MicroRNA-185 downregulates androgen receptor expression in the LNCaP prostate carcinoma cell line

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Abstract. The present study aimed to investigate whether microRNA (miR)-185 downregulated androgen receptor expression in the LNCaP prostate carcinoma cell line. Human prostate cancer (PCa) LNCaP cells were cultured and transfected with synthetic has-miR-185 mimic or inhibitor. The transfected cells were subsequently evaluated with a viability assay, nuclear staining, reverse transcription quantitative polymerase chain reaction (RT-qPCR), dual luciferase assay and western blot analysis. The results of the western blot analysis and RT-qPCR indicated that transfection with an miR-185 mimic markedly reduced the androgen receptor (AR) protein expression levels in LNCaP cells, whereas transfection with an miR-185 inhibitor increased the protein expression of AR in the LNCaP cells. The results of the luciferase reporter assay demonstrated that the predicted target site in the AR 3’ untranslated regions was a specific functional binding site for miR‑185, and that AR was a direct target of miR-185. In addition, downregulation of AR by miR-185 impaired the interaction between AR and androgen response element, and downregulated the expression of the AR target gene prostate specific antigen. Data also suggested that the downregulation of AR mediated by miR-185, impaired the proliferation and induced the apoptosis of the LNCaP cells. Therefore, the results of the present study suggested that miR-185 may be a potential negative modulator of AR-mediated signaling and may act as a tumor suppressor in prostate cancer cells.

Introduction

Prostate cancer (PCa) is the second most common cause of cancer-associated mortality, and the most frequently diagnosed malignancy in males in the USA (1,2). Androgen receptors (AR) have the most significant functions in PCa, in disease initiation and progression (3,4). Targeting the expression of AR and inhibiting AR activity are important in androgen-dependent and androgen-independent diseases. Initially, AR is expressed by the PCa cells, which are dependent on androgens to facilitate growth (5). Common treatments involving androgen ablation have been demonstrated to temporarily alleviate the disease, however, they lead to the recurrence of highly aggressive and androgen-independent types of metastatic cancer (6,7). Previous evidence has suggested that the progression of PCa to the androgen-independent stage does not involve the loss of AR, but is induced by the restoration of AR signaling in the PCa cells (6). During the transformation from the androgen-dependent stage to the androgen-independent stage, PCa cells develop several mechanisms to facilitate the activation of AR in the androgen-depleted environment, including amplification or mutation of the AR gene, overexpression of AR co-activators and the ligand-independent activation of AR (7-9). As the development of PCa progresses, AR activity may evade regulation by ligand binding, resulting in tumor overgrowth (7). Following the activation of molecular pathways to restore AR signaling by PCa cells during the androgen-independent phase, no single therapeutic agent is able to eliminate PCa, resulting in poor patient prognosis (10). As AR is important in the development and progression of PCa, a strategy to downregulate the expression of AR and the inhibition of AR activity, in combination with anti-androgen therapy may prevent or delay the development of androgen-independent PCa (11-16).

Micro (mi)RNAs are short, endogenous, non-coding RNA molecules, which bind to the 3’-untranslated regions (UTRs) of target mRNAs, resulting in translational repression or message degradation (17-18). miRNAs are important in physiological cellular processes, including differentiation, proliferation and apoptosis (19-22), and have also been implicated in types of cancer (23,24). Several studies have demonstrated that miRNA (miR)-185 is able to suppress
tumor growth and progression in non-small cell lung cancer (25), ovarian, pediatric renal and breast cancer cell lines (26,28), and it has been suggested that miR-185 may be important in cell proliferation.

The present study aimed to investigate whether miR-185 affected the expression of AR, cell proliferation or apoptosis of PCa LNCaP cells. The results may provide evidence for the utilization of miRNAs as novel therapeutics to target AR in PCa.

Materials and methods

Cell culture. The human LNCaP-AD (androgen-dependent LNCaP) PCA cell line was obtained from American Type Culture Collection (ATCC; Manassas, VA, USA) and cultured in RPMI-1640 medium (HyClone, GE Healthcare Life Sciences, Little Chalfont, UK) supplemented with 10% charcoal-treated fetal bovine serum (FBS; Sigma-Aldrich, St. Louis, MO, USA), 2.05 mM L-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin and 10^4 mol/l R1881 (Sigma-Aldrich).

