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MicroRNA-190b inhibits tumor cell proliferation and induces apoptosis by regulating Bcl-2 in U2OS osteosarcoma cells

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Osteosarcoma (OS) is one of the most prevalent malignancies in bone with no established therapy so far. This study was aimed to clarify the role of miR-190b in tumor cell growth of OS. The miR-190b mimic, inhibitor and miR-control were transfected into human OS U2OS cells. Then U2OS cell proliferation was tested by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and bromodeoxyuridine (BrdU) incorporation assay. The apoptotic U2OS cells were detected by flow cytometry. Additionally, cell-cycle regulators p27, p21 and apoptosis factors B-cell lymphoma-2 (Bcl-2), Bcl-2 associated X (Bax), caspase-3 were examined by western blotting. Overexpressing miR-190b observably reduced cell viability, BrdU-positive cells (both $P < 0.05$) and caused strong accumulation of cell-cycle inhibitor p27 in U2OS cells compared with the miR-control, whereas the miR-190b inhibitor exerted opposite effects. Further, a marked increase of 18% rate of apoptotic cells by the overexpressing miR-190b ($P < 0.01$) and 4% decrease by miR-190b inhibitor ($P < 0.05$) were detected. The protein expressions of Bcl-2 were downregulated, Bax, pro-caspase-3 and active caspase-3 were upregulated by overexpressing miR-190b in U2OS cell line, while miR-190b inhibitor achieved opposite effects. The present study demonstrates that miR-190b inhibits tumor cell proliferation and induces apoptosis by regulating Bcl-2 in U2OS cells, which points to miR-190b as a novel oncosuppressor for OS. The identified tumor suppressive capacity of miR-190b provides novel avenues for achieving better OS therapy.

1. Introduction

Osteosarcoma (OS) is the most prevalent malignancy of bones, which accounts for 42% of primary bone sarcoma and 2.4% of all tumors in pediatric patients worldwide (Nouri et al. 2015; Ottaviani and Jaffe 2009). It is commonly characterized by a high incidence in children and adolescents, early pulmonary metastasis as well as poor prognosis (Chen et al. 2014; Zhu et al. 2012). OS has been frequently found to reside in the proximal tibia and distal femur region, mainly at the knee, and the five-year survival rate of OS patients is 60-70% (Yang and Zhang 2013). Although a host of attempts, chemotherapy, surgery and drugs, have been applied to protect against OS recurrence or metastases, to date, there remains no established therapy for clinical use (Berlanga et al. 2016; Hirahata et al. 2016). Thus, a better detection of molecular mechanisms underlying OS initiation, development and progression is urgently required to optimize therapeutic strategies.

MicroRNAs (miRNAs) are a highly conserved class of small non-coding RNAs that are 20-25 nucleotides in length (Muhammad et al. 2016). There is overwhelming evidence corroborating that miRNAs are involved in various biological processes, especially human cancers, by regulating the expression of target genes at the post-transcriptional level (Iorio and Croce 2012a, b; Shimono et al. 2016). A growing body of studies serve as the basis for the regulatory effect of miRNAs on tumor growth, invasion and diagnosis of OS, such as miR-33a, miR-93, miR-143, miR-183, miR-29b and miR-214 (Li et al. 2016; Montanini et al. 2012; Xu and Wang 2014; Zhou et al. 2014; Zhu et al. 2012; Zhu et al. 2016). miR-190b was detected as a biomarker for the diagnosis of Merkel cell carcinoma among seven others miRNAs (Ning et al. 2014). Analogously, miR-190b was the highest upregulated miRNA and thought to be a promising target in hormone dependent breast cancers (Cizeron-Clairac et al. 2015). Moreover, in hepatocellular carcinoma (HCC), miR-190b has been detected to induce insulin resistance (IR) and facilitate diagnosis (Hung et al. 2014; Patnaik et al. 2012). Nevertheless, to our current knowledge, properties and mechanisms of miR-190b have not been well described in OS.

The present study was aimed to clarify the role of miR-190b in tumor cell growth of OS. We explored whether aberrant miR-190b played a role in cell proliferation and apoptosis of U2OS cell line, as well as its underlying mechanism. This study was expected to decipher miR-190b properties and offer a novel miR-190b-based therapeutic target of OS.

