlation analysis. P<0.05 was considered statistically significant.

Results

Decreased miR-212 and increased ZEB2 are detected and inversely correlate in NSCLC

Using 26 NSCLC specimens, we detected significantly higher levels of ZEB2 (**Figure 1A**) and significantly lower levels of miR-212 in NSCLC tissues (**Figure 1B**) compared to the adjacent non-tumor tissues (NT) from the same patient.

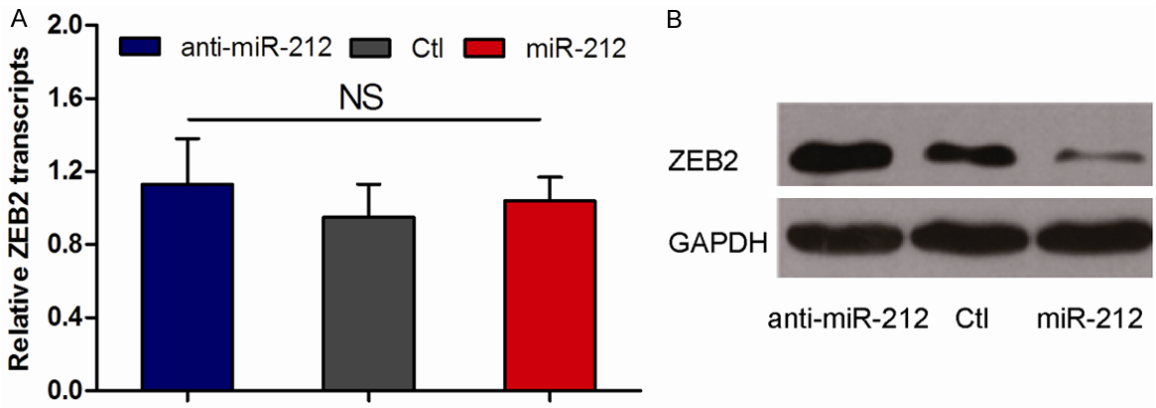

Figure 3. miR-212 decreased ZEB2 protein levels in NSCLC cells. A. We found that alteration of miR-212 in A549 cells did not change mRNA of ZEB2. B. Overexpression of miR-212 significantly decreased ZEB2 protein levels in A549 cells, while inhibition of miR-212 significantly increased ZEB2 protein levels in A549 cells, by western blot. *P<0.05.

To figure out the relationship between miR-212 and ZEB2, we explored the correlation using the 26 NSCLC specimens. A strong inverse correlation between miR-212 and ZEB2 was detected (**Figure 1C**, P<0.05), indicating the presence of a causal link between miR-212 and ZEB2 in NSCLC.

MiR-212 targets 3'-UTR of ZEB2 mRNA to inhibit its translation

Since we found a relationship between miR-212 and ZEB2 in MSCLC, we investigated whether miR-212 could target and regulate ZEB2 mRNA to inhibit its translation. Bioinformatics analyses showed that the miR-212 binding sites in the 3'-UTR of ZEB2 mRNA ranged from the 1303th base site to the 1310th base site (**Figure 2A**). In order to examine whether miR-212 may regulate the translation of ZEB2 in NSCLC, we used a human NSCLC cell line A549, to either over-express miR-212 or to inhibit miR-212 through transfection of the cells with a miR-212-expressing plasmid (miR-212), or with a plasmid carrying miR-212 antisense (anti-miR-212), respectively. The A549 cells were also transfected with a plasmid carrying a scrambled sequence as a control (Ctl). The overexpression or inhibition of miR-212 in A549 cells was confirmed by RT-qPCR (**Figure 2B**). MiR-212-modified A549 cells were then transfected with 1 μg of ZEB2 3'-UTR luciferase reporter plasmid. The luciferase activities were quantified in these cells, indicating that miR-212 targets 3'-UTR of ZEB2 mRNA to inhibit its translation (**Figure 2C**).

MiR-212 decreases ZEB2 protein levels in NSCLC cells

We found that the modification of miR-212 levels in A549 cells did not change the mRNA levels of ZEB2 (**Figure 3A**). However, the overexpression of miR-212 significantly decreased ZEB2 protein levels in A549 cells, while inhibition of miR-212 significantly increased ZEB2 protein levels in A549 cells by western blot (**Figure 3B**). These data indicated that miR-212 suppressed ZEB2 protein translation in A549 cells

MiR-212 inhibits NSCLC cell migration in a scratch wound healing assay

Next, we explored the effects of miR-212 modification in NSCLC cells on cell migration. Our data showed that up-regulated expression of miR-212 resulted in decreases in cell migration ability of A549 cells (**Figure 4**). Similarly, depletion of miR-212 resulted in increases in cell invasion ability of A549 cells (**Figure 4**).

