MiR-155 Negatively Regulates c-Jun Expression at the Post-transcriptional Level in Human Dermal Fibroblasts in vitro: Implications in UVA Irradiation-induced Photoaging

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Key Words
Ultraviolet A • c-Jun • miR-155 • Photoaging • Dermal fibroblasts

Abstract
Objective: C-Jun plays a critical role in ultraviolet A (UVA) irradiation-induced photoaging. The exact mechanisms by which UVA irradiation up-regulates c-Jun expression in human dermal fibroblasts (HDFs) are still not completely understood. We undertook this study to investigate whether microRNA-155 (miR-155) directly regulates the expression of c-Jun in HDFs in vitro. Methods: Expression of c-Jun mRNA and protein and miR-155 in UVA-irradiated HDFs were detected using quantitative real-time RT-PCR and Western blotting. Luciferase reporter assays were performed to examine whether a miR-155 binding site in the 3’-untranslated region (3’-UTR) of the c-Jun gene is responsible for miR-155-mediated c-Jun regulation in HEK293A cells, and expression of c-Jun mRNA and protein in UVA non-exposed and exposed HDFs transfected with a miR-155 mimic or a miR-155 inhibitor was detected by quantitative real-time RT-PCR and Western blotting. Results: Expression of miR-155 was significantly up-regulated in UVA-irradiated HDFs. Luciferase reporter assays indicated that c-Jun is a direct target of miR-155 in HEK293A cells. In both UVA non-exposed and exposed HDFs, miR-155 mimic decreased c-Jun protein levels, while miR-155 inhibitor increased c-Jun protein levels, but both had no effect on c-Jun mRNA expression, which suggest that miR-155-induced c-Jun inhibition occurs at the post-transcriptional level. Conclusions: Our results demonstrate that miR-155 directly controls c-Jun expression in HDFs at the post-transcriptional level and might function as a protective miRNA in HDFs.

Introduction
The primary environmental factor that causes premature skin aging (photoaging) is solar ultraviolet (UV) radiation [1]. UV radiation is divided into three wavelength ranges: UVA, UVB and UVC. UVA covers the wavelength range 320–400 nm and is the major component of daily UV radiation [2]. Although not as
energetic as UVB and UVC, UVA can penetrate more deeply into skin and contribute to photoaging by invoking a complex sequence of specific molecular responses in both the epidermis and dermis [3]. Actually, it has been found that human dermal fibroblasts (HDFs) were more susceptible to UVA exposure than epidermal keratinocytes in a reconstructed human skin model [2]. In this regard, it is of interest to investigate the mechanisms underlying the role of HDFs in the pathogenesis of UVA-induced photoaging.

Photoaging is caused in part by damage to skin connective tissue, which is made up primarily of collagen fibers [1]. UVA radiation induces connective tissue damage not only by activating signaling pathways within the epidermis to induce the degradation of collagen fibers but also by directly interfering with collagen gene expression in HDFs [1, 3, 4]. Several studies have found that c-Jun is involved in UVA radiation-induced abnormal collagen gene expression in HDFs [5-8]. UVA irradiation rapidly induces the activator protein-1 (AP-1) in human skin in vivo [7, 9]. By binding and sequestering factors that are required for procollagen transcription, AP-1 interferes with collagen gene expression in HDFs [1]. Since AP-1 is a transcription factor composed of Jun and Fos proteins, the induction of AP-1 may result from increased c-Jun and c-Fos expression. At present, although there are still conflicting results as to whether c-Fos expression is up-regulated in HDFs following UVA radiation, UVA radiation indeed activates c-Jun in cultured HDFs [6, 7, 10]. However, the exact mechanisms by which UVA up-regulates c-Jun expression are still not completely understood.

MicroRNAs (miRNAs) are a class of highly evolutionarily conserved small non-coding RNA molecules (19–24 nucleotides) that modulate gene expression at a post-transcriptional level [11]. They function to degrade mRNAs or inhibit their translation by recognizing target sites, most commonly found in the 3′-untranslated regions (UTRs), through specific but imperfect base pairing [12, 13]. MiR-155 is a typical multifunctional miRNA that plays a crucial role in various physiological and pathological processes [14, 15]. A recent study shows that miR-155 is lowly expressed in dermal fibroblasts but highly expressed in atopic dermatitis, implicating the role of miR-155 in the pathogenesis of dermal diseases [16]. Currently, it remains unclear whether miR-155 is involved in UVA-induced photoaging. Identification of new miR-155 targets in HDFs may provide new clues to the molecular pathogenesis of this process.