2. Investigations and results

2.1. MiR-190b suppressed cell proliferation of U2OS cells

To investigate the role of miR-190b in OS, cell growth was observed firstly since uncontrolled proliferation was a primary feature of malignancies. The quantification of cell proliferation in transfected U2OS cells was performed with MTT assay for cell viability and BrdU assay for DNA synthesis.

We found that overexpressing miR-190b reduced cell viability in a time-dependent fashion and caused a striking decrease on day 4 and 5 (both $P < 0.05$, Fig. 1A), while the cell viability was enhanced by miR-190b inhibitor in the same manner (both $P < 0.05$). In addition, the effect of aberrant miR-190b expression on DNA synthesis in proliferating U2OS cells was further analyzed by the colorimetric measurement of BrdU incorporation: a 19% decrease of BrdU-positive cells was identified in response to miR-190b mimic, whereas the miR-190b inhibitor caused a 7% increase (Fig. 1 B and C), which was statistically different with the miR-control, respectively (both $P < 0.05$). Taken together, our data claimed that miR-190b suppressed cell proliferation and induced growth arrest in U2OS cell line.

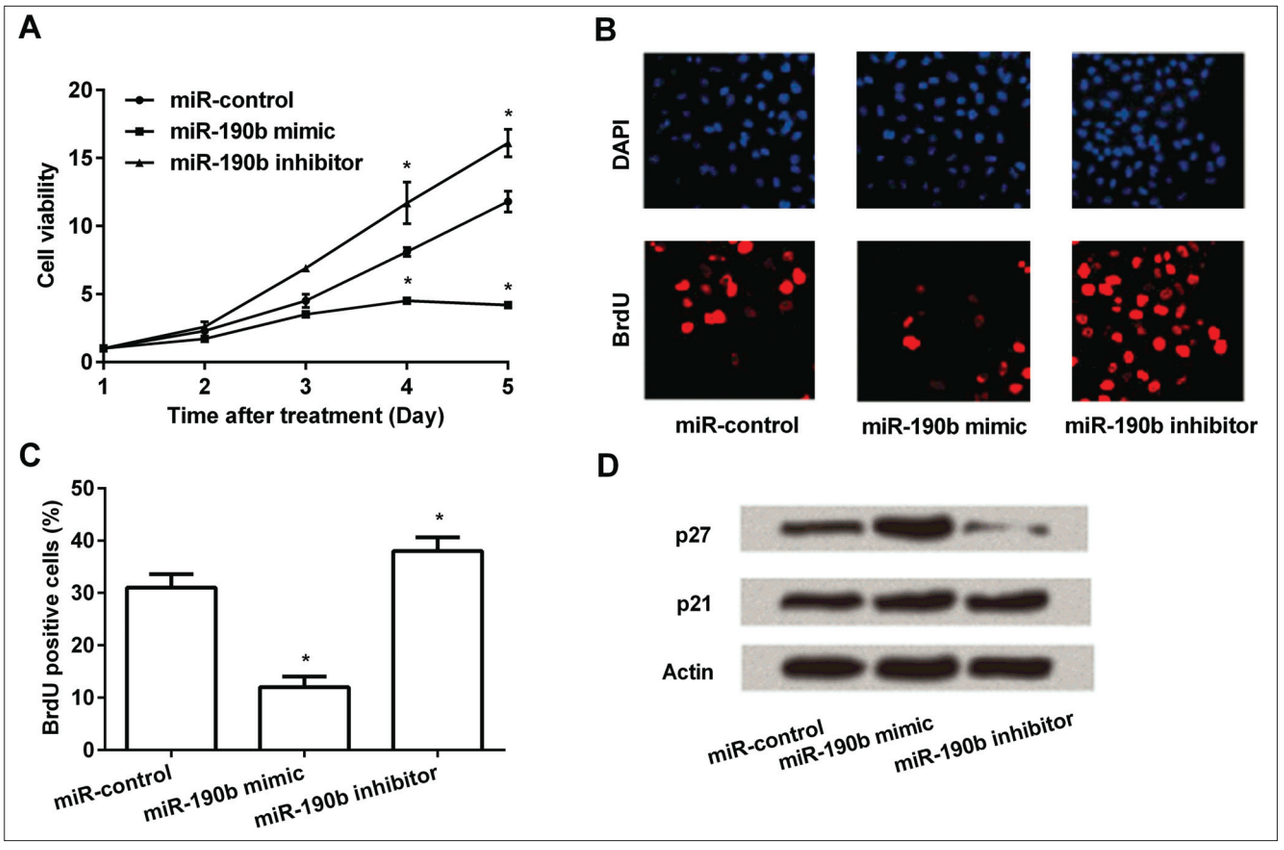

Fig. 1: MiR-190b suppressed cell proliferation of U2OS cells. A. The cell viability of U2OS cells was detected by MTT assay; B. Immunofluorescence assay of BrdU incorporation in transfected U2OS cells; C. The BrdU positive cells were quantified in proliferating U2OS cells; D. The effects of aberrant miR-190b on cell-cycle regulators p21 and p27 expressions. MiR, microRNA; MTT, 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide; DAPI, 4', 6-diamidino-2-phenylindole; BrdU, bromodeoxyuridine.*, $P < 0.05$.

2.2. *MiR-190b-induced growth inhibition was related to upregulation of p27 in U2OS cells*

Next, we assessed the effects of miR-190b on cell cycle-related molecules of U2OS cells by western blot, which was expected to dissect the mechanism of miR-190b -induced suppressed proliferation in OS. For U2OS cell cycle, strong accumulation of cell-cycle inhibitor p27 was detected in response to overexpressing miR-190b as compared to the control, whereas silencing miR-190b remarkably suppressed the protein expressions of p27 (Fig. 1D). No difference was witnessed in the other cell-cycle regulator p21 expressions between overexpressing miR-190b, silencing miR-190b and miR-control, respectively. Thereby, miR-190b-induced growth inhibition was related to the upregulation of p27 in U2OS cells.