MiR-212 suppresses NSCLC cell invasion in transwell cell migration assay

Furthermore, we tested the effects of miR-212 modification in NSCLC cells on cell invasion. We found that overexpression of miR-212 resulted in decreases in cell invasiveness of A549 cells in a transwell cell migration assay (**Figure 5**). Similarly, depletion of miR-212 resulted in increases in cell invasiveness of A549 cells (**Figure 5**). Together, these results suggested

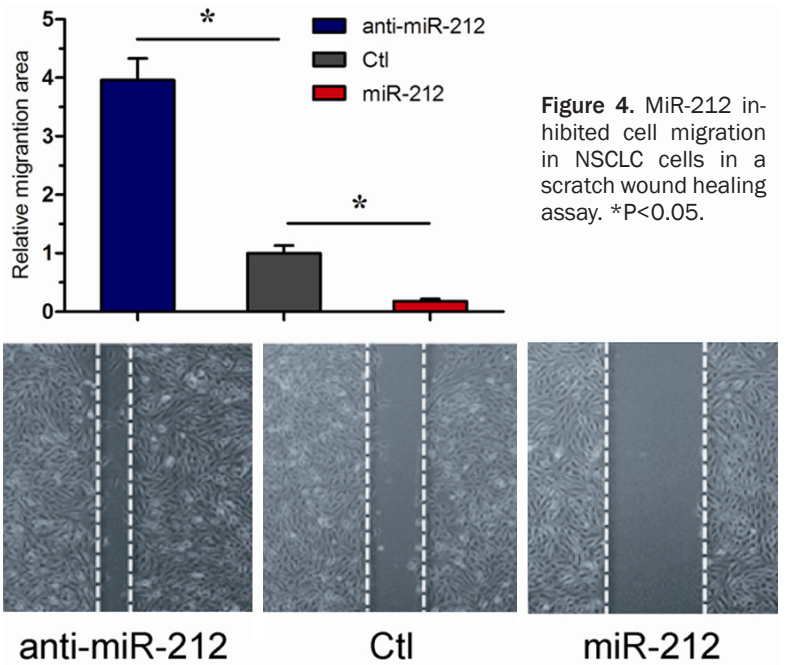

Figure 4. MiR-212 inhibited cell migration in NSCLC cells in a scratch wound healing assay. *P<0.05.

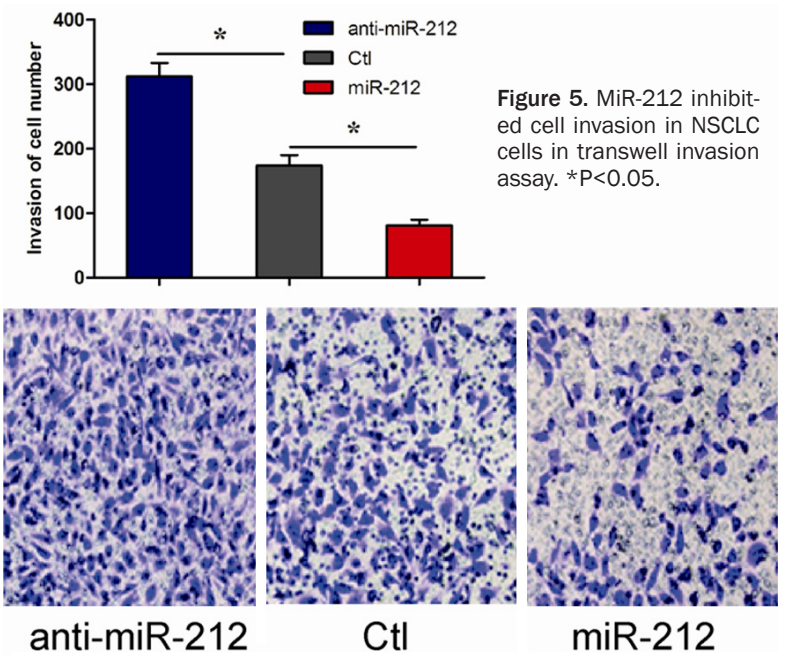

Figure 5. MiR-212 inhibited cell invasion in NSCLC cells in transwell invasion assay. *P<0.05.

that miR-212 inhibited NSCLC metastases through ZEB2 suppression (**Figure 6**).