In a preliminary investigation of possible target genes of miR-155, we identified one site in the c-Jun 3′-UTR which has high match target sequence for the miR-155 seed region. We therefore hypothesized that miR-155 might directly regulate the expression of c-Jun at the posttranscriptional level. To test this hypothesis, we performed luciferase assays to validate if miR-155 can efficiently target the c-Jun 3′-UTR. Moreover, we evaluated the implications of miR-155-regulated c-Jun expression in UVA-induced photoaging. Our data provide new insights into the post-transcriptional regulation of c-Jun expression in HDFs.

### Materials and Methods

#### Cell culture

Human embryonic kidney 293A (HEK293A) cells were obtained from the American Type Culture Collection (ATCC). Primary HDFs were isolated from the foreskin of healthy adult donors. Both HEK293A cells and HDFs were cultured in dishes containing Dulbecco’s modified Eagle’s media (DMEM, Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin at 37°C in a humidified atmosphere containing 5% CO₂. Cultured HDFs at passages 3–6 and HEK293A at passages 5–10 were used for subsequent experiments.

#### UVA irradiation

Non-transfected HDFs were plated at 5 × 10⁵ cells per well in 9.08 cm² dishes, while transfected HDFs (for details on transfection procedure see below) were seeded in six-well plates at 1 × 10⁵ cells per well. When cells were grown to 80–90% confluence or transfected for 48 h, the medium was discarded and UVA irradiation was performed with a UV 181 AL unit (Waldman, Villingen-Schwenningen, Germany) emitting UVA exclusively in the wavelength range of 320–400 nm with a peak at 365 nm. The power density at the level of the cells was calculated to be 20 mW/cm². The average irradiation time for 2.5 J/cm² was calculated at 2 min 5 s and 6 min 15 s for 7.5 J/cm². After irradiation, the cells were cultured in the complete culture medium again for various time periods.

#### Cell viability assay

The 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was used to assess cell viability. Cells that had been grown in a 96-well plate to about 80–90% confluence were exposed to different doses of UVA irradiation and incubated in the complete medium for 2 h. Then, 100 µL of MTT solution (0.5 mg/mL) was added to each well, and the cells were incubated at 37°C for 4 hours. The reaction was stopped by adding 150 µL of 100% dimethyl sulfoxide to dissolve the formazan crystals. The absorbance of the solution in each well was measured at 570 nm using an enzyme-linked immunosorbent assay reader (BioTek ELX800, Winooski, VT, USA).
Construction of a luciferase reporter vector

A 482-bp fragment of the 3'UTR of the c-Jun gene, which contains the putative miR-155 binding site, was amplified by PCR by using human genomic DNA as a template. Table 1 shows the sequences of the primers used. The resulting PCR product was double digested with SacI and Hind III and cloned into the pMIR-Report luciferase vector (Ambion, Austin, TX, USA), yielding the recombinant vector pMIR-Report-c-Jun. The identity of the recombinant plasmid was verified by direct sequencing.

Oligonucleotide synthesis and transfection

A miR-155 mimic and a negative control RNA oligonucleotide (miR-control) were obtained commercially (Genepharma, Shanghai, China). To inhibit the function of miR-155, a miR-155 inhibitor (anti-miR-155) was used, along with a negative control (anti-miR-control). The sequences of these RNA oligonucleotides were as follows: miR-155 mimic: sense, 5'-UUA AUG CUA AUC GUG AUA GGG GU-3'; antisense, 5'-CCC UAU CAC GAU UAG CAU UAA UU-3'; miR-control: sense, 5'-UUC UCC GAA CGU GUC ACG UTT-3'; antisense, 5'-ACG UGA CAC GUU CGG AGA ATT-3'; anti-miR-155: 5'-ACC CCU AUC ACG AUU AGC AGG CCA-3'; and anti-miR-control: 5'-CAG UAC UCU GUA GUA GUU CAA-3'. For transfection, 2 × 10^4 HEK293A cells or 1 × 10^5 HDFs were seeded into each well of 48- or 6-well plates and incubated overnight. Transfection of RNA oligonucleotides was carried out using Lipofectamine 2000 according to the manufacturer's instructions (Invitrogen). The miR-155 mimic and miR-control were transfected at a final concentration of 10, 20 or 80 nM while the anti-miR-155 and anti-miR-control at a final concentration of 80 or 200 nM. Cells transfected with empty liposomes were also used as controls (liposome control).