2.3. *MiR-190b induced apoptosis by regulating Bcl-2 in U2OS cells*

Since miR-190b suppressed cell proliferation and induced growth arrest in the U2OS cell line, we tested the role of miR-190b in cell apoptosis. The flow cytometry assay showed a marked increase of 18% rate of apoptotic cells by the overexpressed miR-190b ($P < 0.01$) and a 4% decrease by the miR-190b inhibitor ($P < 0.05$, Fig. 2 A and B) in U2OS cells. Further, protein expressions of key apoptotic factors affected by aberrant miR-190b expression were evaluated (Fig. 2C). The protein expressions of Bcl-2 were downregulated by overexpressing miR-190b and were upregulated by miR-190b inhibitor. The miR-190b overexpression in U2OS cell line enhanced protein expressions of Bax, pro-caspase-3 and active caspase-3, whereas miR-190b inhibitor achieved opposite effects. Accordingly, miR-190b induced apoptosis by regulating Bcl-2 in U2OS cells.

3. Discussion

Given the fact that the effects of miR-190b on tumors are not well understood up to now, this study investigated whether dysregulation of miR-190b modulated cell proliferation and apoptosis, and if so, what the molecular consequences might be. Results of the current study have shown that overexpressed miR-190b induced inhibitions on cell viability, DNA synthesis and cell cycle of U2OS cells. Further, overexpressing miR-190b greatly enhanced cell apoptosis in U2OS cells. Our findings were indicative of miR-190b as a novel therapeutic biomarker for OS by providing the first insight that miR-190b suppressed OS cell growth.

To address the mechanisms implicated in the miR-190b-mediated cell proliferation inhibition, miR-190b-mediated OS cell motility cell viability, DNA synthesis and cell cycle of U2OS cells were determined. The overexpression of miR-190b strongly diminished whereas the miR-190b inhibitor enhanced U2OS cell viability, which was consistent with results in breast cancer MCF-7 and T-47D cells (Cizeron-Clairac et al. 2015). The miR-190b-mediated inhibition of cell viability was considered to correlate with the inhibition of DNA replication (Fernandez et al. 2015). Thereafter, a potent reduction of BrdU-positive cells in response to miR-190b mimic reflected miR-190b-mediated weakened DNA synthesis in proliferating U2OS cells.