Discussion

Accumulating evidence showed that alterations in miRNA expression levels could affect NSCLC physiology and tumourigenesis. For example, Dong et al. showed that miR-630 suppressed cell growth and invasion by down-regulating the expression of LMO3 in NSCLC [12]. Zhen et al. indicated that miR-200a targeted EGFR and c-Met to inhibit migration, invasion, and gefitinib resistance in NSCLC [13]. Yang et al. found that miR-26a was responsible for NSCLC cell sensitivity in the treatment of CDDP through regulating HMGA2-mediated E2F1-Akt pathway [14]. These findings demonstrated that miRNAs might play multiple roles in NSCLC progression.

Aberrant expression of miR-212 has been observed in several cancers. For example, Tu et al. found that miR-212 inhibited hepatocellular carcinoma cell proliferation and induced apoptosis by targeting FOXA1 [15]. Luo et al. revealed that miR-212 inhibited osteosarcoma cells proliferation and invasion by down-regulation of Sox4 [16]. Zhao et al. reported that miR-212 could down-regulate SMAD2 expression to suppress the G1/S phase transition of the cell cycle and the epithelial to mesenchymal transition in cervical cancer cells [17]. Recently, Jiang et al. found that miR-212 could act as a tumor suppressor in NSCLC progression [18]. However, the detailed role of miR-212 in NSCLC carcinogenesis is still unclear.

ZEB2 is a member of the ZEB family, which could induce epithelial to mesenchymal transition (EMT) through the inhibition of E-cadherin expression and promotion of tumor progression and metastasis [19]. The expression of ZEB2 has been found to be elevated in a variety of cancers, and ZEB2 plays critical roles in the development of many cancers. For example, Prislei et al. showed that nuclear ZEB2 might enhance progression of EMT transition and

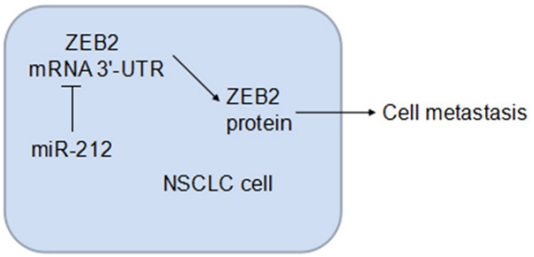

Figure 6. Schematic miR-212 suppressed NSCLC cell metastasis through ZEB2 suppression.

acquisition of an aggressive phenotype in ovarian cancer [20]. Yang et al. demonstrated that ZEB2 could promote vasculogenic mimicry by TGF-β1 induced EMT transition in hepatocellular carcinoma [21]. Yue et al. suggested that miR-139-5p suppressed cancer cell migration and invasion through targeting ZEB1 and ZEB2 in glioblastoma multiforme [22]. These data provide compelling data to demonstrate a critical role of ZEB2 as a promoter for EMT and cancer metastasis. However, how ZEB2 was regulated in NSCLC has not been studied.

In the present study, by sequence matching, we found a number of candidate miRNAs that target ZEB2. Among these miRNAs, we specifically found that the levels of miR-212 significantly decreased in NSCLC specimens compared to the adjacent non-tumor tissue. Hence, we hypothesized that miR-212 might target and regulate ZEB2 in NSCLC cells. Correlation test further supported this hypothesis in that the levels of miR-212 in NSCLC tissues were inversely correlated with the levels of ZEB2. Moreover, overexpression of miR-212 inhibited cell metastasis, while depletion of miR-212 increased cell metastasis. And in miR-212-modified NSCLC cells, we found that the modification in miR-212 levels did not affect ZEB2 mRNA but regulated the protein level. Using luciferase reporter activity assay, we found that miR-212 suppressed ZEB2 through translation suppression. In addition, miR-212 mediated changes in ZEB2 seemed to result in changes in cell metastasis. We have examined several other NSCLC cell lines and got essentially similar results. Thus, a possibility of the results to be cell-line-dependence could be excluded.

In summary, we revealed the tumor suppressive role of miR-212 in NSCLC. MiR-212 suppressed the metastasis of NSCLC cells by targeting ZEB2. Our findings indicated that miR-212 might provide a potential therapeutic target for NSCLC treatment.

Disclosure of conflict of interest

None.

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