Luciferase reporter assay

After HEK 293A cells (2 × 10^4) were seeded into each well of 48-well plates, the cells in each well were transfected with 500 ng of pMIR-Report-c-Jun plasmid together with 50 ng of pRL-TK (a construct containing the Renilla luciferase gene; Promega, Madison, WI, USA) and either 10 nM miR-155 mimic or miR-control or 200 nM anti-miR-155 or anti-miR-control. Both firefly and Renilla luciferase activities were measured 24 hours after transfection using the Dual-Luciferase Reporter Assay System (Promega, Madison, WI, USA). Firefly luciferase activity was normalized to renilla luciferase activity, and relative luciferase activity (RLA) was calculated using the following formula: RLA = firefly luciferase activity/renilla luciferase activity.

Quantitative real-time RT-PCR analysis

Total RNA was extracted from HDFs 2 hours after irradiation with different doses or 48 hours after transfection using Trizol Reagent (Invitrogen) following the manufacturer’s protocol. First-strand cDNA was synthesized using the PrimeScript RT reagent Kit (Fermentas Life Sciences, Burlington, ON, Canada). The expression of c-Jun mRNA was then detected by quantitative real-time polymerase chain reaction (RT-PCR), while the expression of mature miR-155 was assayed using stem-loop RT followed by quantitative real-time PCR analysis. The primers used are listed in Table 1. Amplification was carried out using the iQ5 real-time PCR detection system (Bio-Rad, Hercules, CA, USA). The expression levels of mRNA and miRNA were measured using the 2^-ΔΔCt method and normalized to those of β-actin and U6, respectively.

Western blotting

Whole cell extracts of HDFs were prepared 2 hours after irradiation with different doses of UVA or 48 hours after transfection. Equal amounts of protein were separated on 10% sodium dodecyl sulfate–polyacrylamide gels and transferred onto nitrocellulose membranes (Millipore Biotechnology, Bedford, MA, USA). The membranes were probed at 4°C overnight with primary antibodies against c-Jun (dilution, 1:500; Cell Signaling Technology, Beverly, MA, USA) or β-actin (dilution, 1:750; Cell Signaling Technology). The membranes were then incubated with a secondary antibody (dilution, 1:5000; Biosynthesis Biotechnology, Beijing, China) for 1 h at room temperature, and the bands were visualized using an

<table>
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<th>Gene</th>
<th>Primer sequence (5’–3’)</th>
<th>Tm (°C)</th>
<th>Cycles</th>
<th>Size (bp)</th>
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<tr>
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<tr>
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<td>F: CTC GCT TCG GCA GCA CA</td>
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</tbody>
</table>

Table 1. Sequences of the primers used in this study. Tm: melting temperature; F: forward; R: reverse.
enhanced chemiluminescence detection kit (Pierce Biotechnology, Rockford, IL, USA). Immune-reactive bands were quantified using Quantity One software (Bio-Rad). β-actin was used as an internal control to normalize the expression of c-Jun protein.

Statistical analysis
Multiple comparisons were determined using one-way analysis of variance with Turkey’s post hoc test. P values less than 0.05 were considered statistically significant.

Results

Effect of UVA irradiation on cell viability in HDFs
To examine the effect of UVA irradiation on cell viability, MTT assays were performed to count viable cells 2 hours after irradiation. Compared to mock irradiated cells, the viability of cells irradiated with 10.0 J/cm² UVA decreased significantly (P < 0.01), but that of cells irradiated with UVA at doses of 7.5 J/cm² and below showed no significant decrease (P > 0.05 for all, Fig. 1). Thus, the dose of UVA used in subsequent experiments was 7.5 J/cm² or below.