Additionally, for cell cycle, our results showed that the negative cell-cycle regulator p21 was unaffected, while p27 was expressed at relatively high levels by miR-190b in U2OS cells. It hinted that p21 and p27 played different roles in the oncogenesis of OS, in line with foregone findings in breast cancer (Wong et al. 2001). p27, a cyclin-dependent kinase (CDK) inhibitor, is known to be a negative cell-cycle regulator *via* a vatiety of functions, including impaired synthesis and elevated protein degradation

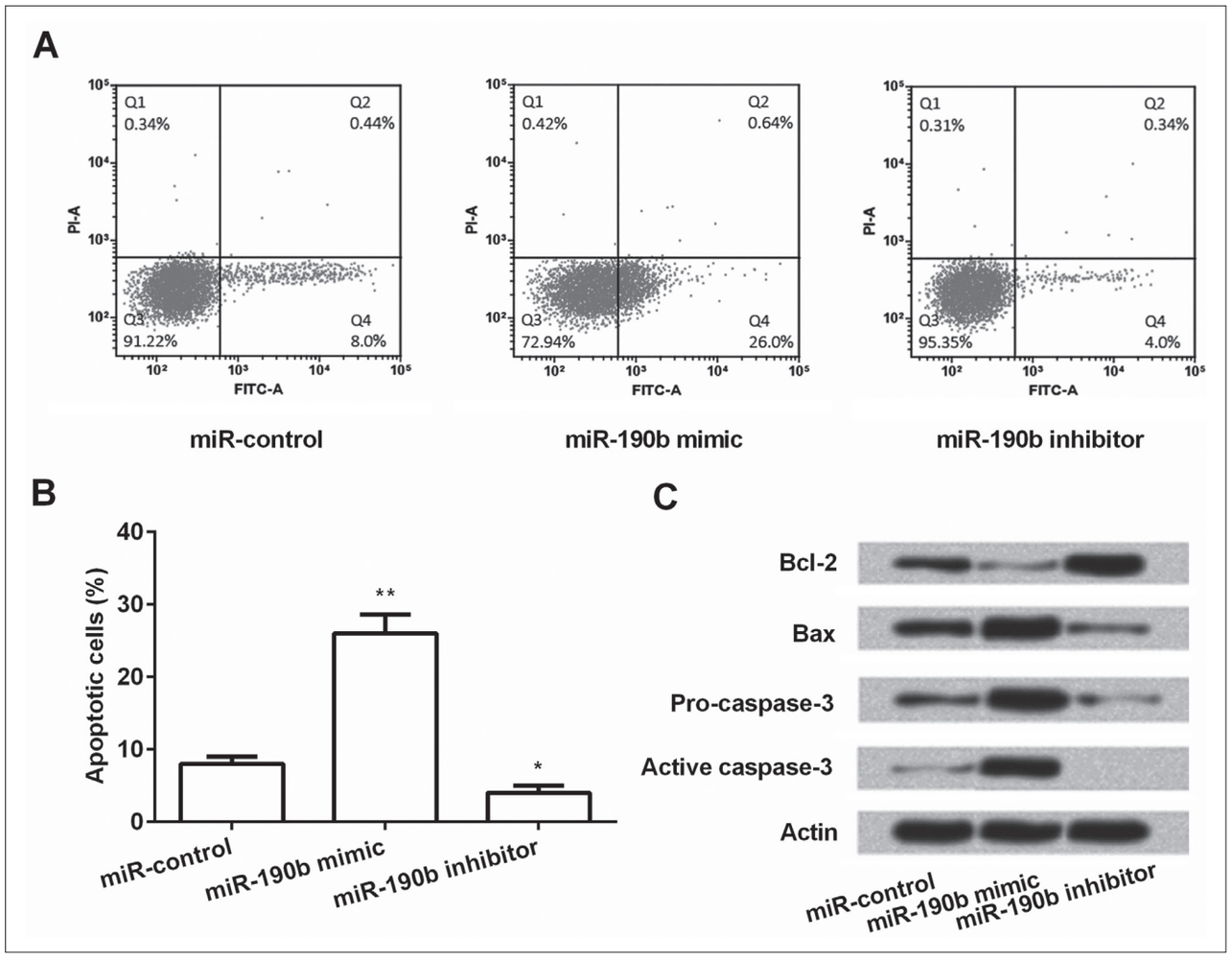

Fig. 2: MiR-190b induced apoptosis by regulating Bcl-2 in U2OS cells. A. The apoptotic cells were assessed by flow cytometry assay; B. The effects of aberrant miR-190b on cell apoptosis; C. The protein expressions of key apoptotic factors affected by aberrant miR-190b. MiR, microRNA; Bcl-2, B-cell lymphoma-2; Bax, Bcl-2 associated X. *, $P < 0.05$; **, $P < 0.01$.

during tumorigenesis (Chu et al. 2008). Many lines of evidence identified p27 as a key tumor suppressor and in OS, Cao et al. (2013) demonstrated that miRNAs targeting p27 could promote cell proleferation, in agreement with similar observations (Micel et al. 2013; Wang et al. 2014; Zhang et al. 2001). Importantly, stable overexpressed p27 brought about apoptotic death in non-small cell lung cancer (NSCLC) A549 cells (Wang et al. 1997). Thus, miR-190b-decreased cell viability in U2OS cells was partially consequent to the accumulation of p27. It is suggested that the enrichment of p27 level was a determinant of the miR-190b-induced growth arrest in OS.

Next, we characterized the apoptosis stimulation of miR-190b in OS. The great increase of apoptotic cells responding to overexpressed miR-190b in U2OS cells was related to dysregulations of corresponding antiapoptotic Bcl-2 and proapoptotic Bax, caspase-3. As we all know, the intrinsic apoptosis mechanism mainly involves the Bcl-2 family and caspase activation. One previous study found that OS cell apoptosis was inducted by the upregulation of Bax and downregulation of Bcl-2, as well as activation of caspase-3, -8, and -9, which subsequently triggered apoptotic cascades (Liang et al. 2012). Since upregulation of Bcl-2 could reduce protein expressions of Bax and induce activation of downstream caspase-3 (Cheng et al. 1997), it was hypothesized that miR-190b might exert its proapoptotic function in OS cells through suppressing Bcl-2 expressions.

