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Original Article

Impact of NPR-A expression in gastric cancer cells

Jia Zhang, Jingkun Qu, Ya Yang, Min Li, Mingxin Zhang, Xiaohai Cui, Jing Zhang, Jiansheng Wang

Second Department of Thoracic Surgery, First Affiliated Hospital, Xi'an Jiaotong University, Xi'an, Shaanxi, China

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Abstract: Background: The receptors for the cardiac hormone atrial natriuretic peptide (ANP), natriuretic peptide receptor A (NPR-A), have been reported to be expressed in lung cancer, prostate cancer, ovarian cancer. NPR-A expression and signaling is important for tumor growth, its deficiency protect C57BL/6 mice from lung, skin, and ovarian cancers, and these result suggest that NPR-A is a new target for cancer therapy. Recently, NPR-A has been demonstrated to be expressed in pre-implantation embryos and in ES cells, it has a novel role in the maintenance of self-renewal and pluripotency of ES cells. However, the direct role of NPR-A signaling in gastric cancer remains unclear. Method: NPR-A expression was downregulated by transfection of shRNA. The proliferation of gastric cancer cells was measured by Hoechst 33342 stain. Cell proliferation and invasion were determined via BrdU and transwell assays, respectively. Results: Down-regulation of NPR-A expression by shNPR-A induced apoptosis, inhibited proliferation and invasion in AGS cells. The mechanism of shNPR-A-induced anti-AGS effects was linked to NPR-A-induced expression of KCNQ1, a gene to be overexpressed in AGS and significantly reduced by shNPR-A. Conclusion: Collectively, these results suggest that NPR-A promotes gastric cancer development in part by regulating KCNQ1. Our findings also suggest that NPR-A is a target for gastric cancer therapy.

Keywords: NPR-A, gastric cancer, KCNQ1

Introduction

Gastric cancer is the fourth most common cancer and the second most common cause of cancer deaths worldwide [1]. In China, the annual number of new cases of gastric cancer ranks the first in the world. Gastric cancer is often the time of diagnosis in advanced gastric cancer (Phase III or IV), which has a poor prognosis [2]. So far, the molecular targeted therapy for gastric cancer is rare [3]. Thus, it is extremely important to get a new molecular marker for gastric cancer, which could improve the rate of early diagnosis of gastric cancer, and find a new target for treatment of gastric cancer.

The receptor for the cardiac hormone atrial natriuretic peptide (ANP), natriuretic peptide receptor A (NPR-A), have been reported to be expressed in lung cancer, prostate cancer, ovarian cancer. NPR-A expression and signaling is important for tumor growth, its deficiency protect C57BL/6 mice from lung, skin, and ovarian cancers, and these result suggest that NPR-A is a new target for cancer therapy [4, 5]. Recently, NPR-A has been demonstrated to be expressed in pre-implantation embryos and in ES cells, it has a novel role in the maintenance of self-renewal and pluripotency of ES cells [6].

On channels in virtually contribute to all basic cellular processes, including crucial roles in maintaining tissue homeostasis such as proliferation, differentiation and apoptosis. Eveidences have shown that ion channels are involved in certain cancer-related cellular behaviors such as proliferation, apoptosis, migration or angiogenesis [7-19].

We previously reported that NPR-A is expressed in the human gastric cancer cell line, ANP modulates AGS cells proliferation by KCNQ1 [19]. However, the direct role of NPR-A signaling in gastric cancer remains unclear. The aim of this study is to examine the mechanism for the down-regulation of NPR-A and the involved ion channels.

Material and methods

Cell lines

AGS cell lines were obtained from American Type Culture Collection (Manassas, VA, USA). The cells were cultured in Ham-12 F medium