UVA irradiation up-regulates the mRNA and protein expression of c-Jun in HDFs
The expression of c-Jun mRNA and protein in HDFs was determined by quantitative real-time RT-PCR and Western blotting 2 h after UVA irradiation. Compared to mock irradiation, UV irradiation up-regulated the expression of c-Jun mRNA in a dose-dependent manner over the dose range 2.5-7.5 J/cm² (P < 0.01 for all, Fig. 2A). Similar results were also obtained in the Western blot analysis of the expression of c-Jun protein in UVA-irradiated HDFs (P < 0.01 for all, Fig. 2B, C).
UVA irradiation down-regulates the expression of miR-155 in HDFs

Determination of miR-155 expression in HDFs 2 h after UVA irradiation was performed using quantitative real-time RT-PCR analysis. Compared to mock irradiated cells, miR-155 expression in HDFs irradiated with UVA at doses of 7.5 J/cm² and below were significantly lower ($P < 0.05$ for all). Unexpectedly, the decrease in miR-155 expression was more significant in cells irradiated with 2.5 J/cm² UVA than in those irradiated with UVA at doses of 5.0 and 7.5 J/cm² (Fig. 3).

c-Jun is a novel target gene of miR-155 in HEK293A cells

By retrieving and comparing the sequences of c-Jun 3'UTR and mature has-miR-155, in the TargetScan database (http://www.targetscan.org/index.html) and miRbase (http://microrna.sanger.ac.uk), we found that c-Jun 3'UTR contains one site (nt 331-337) which has high match target sequence for the miR-155 seed region (Fig. 4A), though the TargetScan program did not predict a miR-155 target site in the c-Jun 3'UTR. To validate whether or not the c-Jun gene is a direct target of miR-155, luciferase reporter assays were performed in HEK293A cells. Cells transfected with the miR-155 mimic showed significantly lower ($P < 0.01$) luciferase activity compared to those transfected with the miR-control or empty liposomes (Fig. 4B). In contrast, cells transfecte
transfected with the anti-miR-155 showed significantly higher ($P < 0.01$) luciferase activity compared to those transfected with the anti-miR-control or empty liposomes (Fig. 4C). There was no significant difference in luciferase activity between the two control groups ($P > 0.05$, Fig. 4B, C). Taken together, these results suggest that c-Jun is a novel target gene of miR-155.

**MiR-155 down-regulates the expression of c-Jun protein in HDFs**

To validate whether c-Jun expression is regulated by miR-155, quantitative real-time RT-PCR and Western blotting were performed in HDFs. Compared to transfection of miR-control or empty liposomes, miR-155 mimic transfection significantly repressed c-Jun protein.
Fig. 6. miR-155 down-regulates c-Jun protein expression in UVA-exposed HDFs. Cells were transfected with empty liposomes, miR-control, miR-155 mimic (80 nM), anti-miR-control, or anti-miR-155 (80 nM). Forty-eight hours after transfection, cells were exposed with 2.5 J/cm² UVA. Two hours after irradiation, quantitative real-time RT-PCR or Western blotting was performed. (A) Quantitative analysis of expression of c-Jun mRNA in cells transfected with empty liposomes, miR-control, or miR-155 mimic. (B) Representative images of the Western blots used to quantify c-Jun protein expression in cells transfected with empty liposomes, miR-control, or miR-155 mimic. (C) Densitometric analysis of expression of c-Jun protein in cells transfected with empty liposomes, miR-control, or miR-155 mimic. (D) Quantitative analysis of expression of c-Jun mRNA in cells transfected with empty liposomes, anti-miR-control, or anti-miR-155. (E) Representative images of the Western blots used to quantify c-Jun protein expression in cells transfected with empty liposomes, anti-miR-control, or anti-miR-155. (F) Densitometric analysis of expression of c-Jun protein in cells transfected with empty liposomes, anti-miR-control, or anti-miR-155. Data shown are the mean ± SD (n = 3).