Androgen-independent LNCaP cells (LNCaP-AI) were generated from LNCaP-AD cells under androgen-depleted conditions. LNCAP-AI cells were produced from LNCaP-AD cells following continuous culture with phenol red-free RPMI-1640 medium (HyClone, GE Healthcare Life Sciences, Logan, UT, USA) supplemented with 10% charcoal-stripped fetal bovine serum (Serana, Bunbury, Australia) for 50 passages. The human PC-3 PCA cell line was obtained from ATCC and cultured in RPMI-1640 medium (HyClone) supplemented with 10% FBS (TBD Science, Tianjin, China), 2.05 mM L-glutamine, 100 U/ml penicillin and 100 µg/ml streptomycin. All the cell lines were maintained at 37°C in a humidified atmosphere containing 5% CO₂.

Plasmid construction. The wild-type (WT) 3'UTR of the human AR gene was amplified from human AR complementary (c)DNA by polymerase chain reaction (PCR) using a PCR kit purchased from Takara (Dalian, China), and the fragments produced by PCR were cut using MluI and HindIII (Thermo Fisher Scientific, Waltham, MA, USA) before being cloned into the 3' end of the luciferase reporter (Eclipse TE2000-U; Nikon Corp., Tokyo, Japan) at 350 nm excitation and 460 nm emission.

Cell transfection. The LNCaP cells were transfected with the miR-185 mimic (160 nM), normal control (NC) mimic (160 nM), miR-185 inhibitor (160 nM) or NC inhibitor (160 nM) at 40% confluency using siPORT NeoFX™ transfection agent (Ambion Life Technologies) at room temperature, according to the manufacturer's instructions. The pMIR-AR-3'UTRw, pMIR-AR-3'UTRm and pGL4-ARE plasmids and miR-185 were transfected into the cells at 90% confluency using FuGENE HD (Roche Diagnostics, Basel, Switzerland) at room temperature, according to the manufacturer's instructions.

Cell viability assay. The LNCaP-AD, LNCaP-AI and PC-3 cells were then determined 24, 48, 72 and 96 h after transfection using an MTT assay (Sangon Biotech Co., Ltd, Shanghai, China), according to the manufacturer's instructions. Three independent experiments were performed.

Nuclear staining with Hoechst 33342. The LNCaP-AD or LNCaP-AI cells were seeded into 24-well plates at 40% confluency and transfected with either the miR-185 mimic, miR-185 inhibitor, NC mimic or NC inhibitor using siPORTNeoFX™ transfection agent at room temperature. The cell viabilities were then determined 24, 48, 72 and 96 h after transfection using an MTT assay (Sangon Biotech Co., Ltd, Shanghai, China), according to the manufacturer's instructions. Images of the stained cells were captured under a fluorescent microscope (Eclipse TE2000-U; Nikon Corp., Tokyo, Japan) at 350 nm excitation and 460 nm emission.

Reverse transcription quantitative polymerase chain reaction (RT-qPCR). The LNCaP-AD or LNCaP-AI cells were seeded into 6-well plates and transfected with either the miR-185 mimic, miR-185 inhibitor, NC mimic or NC inhibitor using siPORTNeoFX™ transfection agent. The total RNA was extracted 72 h after transfection using TRIzol reagent (Invitrogen Life Technologies, Carlsbad, CA, USA).
First strand cDNA was synthesized from 10 ng total RNA using gene specific primers. RT-qPCR was performed using MonsterScript™ Reverse Transcriptionase (Epicentre, Illumina, Inc., Madison, WI, USA) and SYBR™ Green I nucleic acid gel stain (Molecular Probes Life Technologies, Carlsbad, CA, USA).

The primers used were as follows: miR-185, forward 5'-GGTGGAGAGAAGGCAGT-3' and reverse 5'-CAGTGGTTGCTGTGGAG-3'; U6 snRNA, forward 5'-CTCGAGCACACATATAAAT-3' and reverse 5'-CGCTCAGATTTCGGTTCAT-3'. U6 snRNA was used as a control gene for normalization. The relative quantity of miR-185 was determined using the 2^ΔΔCt method (29).