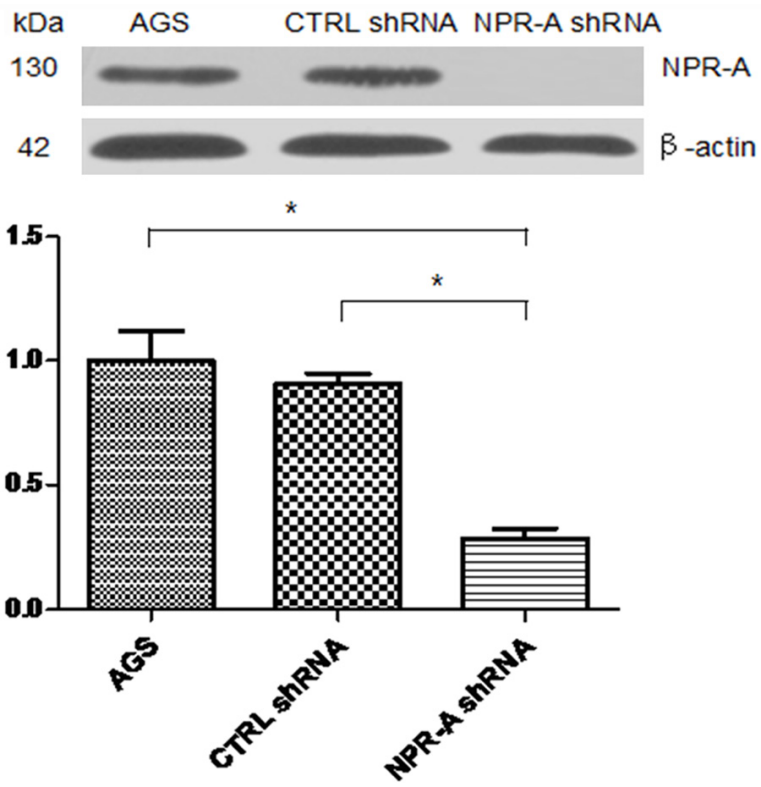

Figure 1. NPR-A depletion in AGS cell line. Expression NPR-A was analyzed by Western blot in AGS cell line and Western blot of NPR-A depletion efficiency in AGS cell line.

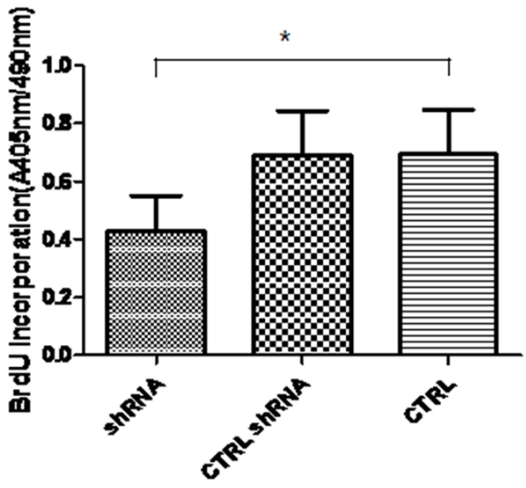

Figure 2. NPR-A depletion impaired cancer cell proliferation. Cell proliferation was performed using BrdU labeling. $^{*}P < 0.05$, compared with shRNA group.

(Invitrogen, Carlsbad, CA, USA) containing 10% fetal calf serum (Invitrogen), 100 IU/ml penicillin (Sigma, St. Louis, MO, USA), and 100 mg/ml streptomycin (Sigma). Cells were grown on sterile tissue culture dishes and were passaged every 2 days using 0.25% trypsin (Invitrogen).

Western blot analysis

Total proteins from cell lines were extracted in lysis buffer (Thermo Fisher Scientific, Rockford, IL) and quantified using the Bradford method. Fifty micrograms of protein were separated by SDS-PAGE (12%). After transferring, the polyvinylidene fluoride (PVDF) membranes (Millipore, Billerica, MA, USA) were incubated overnight at 4°C with the following NPR-A (1:1000; Proteintech, Chicago, IL, USA) antibody. After incubation with peroxidase-coupled anti-mouse/rabbit IgG (Santa Cruz Biotechnology) at 37°C for 2 hours, bound proteins were visualized using ECL (Thermo Fisher Scientific) and detected using BioImaging Systems (UVP Inc., Upland, CA, USA). The relative protein levels were calculated based on -actin as the loading control.

Small interfering RNA treatment

Confluent cells were incubated in complete medium with 5 μg/ml Polybrene (Santa Cruz Biotechnoloty, Inc.) and were transfected with NPR-A shRNA (h) Lentiviral Particles by adding the shRNA Lentiviral Particles for overnight. Select stable clones expressing the shRNA via Puromycin dihydrochloride (Santa Cruz Biotechnoloty, Inc.) selection. After the incubation, the cell lysates were prepared and used for western blotting. Control shRNA Lentiviral Particles-A (Santa Cruz Biotechnoloty, Inc.) was used as a negative control. Transgene expression was confirmed at 48 h post-infection by green fluorescent protein (GFP) expression, immediately followed by drug selection using puromycin.