expression (Ps < 0.01 for both, Fig. 5B, C). In contrast, anti-miR-155 transfection significantly increased c-Jun protein expression (Ps < 0.01 for both, Fig. 5E, F). However, transfection with either miR-155 or anti-miR-155 had no significant impact (Ps > 0.05 for all) on the expression of c-Jun mRNA compared to control cells (Fig. 5A, D). There were no significant differences in either c-Jun mRNA or protein expression between the two control groups (Ps > 0.05 for both, Fig. 5). Collectively, these results suggest that miR-155 can negatively control
MiR-155 down-regulates the expression of c-Jun protein in UVA-exposed HDFs

To examine whether c-Jun expression is regulated by miR-155 in UVA-exposed HDFs, quantitative real-time RT-PCR and Western blotting were performed in HDFs exposed to UVA. Compared to transfection of miR-control or empty liposomes, miR-155 mimic transfection significantly repressed c-Jun protein expression (Ps < 0.01 for both, Fig. 6B, C). In contrast, anti-miR-155 transfection significantly increased c-Jun protein expression (Ps < 0.01 for both, Fig. 6E, F). However, transfection with either miR-155 or anti-miR-155 had no significant impact (Ps > 0.05 for all) on the expression of c-Jun mRNA compared to control cells (Fig. 6A, D). There were no significant differences in either c-Jun mRNA or protein expression between the two control groups (Ps > 0.05 for both, Fig. 6). Taken together, these results suggest that miR-155 can negatively control c-Jun expression at the post-transcriptional level in UVA-exposed HDFs.

Discussion

We demonstrate here for the first time that miR-155 could directly control the protein expression of c-Jun in both UVA non-exposed and exposed HDFs. UVA irradiation down-regulates the expression of miR-155 and up-regulates the mRNA and protein expression of c-Jun in HDFs. Since c-Jun is involved in UVA radiation-induced abnormal collagen gene expression in HDFs [5-8], our data suggest that down-regulated miR-155 expression may partially mediate UVA-induced photoaging by repressing c-Jun protein expression.

MiRNAs regulate the gene expression by incorporating into a RNA-induced silencing complex (RISC) to guide it to the appropriate mRNAs [17]. It is clear that miRNA-mediated repression can be manifested by inhibition of translation and/or by increased mRNA degradation [13]. In the present study, we found that transfection with miR-155 or anti-miR-155 significantly altered c-Jun protein expression but had no significant impact on c-Jun mRNA expression in both UVA non-exposed and exposed HDFs, suggesting that miR-155 regulates c-Jun expression by inhibiting its translation.

UV radiation can activate growth factor receptors and induce the activation of protein kinase cascades to up-regulate the expression of c-Jun and c-Fos, which form an active AP-1 complex [1, 3]. AP-1 not only stimulates transcription of matrix metalloproteinase (MMP)-1, MMP-3, and MMP-9 to induce the degradation of extracellular matrix proteins, but also inhibits the expression of type I procollagen and type III procollagen in HDFs [1]. The transcriptional activity of AP-1 is dependent on both the degree of phosphorylation and abundance of c-Jun and c-Fos [18]. Our finding that miR-155 could directly down-regulate the protein expression of c-Jun in both UVA non-exposed and exposed HDFs provides a novel mechanism for translation-dependent post-transcriptional regulation of c-Jun expression. Consistent with our finding, Polak et al. [19] found that the cytoskeletal network controls c-Jun translation in a UTR-dependent manner in different cell types. Interestingly, several previous studies [20-22] have demonstrated that the c-fos 3’-UTR also contains a miR-155 seed and that miR-155 could silence c-Fos expression in dendritic cells. Thus, it appears reasonable to speculate that miR-155 can directly down-regulate the expression of c-Jun in HDFs and that this mechanism may also be involved in UVA irradiation-induced photoaging. Further experiments are needed to prove this hypothesis.