The primers used for AR were: Forward 5'-CTTCCCTCTCC ATCTAAACC-3' and reverse 5'-TCTAAAC TTCCCGTGGAATAA-3'; for PSA were: Forward 5'-AGGTGTTGC TAGACTTGTGGTGC-3' and reverse 5'-GGTGGAGGTCCCAGGGCTT-3'; and for GAPDH were: Forward 5'-GGGAAGCTGTGGGTGTCGAT-3' and reverse 5'-GAGTGTTGTGCTGTGGTA-3'. PCR conditions for AR were: initial denaturation at 95°C for 5 min; 35 cycles of 95°C for 10 sec, 59°C for 15 sec, 72°C for 20 sec and 82°C (fluorescence collection) for 5 sec. PCR conditions for PSA and GAPDH were: initial denaturation at 95°C for 5 min; 35 cycles of 95°C for 10 sec, 59°C for 15 sec, 72°C for 20 sec and 80°C (fluorescence collection) for 15 sec. GAPDH was used as a control for normalization, and the data were analyzed with Rotor-Gene Real-Time Analysis software 6.0 (Corbett Research, Mortlake, Australia) according to the standard curve.

Dual-luciferase assay. The LNCAp-AD cells were seeded in 24-well plates and transfected for 24 h with either the miR-185 mimic, miR-185 inhibitor, NC mimic or NC inhibitor using siPORT™ NeoFX™ transfection agent. Subsequently, the LNCAp-AD cells were co-transfected with either pMIR-AR-3'UTR (0.04 µg; Promega Corporation). Three independent experiments were performed in duplicates.

Western blot analysis. The LNCAp-AD or LNCAp-AI cells were seeded into 6-well plates and transfected with either the miR-185 mimic, miR-185 inhibitor, NC mimic or NC inhibitor using siPORT™ NeoFX™ transfection agent. The cells were harvested 48 and 72 h after transfection for protein extraction. The protein (20 µg) was resolved on an 10% SDS-PAGE gel and electrotransferred onto nitrocellulose membranes (Bio-Rad Laboratories, Inc., Hercules, CA, USA) using a DYCZ-40D (Beijing Liuyi Instrument Factory, Beijing, China). Following blocking with 5% milk in Tris-buffered saline with 0.05% Tween-20 (TBST) buffer (Sangon Biotech Co., Ltd) and washing with TBST buffer, the membrane was incubated with mouse monoclonal anti-human AR (1:1,000; BD Biosciences, Franklin Lakes, NJ, USA) or mouse anti-β-actin (1:1,000; Santa Cruz Biotechnology, Inc., Dallas, TX, USA) at 4°C for 12 h, followed by incubation with horseradish peroxidase-labeled goat anti-mouse polyclonal secondary antibody (1:2,000; Zhongshan Golden Bridge Biological Technology, Beijing, China) for 1 h at room temperature. The immunoreactive bands were visualized by enhanced chemiluminesence (Santa Cruz Biotechnology, Inc.). The protein expression levels of AR in each sample were determined by normalizing AR band intensity to that of β-actin.

Sequence Analysis. TargetScan predicts the biological targets of miRNAs. This is via searching a database for conserved 8mer and 7mer sites, which match seed regions of the miRNA being screened, nonconserved sites are also predicted. In addition sites with mismatches in the seed region that are compensated by conserved 3’ pairing are also identified. In mammals, the predictions are ranked based on the predicted targeting efficacy as calculated using context+ scores of the sites. The predictions are also ranked by their probability of conserved targeting. Conserved targeting has also been detected within open reading frames.

Results

miR-185 downregulates protein expression levels of AR in LNCaP cells. To investigate the potential effects of miR-185 on the regulation of AR expression, LNCaP-AD and LNCaP-AI cells were transfected with an miR-185 mimic or inhibitor for 48 and 72 h, and the expression levels of AR were detected using western blot and qPCR analyses. The results indicated that transfection with the miR-185 mimic markedly reduced the protein levels of AR, while transfection with the miR-185 inhibitor increased the protein levels of AR in the LNCaP-AD (Fig. 1A) and LNCaP-AI (Fig. 1B) cells.