Cell proliferation test

Cell proliferation was measured using 5-Bromo-2'-deoxy-uridine Labeling and Dectection Kit III (Roche Applied Science) in accordance with the manufacturer's instructions. Briefly, 1×10^4 cells were plated in 96-well plate and grew to conflu-

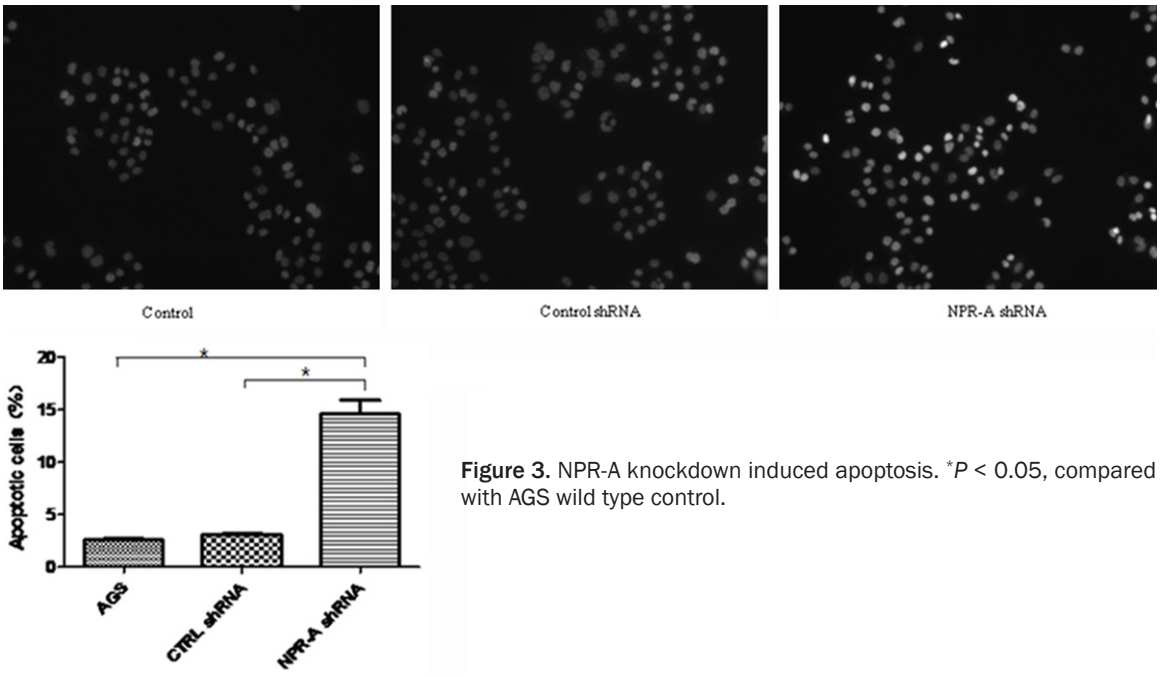

Figure 3. NPR-A knockdown induced apoptosis. $^{*}P < 0.05$, compared with AGS wild type control.

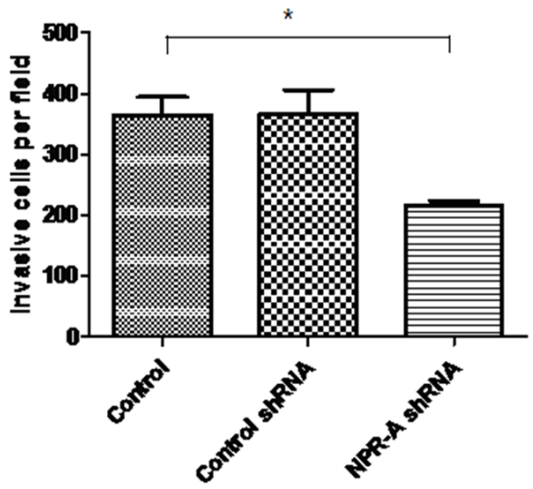

Figure 4. Invasion assays of AGS cells transfected with control and NPR-A-specific shRNA. NPR-A shRNA treatment significanly inhibit the cell invasion in AGS cells. Numbers of cells invading onto the lower surface of the filter were counted, $^{*}P < 0.05$, compared with control group.

ence. The medium was switched to culture medium containing 10 μM BrdU, and cells were incubated for an additional 2 hours. BrdU incorporation into cellular DNA was measured by using a microplate reader (Safire II, Tecan). Three independent experiments were performed, and each assay was carried out in triplicate.