Our results showed that UVA irradiation also up-regulated the expression of c-Jun mRNA in HDFs, suggesting that c-Jun can be regulated at both the transcriptional and post-transcriptional levels in response to UVA. Actually, a previous study has shown that c-Jun mRNA expression was increased following exposure to UVA in HDFs [6]. Our finding that the increase in mRNA expression levels of c-Jun in response to UVA does not match well with the protein levels provides further support for the presence of other mechanisms to regulate c-Jun expression. Interestingly, we also found that, with the increase in the dose of UVA irradiation over the dose range of interest, the decrease in the expression of miR-155 was not parallel to the increase in c-Jun protein expression and became less significant. At present, the mechanism behind UVA irradiation-induced miR-155 down-regulation is unknown. However, the increase in miR-155 expression with increasing UVA doses may be partially attributed to a negative feedback mechanism. In previous studies, miRNAs have been implicated in the cellular stress response [23], and it has been demonstrated that miR-155 expression can be up-regulated by stimulation with lipopolysaccharide in macrophages through the JNK-AP-1-dependent pathway [24, 25]. Since UVA irradiation can negatively regulate miR-155 expression and up-regulate c-Jun (and possibly c-Fos) protein expression in HDFs, activation of the AP1/JNK

Cell Physiol Biochem 2012;29:331-340

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pathway may in turn induce miR-155 up-regulation. As higher doses of UVA were associated with higher levels of c-Jun expression, increased miR-155 expression was induced in HDFs exposed to higher doses of UVA. We surmise that such negative feedback loop may represent a self-defense mechanism of HDFs that occurs in response to UVA irradiation. This surmise, together our observation that miR-155 can negatively regulate the expression of c-Jun, a component of the heterodimeric AP-1 transcription factor that can interfere with collagen synthesis and mediate UVA radiation-induced connective tissue damage, led us to speculate that miR-155 may function as a protective miRNA in HDFs.

MiR-155 is a typical multifunctional miRNA that has been implicated in inflammation, immunity and carcinogenesis [26-28]. However, there have been very few studies evaluating its role in human dermis. It is now known that miR-155 is lowly expressed in HDFs and highly expressed in atopic dermatitis [16]. Ectopic expression of miR-155 in human fibroblasts induces modulations of a large set of genes important for cell functions [29]. In this study, we provide the first evidence that miR-155 may mediate UVA radiation-induced connective tissue damage. Consistent with our finding, a previous study shows that enforced expression of miR-155 could repress the levels of MMP-1 and MMP-3 in rheumatoid arthritis synovial fibroblasts [26].

Several previous studies demonstrated that treatment with all-trans retinoic acid (tRA) reverses c-Jun-dependent inhibition of cutaneous procollagen transcription following UV irradiation in human skin [7, 8]. However, tRA does not inhibit UV induction of c-Jun mRNA, but rather blocks accumulation of c-Jun protein [7], indicating that tRA may inhibit c-Jun protein synthesis and/or stimulate c-Jun protein degradation. Intriguingly, in the skin of patients with atopic dermatitis, intrinsic retinoid transport, synthesis, concentrations and signaling are strongly changed and miR-155 is highly expressed [16, 30]. These findings, together with our observation that miR-155 can directly regulate c-Jun expression in HDFs, suggesting that this novel mechanism for post-transcriptional regulation of c-Jun expression may be involved in tRA signaling in the skin.

In the present study, we identified a miR-155 binding site in the c-Jun 3’-UTR. Indeed, the TargetScan program did not predict a miR-155 target site in the c-Jun 3’-UTR. However, luciferase reporter assay proved that this site is responsible for miR-155-mediated c-Jun regulation. Our results highlight the deficiency of predicting candidate miRNA targets through bioinformatic approaches. Because of miRNA targets generally are only partially complementary to the mature miRNA sequence in animal cells [31, 32], the miRNA binding sites predicted using bioinformatic tools may be false-negatives or false-positives.

In conclusion, our study presents evidence that miR-155 directly controls c-Jun expression in HDFs at the post-transcriptional level. To the best of our knowledge, this is the first work demonstrating that c-Jun is a direct target gene of miR-155. Our findings provide new insights into the pathogenesis of UVA-induced photoaging. MiR-155 might function as a protective miRNA in HDFs and serve as a potential target for the treatment of photoaging.

Acknowledgements

This work was supported by a grant (No. 30600764) from the National Natural Science Foundation of China.

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