The mature miR-185 levels in the miR-185-transfected LNCaP cells measured by qPCR. The mature levels of miR-185 in the LNCaP cells transfected with the miR-185 mimic were significantly higher compared with those in the NC mimic-treated cells (Fig.1C), which indicated that the synthetic miR-185 mimic was effectively transfected and matured in the LNCaP cells. However, differences in the mRNA expression levels of AR were not detected in all types of transfected LNCaP cells (Fig. 1D). The results demonstrated that miR-185 reduced the protein levels of AR by inducing translational repression, but not AR mRNA degradation.

miR-185 interacts with the putative target site in the AR-3' UTR. Sequence analysis, using the TargetScan tool (version 5.2; http://www.targetscan.org/vert_50/), indicated that a putative binding site for miR-185 was located at 173-179 bp of the AR-3' UTR. To assess whether miR-185 interacted with this predicted binding site in the AR-3' UTR to reduce the expression of AR, wild-type 3'UTR (3'UTRw) and the 3'UTR of the AR gene, containing deletion mutations (3'UTRm), were cloned and inserted downstream of the luciferase gene in a reporter plasmid (pMIR-REPORT Luciferase), and named pMIR-AR-3'UTRw and pMIR-AR-3'UTRm. Each of these constructs were cotransfected into the LNCaP-AD cells with either the miR-185 mimic, miR-185 inhibitor, NC mimic or
Figure 1. miR-185 targets the expression of AR in LNCaP cells. LNCaP-AD and LNCaP-AI cells were transfected with 160 nM miR-185 mimic, miR-185 inhibitor, NC mimic or NC inhibitor for 48 and 72 h. Western blot analysis was performed to detect the effects of miR-185 on the protein expression of AR in the (A) LNCaP-AD and (B) LNCaP-AI cells. β-actin was used as a loading control. (C) Expression of mature miR-185 in the miR-185-transfected LNCaP cells. U6 small nuclear RNA or GAPDH was used as the internal control for qPCR analysis. qPCR was performed to evaluate the effects of miR-185 on the mRNA expression levels of AR 72 h after transfection in the (D) LNCaP-AD and LNCaP-AI cells. Values are expressed as the mean ± standard deviation. Experiments were repeated more than three times. *P<0.05. miR-185, microRNA-185; AR, androgen receptor; AD, androgen-dependent; AI, androgen independent; NC, normal control; qPCR, quantitative polymerase chain reaction.

Figure 2. miR-185 directly targets the AR-3'UTR. (A) Schematic representation of AR mRNA, indicating the positions and sequences of the predicted miR-185 binding sites located in the AR-3'UTR. (B) LNCaP cells were transfected with 160 nM miR-185 mimic, miR-185 inhibitor, NC mimic or NC inhibitor for 48 h, and subsequently cotransfected with pMIR-AR-3'UTRw or pMIR-AR-3'UTRm for another 48 h. Luciferase activity was detected using a Dual Luciferase Assay system and was plotted as the ratio of firefly (M1) to Renilla (M2) luciferase activity (M1/M2). Data are expressed as the mean ± standard deviation of six individual values. Experiments were repeated more than three times. *P<0.05. miR-185, microRNA-185; AR, androgen receptor; 3'UTR, 3' untranslated region; CDS, coding sequence; NC, normal control; pMIR-REPORT, luciferase reporter gene plasmid; pMIR-AR-3'UTRw, plasmid containing wild-type 3'UTR of human AR gene; pMIR-AR-3'UTRm, plasmid containing mutant 3'UTR of human AR gene.
NC inhibitor. Luciferase activity was measured 48 h after transfection. The results (Fig. 2) indicated that transfection with the miR-185 mimic markedly reduced luciferase activity, while the miR-185 inhibitor enhanced luciferase activity of the pMIR-AR-3'UTRw. However, neither the miR-185 mimic nor miR-185 inhibitor altered the luciferase activities of the pMIR-AR-3'UTRm or pMIR-Report plasmids. Therefore, these data indicated that the predicted target site in the AR

Figure 3. miR-185 impairs the interaction between AR and ARE. (A) LNCaP-AD and (B) LNCaP-AI cells were transfected with miR-185 mimic, miR-185 inhibitor, NC mimic or NC inhibitor for 48 h, and subsequently cotransfected with the pGL4-ARE or pGL4 vector for another 48 h. Luciferase activity was evaluated using a Dual Luciferase Assay system and was plotted as the ratio of firefly (M1) to Renilla (M2) luciferase activity (M1/M2). Data are expressed as the mean ± standard deviation of six individual values. Experiments were repeated more than three times. *P<0.05. AR, androgen receptor; ARE, androgen response element; AD, androgen dependent; AI, androgen independent; miR, microRNA; NC, normal control; pGL4-ARE, pGL4 ARE reporter plasmid; pGL4 vector, control plasmid.