Matrigel invasion assay

Cell invasion assay was performed using a 24-well Transwell chamber with a pore size of 8 mm (Costar, Cambridge, MA). The inserts were coated with 20 ml Matrigel (1:3 dilution, BD Bioscience, San Jose, CA, USA). Forty-eight hours after the transfection, cells were trypsinized and 36105 cells in 100 ml of serum-free medium were transferred to the upper Matrigel chamber and incubated for 16 hours. Medium supplemented with 10% FBS alone or containing 100 ng/ml EGF (Invitrogen, -Carlsbad, Carlsbad, CA) was added to the lower chamber as the chemoattractant. After incubation, the non-invaded cells on the upper membrane surface were removed with a cotton tip, and the cells that passed through the filter were fixed with 4% paraformaldehyde and stained with hematoxylin. The number of invaded cells was counted in 10 randomly selected high power fields under the microscope. This experiment was performed in triplicate.

Apoptosis analysis

For detection of apoptosis, adherent cells were both collected and resuspended in cold PBS for analysis. Cells were stained with Hoechst 33342 (BD Pharmingen, USA) to monitor apoptosis cells. Data were collected using BD systems.

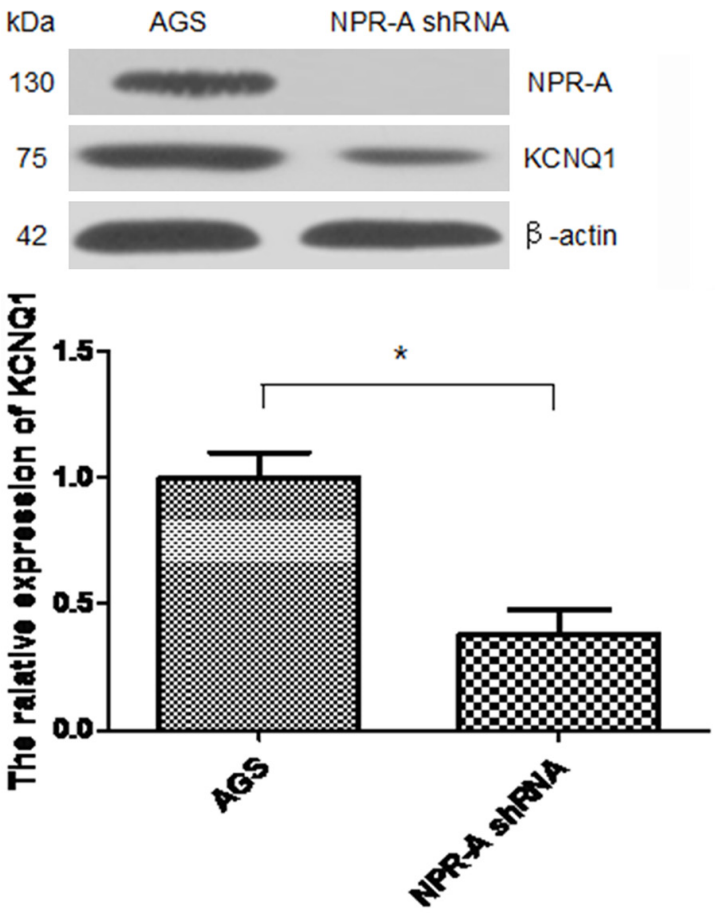

Figure 5. Expression of KCNQ1 in NPR-A depleted AGS cells. Western blot analysis showed the protein level of KCNQ1 was decreased in AGS cells $^{*}P < 0.05$, compared with AGS wild type.