Our results suggest that the restraining function of cell growth by miR-190b in U2OS cells was mainly by means of impaired Bcl-2. However, we are still far from uncovering the last target of miR-190b in OS. The identified targeted genes of miR-190b were insulin-like growth factor (IGF-1) in HCC and myotubularin-related protein 6 (MTMR6) in response to simian immunodeficiency virus (SIV) (Hung et al. 2014; Mohan et al. 2014), which might serve as positive prognosis factors. Given the miR-190b-mediated OS alleviation evidenced by our work, future research is warranted to determine the target genes and entire effects of miR-190b on OS progression.

Collectively, the present study demonstrates that miR-190b inhibits tumor cell proliferation and induces apoptosis by regulating Bcl-2 in U2OS cells, which points to miR-190b as a novel oncosuppressor for OS. The indentified tumor suppressive capacity of miR-190b provides promising avenues for achieving better OS therapy.

4. Experimental

4.1. U2OS cell culture

The human OS cell line U2OS (American Type Culture Collection, Rockville, USA) was grown in Dulbecco's modified Eagle's medium (DMEM; Gibco-BRL, Invitrogen, Carlsbad, USA) supplemented with 10% fetal bovine serum (FBS; Gibco, Grand Island, NY, USA). All U2OS cells were incubated in a humidified atmosphere with 5% CO₂ at 37 °C until reaching 80% confluency under the phase-contrast microscopy (Olympus Optical Co., Tokyo, Japan).

4.2. Transfection of U2OS cells with aberrant miR-190b

After U2OS cells were maintained at 1×10^5 cells/well, miR-190b mimic, inhibitor and the corresponding controls (RiboBio, Guangzhou, China) were transfected into U2OS cells on the next day. Cell transfections were performed using Lipofectamine reagent (Invitrogen, San Diego, USA) according to manufacturer's protocol.