Figure 4. miR-185 downregulates the mRNA expression of PSA. LNCaP-AD and LNCaP-AI cells were transfected with miR-185 mimic, miR-185 inhibitor, NC mimic or NC inhibitor, and the cells were harvested 72 h after transfection. Total RNA was extracted and a quantitative polymerase chain reaction was performed to detect the effects of miR-185 on the mRNA expression of PSA. GAPDH was used as a control for normalization, and the relative mRNA expression of PSA was relative to that of GAPDH. Data are expressed as the mean ± standard deviation of three individual values. Experiments were repeated more than three times. *P<0.05. miR, microRNA; PSA, prostate specific antigen; AD, androgen dependent; AI, androgen independent; NC, normal control.

Figure 5. miR-185 inhibits the growth of LNCaP cells. (A) LNCaP-AD, (B) LNCaP-AI and (C) PC-3 cells were transfected with miR-185 mimic, miR-185 inhibitor, NC mimic or NC inhibitor, and cell viability was detected 24, 48, 72 and 96 h after transfection using an MTT assay. Data are expressed as the mean ± standard deviation of six individual values. miR, microRNA; AD, androgen dependent; AI, androgen independent; NC, normal control.
3'UTR was a specific functional binding site for miR-185, and that AR was a direct target of miR-185.

**miR-185 impairs the interaction of AR with ARE.** AR is a ligand-activated transcription factor, which induces the transcription of genes exhibiting ARE in their regulatory regions (3). In order to examine whether the downregulation of AR by miR-185 impaired the interaction between AR and ARE, an ARE sequence was synthesized and inserted upstream of the TATA box in pGL4.23 [luc2/minp] plasmids, named pGL4-ARE. pGL4-ARE was cotransfected into the LNCaP-AD and LNCaP-AI cells with either the miR-185 mimic or inhibitor. Luciferase activity was measured 72 h after transfection. The results indicated that transfection with the miR-185 mimic reduced luciferase activity, while miR-185 inhibitor increased the luciferase activity of pGL4-ARE in LNCaP-AD (Fig. 3A) and LNCaP-AI (Fig. 3B) cells, demonstrating that downregulation of AR by miR-185 may impair the interaction between AR and ARE.

**miR-185 down-regulates the expression of AR target gene prostate specific antigen (PSA).** PSA is a prostate specific gene, regulated by AR. In the present study, the effects of AR downregulation by miR-185 on the expression of PSA mRNA in LNCaP cells was evaluated. The results of PCR analysis indicated that the expression of PSA mRNA in LNCaP-AD and LNCaP-AI cells transfected with miR-185 mimic was reduced compared with that in cells transfected with the NC mimic, while the mRNA expression of PSA in the miR-185 inhibitor-transfected LNCaP cells was increased compared with the NC inhibitor-transfected cells (Fig. 4).

**miR-185 inhibits the proliferation of LNCaP cells.** To investigate the potential of miR-185 to inhibit the growth of LNCaP cells by downregulation the expression of AR, the LNCaP-AD, LNCaP-AI and PC-3 cells were transfected with either the miR-185 mimic or inhibitor, and the numbers of viable cells were measured 24, 48, 72 and 96 h after transfection using an MTT assay. The results indicated that the miR-185 mimic reduced, while the miR-185 inhibitor increased the numbers of viable LNCaP-AD (Fig. 5A) and LNCaP-AI (Fig. 5B) cells. The same transfection procedure was performed in PC-3 cells, not expressing AR, in which the miR-185 mimic or inhibitor had no significant effects on cell proliferation (Fig. 5C). These data demonstrated that the effect of miR-185 on cell growth was associated with downregulation of AR in the LNCaP cells.