Patch-clamp recordings

The voltage clamp technique was performed using the whole-cell configuration at room temperature (22~25°C). The normal Tyrode's solution used in the experiments contained the following (in mM): 140 NaCl, 5.4 KCl, 1.2 $MgCl_2$, 5 HEPES, 1.8 $CaCl_2$, and 10 Glucose, and was titrated to PH 7.4 with NaOH. The pipette solution used in the experiments contained the following (in mM): 120 K-Asparatate, 10 Na_2A-TP/$2H_2O$, 2 $MgCl_2$, 10 EGTA, and 10 HEPES, and was titrated to PH 7.4 with KOH. The glass pipette electrodes were made from Corning 7056 glass capillaries (Warner Instrument, Hammed, CT) with a pipette resistance of 2~3 MΩ in the bath solution. All recordings were initiated at least 10 min after rupture of the membrane. Signals were measured with an Axopatch 700A amplifier using pclamp 9 software (Molecular Device, Sunnyvale, CA), with Bessel low-pass filter (cut-off frequency: 10 kHz) and sampling frequency at 10 kHz.

Statistical analysis

SPSS version 16.0 for Windows was used for all analyses. *P* value was based on the two-sided statistical analysis. *P* values of < 0.05 was considered to indicate statistical significance.

Results

NPR-A depletion inhibits proliferation, invasion and induces apoptosis in gastric cancer cell lines

In order to explore the biological function of NRR-A in gastric cancer, we employed shRNA to knockdown NPR-A expression in AGS cell line. NPR-A-specific shRNA reduced NPR-A protein expression after 48 hours of shRNA treatment (**Figure 1**). Our cell proliferation analysis showed that depletion of NPR-A in AGS cells led to a significant reduction of proliferation rate (AGS control vs NPR-Ash: 0.7±0.12 vs 0.4±0.09, $P < 0.05$) , suggesting that NPR-A modulates proliferation of gastric cancer cells (**Figure 2**). In addition, Hoechst 33342 staining was employed to detect the apoptosis of AGS cells with NPR-A knockdown (**Figure 3**). A significant increased apoptosis rate was observed in cells with NPR-A knockdown compared with scramble controls (NPR-Ash vs AGS control: 14% vs 3%, $P < 0.05$), demonstrating that NPR-A knockdown results in apoptosis of the gastric cancer cells.

To investigate whether NPR-A contributes to the invasion of gastric cancer cells, we conducted matrigel invasion assays. As shown in **Figure 4**, NPR-A knockdown inhibited cell invasion (AGS control vs NPR-Ash, 370±20 vs 205±16, $P < 0.05$).

Depletion of NPR-A downregulated KCNQ1 and decreased K+ current in gastric cancer cells

Our previous study has shown ANP modulates AGS cells proliferation by KCNQ1 [19]. In this

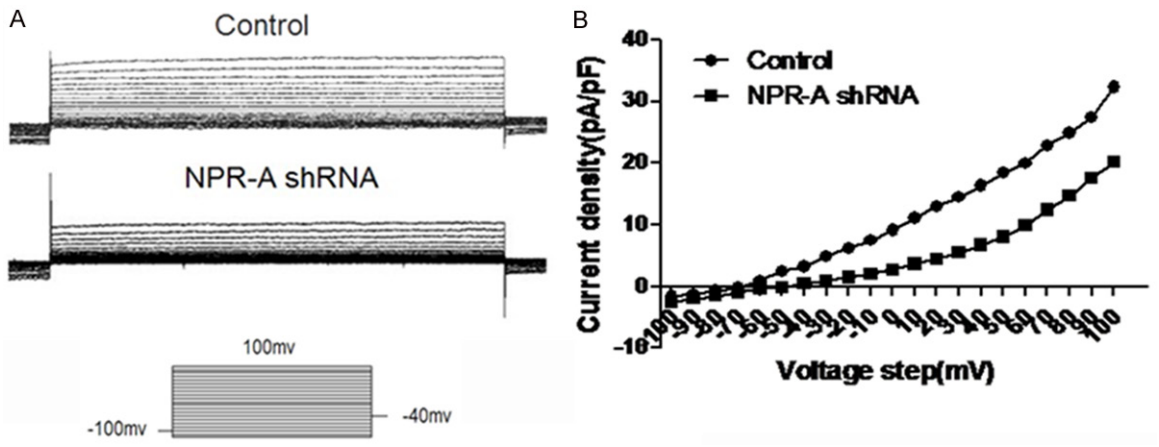

Figure 6. NPR-A shRNA decreased the steady-state activation property of Ik in AGS. A: NPR-A knockdown significantly decreased voltage-gated outward K+ current (n=20, $P < 0.05$). B: Voltage-dependent activation curve of Ik obtained in AGS control and AGS NPR-Ash. Current density (in pA/pF) is plotted against the voltage step. the activation curve was significantly shifted towards right by NPR-A knockdown (n=20, $P < 0.05$).