4.3. Cell viability assay

Two days after transfection, U2OS cell line (1×10^3 cells/well) was seeded on 96-well plates in triplicate. Cell viability assay was carried out by adding 10 mg/mL 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Sigma-Aldrich, USA) to 0.2 mL of DMEM medium for 3 h. Then after removing the medium, 100 µL dimethylsulfoxide (DMSO; Lonza, USA) were added to dissolve the blue formazan (Sigma, USA) product for 1 h. The percentage of living U2OS cells was determined for five consecutive days at an optical density of 590 nm using a Multiskan EX (Thermo Scientific, Helsinki, Finland).

4.4. Bromodeoxyuridine (BrdU) assay

The U2OS cell line (2×10^4 cells/well) was seeded in 6-well plates on sterilized coverslips after transfection for 2-3 d. For immunofluorescence assay of BrdU incorporation in U2OS cells, we added 10 µM BrdU (Sigma-Aldrich, St. Louis, USA) in the DMEM media and maintained overnight. After cells were fixed and DNA was denaturated by 1.5 M HCl, the incorporation of BrdU into DNA was visualized by a human anti-BrdU antibody (Abcam; Cambridge, United Kingdom). The antibody was incubated for 45 min with a secondary antibody conjugated to green-fluorescence dye Alexa Fluor 488 (1:500; Invitrogen, Carlsbad, USA). Finally, U2OS nuclei were stained by 4', 6-diamidino-2-phenylindole (DAPI; Vector Laboratories, Burlingame, USA) in Vectashield mounting medium. Pictures were captured by a Leica DMI6000B inverted microscope and DFC420 RGB digital camera (Leica Microsystems, Wetzlar, Germany).

4.5. Apoptosis assay

The Annexin V-FITC/Propidium iodide (PI) apoptosis detection kit (Becton Dickinson Vacutainer Systems, Rutherford, USA) was applied to detect apoptotic U2OS cells by flow cytometry. After transfection for 3 d, U2OS cells with miR-190b mimic, inhibitor and miR-control were seeded in 6-well culture plate respectively and washed twice with ice-cold phosphate buffer saline (PBS). Briefly, they were incubated with serum-free culture medium containing 1 µmol/L dichlorofluorescein diacetate (DCFH-DA; Jiancheng Bioengineering Institute, Nanjing, China) for 30 min. Subsequently, samples were collected by a trypsin digestion approach and centrifuged. Then U2OS cells were collected as a pellet and resuspended in 100 µL annexin-binding buffer. The percentage of apoptotic U2OS cells was calculated with a flow cytometer based on the manufacturer's protocol.

4.6. Western blot analysis

The protein used for western blotting was extracted from U2OS cells with miR-190b mimic, inhibitor and miR-control respectively using RIPA lysis buffer (Beyotime, Shanghai, China). Thereafter, proteins concentration was determined by Pierce Bicinchoninic Acid (BCA) Protein Assay Kit (Appleton, USA). For western blot, the Bio-Rad Bis-Tris Gel system was performed, in which primary antibodies p27 (ab32034), p21 (ab109520), B-cell lymphoma-2 (Bcl-2; ab32124), Bcl-2 associated X (Bax; ab32503), pro-caspase-3 (ab32150), active caspase-3 (ab2302) and the internal control actin were obtained from Abcam (Cambridge, United Kingdom). Membranes were incubated at 4 °C overnight with the indicated primary antibodies and thereafter revealed using secondary antibodies with horseradish peroxidase (1:5000) for 1 h. Then, reaction products and antibodies were transferred onto the polyvinylidene fluoride (PVDF; Millipore Co., Bedford, USA) membrane. Pictures were scanned from the stained gel with Gel Doc 2000 Gel documentation system (Bio-Rad, Hercules, USA).

4.7. Statistical analysis

All experiments were performed in three times, and the results were presented as mean±standard deviation (SD). Differences were compared by one-way analysis of variance (ANOVA) with SPSS 19.0 software (SPSS, Chicago, USA). $P < 0.05$ was considered as statistical significance.

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Conflicts of interest: Authors declare no conflict of interest.

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