**miR-185 induces the apoptosis of LNCaP cells.** The LNCaP-AD and LNCaP-AI cells were transfected with either miR-185 mimic or inhibitor for 48, 72 and 96 h. Apoptosis was assessed using Hoechst 33258 staining. As shown in Fig. 6, following transfection with miR-185 mimic, significantly more LNCaP-AD and LNCaP-AI cells exhibited chromatin condensation and marginalization compared with the NC mimic-transfected cells. By contrast, transfection with the miR-185 inhibitor resulted in a decreased number of LNCaP-AD and LNCaP-AI cells with nucleic chromatin condensation compared with the cells transfected with the NC mimic.
inhibitor. These data demonstrated that the miR-185-mediated downregulation of AR induced apoptosis of the LNCaP cells.

Discussion

In the past few years, numerous studies have demonstrated that miRNAs are important in the developmental process of cancer (30-33). They may function as oncogenes or tumor suppressor genes (34) in tumorigenesis, and regulate multiple cellular processes involved in the progression of cancer. Due to each miRNA regulating numerous potential targets, identification of the true target genes that are involved in cancer cell behaviors is important for understanding the mechanisms underlying the functional contributions of miRNAs in tumor development and progression.

Several studies have demonstrated that miR-185 functions as a tumor suppressor, which is able to suppress tumor growth and progression in non-small lung carcinoma, ovarian, pediatric renal and breast cancer cell lines (25-28). It has also been suggested that miR-185 may be important in cell proliferation (25-28). However, the mechanisms by which miR-185 inhibits cell proliferation in different cancer cells vary. miR-185 suppresses the growth of the human non-small cell lung cancer cell lines and induces cell cycle arrest by suppressing the mRNA expression of cell cycle regulating genes, including CDK6 and AKT1 (25). Additionally, miR-185 suppresses tumor growth and progression by targeting the Six1 oncogene in multiple types of human cancer, including pediatric renal tumors, aggressive ovarian cancer and breast cancer (26). In colorectal cancer cells, miR-185 directly regulates the expression of RhoA and Cdc42 and their associated functions, including proliferation and invasion (35). In contrast to its role in tumor suppression, miR-185 was also demonstrated to induce tumor growth and progression in clear cell renal cell carcinoma (36). The expression of miR-185 was inversely correlated with its putative target PTPN13, which suppressed cell growth and induced apoptosis as a tumor suppressor gene by inhibiting phosphoinositide 3-kinase (PI3K)/AKT signaling (37). These findings suggest that miR-185 may target differing genes in various cell types, which contribute to different biological processes.

The results of the present study confirmed miR-185 as a tumor suppressor gene, which exerted its effect through modulating the expression of AR, inhibiting cell proliferation and enhancing apoptosis of the LNCaP cells. Computational analysis revealed a potential binding site for miR-185 in the 3′UTR of AR. Deletion mutagenesis and a luciferase-based reporter gene assay demonstrated that the predicted miR-185 target sites in the AR 3′UTR were functional. Furthermore, data indicated that miR-185 effectively downregulated the protein expression of AR, impaired the interaction of AR with ARE and downregulated the expression of the AR target gene, PSA.

AR is a ligand-dependent transcription factor belonging to the nuclear hormone receptor superfamily (38). AR functions as a ligand-dependent transcription factor by binding to ARES in the regulatory regions of specific androgen-regulated target genes (39). The development of PCa and growth of prostate tissue depend on androgen signaling via the AR (14,40); therefore, downregulation of the expression levels of AR and androgens is a basic therapeutic strategy in preventing the development of PCa.

Based on the significance of AR for the androgen-dependent and androgen-independent growth of PCa cells (6), understanding the association between miR-185 and AR is important for investigation of prostate carcinogenesis and therapeutics. An increasing number of studies have focused on improving PCa treatment by developing more effective strategies for silencing AR (41-44). A combinatorial approach, involving the simultaneous targeting of multiple pathways, is an accepted approach in the development of PCa therapy and the development of numerous therapeutic options, which can be evaluated in combinatorial therapy settings is being emphasized at present (10). Therefore, the results of the present study, revealing that miR-185 mediated the repression of AR expression and activity, are of significance.

In conclusion, the present study demonstrated that miR-185 directly targeted the AR-3′UTR, to inhibit the expression of AR, and acted as a tumor suppressor in the PCa cells. miR-185 is, therefore, a potential negative modulator of AR-mediated signaling with potential for use in PCa therapeutics.

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