study, we investigate the role of KCNQ1 and K+ current in the effects of NPR-A knockdown on the AGS cells. As shown in **Figure 5**, Western blot analysis revealed that knockdown of NPR-A decreased the protein level of KCNQ1. I_K was evoked by a 500 msec depolarizing pulse from a first pulse potential of -100 to 100 mV, in 10 mV steps at 10 sec intervals (**Figure 6**). NPR-A knockdown significantly decreased voltage-gated outward K+ current (**Figure 6A**, n=20, $P < 0.05$). By plotting normalized conductance as a function of the command potential, we obtained the I_K activation curve. As shown in **Figure 6B**, the activation curve was significantly shifted towards right by NPR-A knockdown (n=20, $P < 0.05$).

Discussion

NPR-A is expressed at a higher level in all tumor cells, including cells of lung carcinoma (A549 and LLC1), melanoma (B16), ovarian cancer (SKOV3 and ID8), and prostate cancer cells (DU145), compared with that in normal human bronchial epithelial cells [4]. NPR-A-KO mice do not permit growth of implanted human lung cancer, melanoma and ovarian cancer cells [4]. In human PCa tissue microarray, NPR-A expression during human PCa development in BPH, high grade PIN (prostatic intraepithelial neoplasm) and prostatic adenocarcinoma showed positive correlation with clinical staging [5]. Previous research showed that NPR-A was overexpressed in prostate cancers and its overexpression was correlated with clinical and pathological factors [5]. To validate the potential role of NPR-A in gastric cancer development, we first checked its expression level in several cell lines and picked up AGS with relatively high NPRA level for further study. We employed shRNA to knockdown NPR-A expression in the cell line. We found an impaired proliferation capacity, increased apoposis of AGS cells after NPR-A knockdown. Furthermore, matrigel invasion assay showed decreased invading ability of shRNA treated cells. Thus, our study suggested that NPR-A functioned as an oncogene in gastric cancer development.

Previous report indicated that NF-KB and pRb are involved in tumor suppression in NPR-A deficient mice, Superarray analysis revealed that the expression of several genes, such as hexokinase 2, glycogen synthase 1, and matrix metallopeptidase 10 were down-regulated from about 4- to 17-fold in the lung of $NPRA^{-/-}$ mice [4]. In prostate cancer, by siRNA and animal model it has shown NPR-A promote PCa development in part by regulating MIF (migration inhibitory factor) [5].

Evidences have been show the crucial role of plasma K+ channels in the regulating of tumor cell proliferation [20, 21]. In human gastric cancer cell, it has been show that delayed rectifier potassium channel subunits Kv1.3, Kv1.5, Kv1.6, Kv2.1 and Kv2.2 expressed, down-regualtion of the expression can significantly inhibit the proliferation of gastric cancer [22]. Our previous study has shown ANP modulates AGS cells proliferation by KCNQ1 [19]. In this study, we investigate the role of KCNQ1 and K+

current in the effects of NPR-A knockdown on the AGS cells. Western blot analysis revealed that knockdown of NPR-A decreased the protein level of KCNQ1. NPR-A knockdown significantly decreased voltage-gated outward K+ current. By plotting normalized conductance as a function of the command potential, we obtained the I_K activation curve. The activation curve was significantly shifted towards right by NPR-A knockdown.

In conclusion, the present study addressed the biological role and potential mechanism of NPR-A in gastric cancer progression.

Disclosure of conflict of interest

None.

Address correspondence to: Jiansheng Wang, Second Department of Thoracic Surgery, First Affiliated Hospital, Xi'an Jiaotong University, 277# West Yanta Road, Xi'an, Shaanxi, China. E-mail: wangjshxjtu@mail.xjtu.edu.cn

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