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MicroRNA-155 promotes atherosclerosis-signaling pathway through targeting gene/SOCS1 and IRAKM

Jack S Teng, Lin T Andrews, Emily H Faraoni, Michele D Arranz, Martin K Heymans, Mark L Möller[1*]

Abstract

MiR-155 plays a role in the regulates various aspects of innate and adaptive immune response, physiological and pathological processes. Exogenous molecular control in vivo of miR-155 expression may inhibit malignant growth, viral infections, and attenuate the progression of cardiovascular diseases. Up-regulation of proinflammatory cytokines plays a central role in atherosclerosis. In this study, we investigated the role of miR-155 in regulating proinflammatory response in atherosclerosis. Hyperlipidemic C57BL/6 male mice model were fed with atherogenic-diet for 12-weeks. MiR-155 positively regulates proinflammatory cytokines and we found increased TNFα, IL-1b, IL-6 mRNA and NF-kB in hyperlipidemic mice. Furthermore, increased miR-155 levels are correlated with proinflammatory cytokine expression in hyperlipidemic mice. To understand the mechanism by which miR-155 regulates proinflammatory cytokines in atherosclerosis, we evaluated the miR-155 target genes SOCS1 and IRAKM. We found increased miR-155 and decreased expression of SOCS1 and IRAKM in hyperlipidemic mice. Interestingly inhibition of miR-155 by using a specific miR-155 silencing, inhibited proinflammatory cytokine in hyperlipidemic mice, suggesting a role of miR-155 in immune response regulation. Based on these observations, we conclude that miR-155 modulates proinflammatory response in hyperlipidemic mice via regulation of SOCS1 and IRAKM expression. Thus, modulation of miR-155 could be a strategy to regulate atherogenic diet-induced atherosclerosis where proinflammatory cytokine plays significant role in disease progression.

Keywords: Proinflammatory cytokines, Atherosclerosis, MiR-155, SOCS1 and IRAKM

*Corresponding author email: Mark.Mölle@ anis.com
[1]Institute of Cardiology, Fondazione Policlinico Universitario, Rome, Italy
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Introduction

MiR-155 plays a role in the regulates various aspects of innate and adaptive immune response, physiological and pathological processes. Exogenous molecular control in vivo of miR-155 expression may inhibit malignant growth, viral infections, and attenuate the progression of cardiovascular diseases [1]. Up-regulation of proinflammatory cytokines plays a central role in atherosclerosis. In this study, we investigated the role of miR-155 in regulating proinflammatory

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response in atherosclerosis. Hyperlipidemic C57BL/6 male mice model were fed with atherogenic-diet for 12-weeks. MiR-155 positively regulates proinflammatory cytokines and we found increased TNFα, IL-1b, IL-6 mRNA and NF-kB in hyperlipidemic mice. Furthermore, increased miR-155 levels are correlated with proinflammatory cytokine expression in hyperlipidemic mice [2].

MicroRNAs are ~23 nt small non-coding RNAs that regulate mRNA expression at the posttranscriptional level by directing mRNA degradation or translational repression. They fine-tune expression of their target genes (by approximately 1.2-fold to fourfold) and correspondingly affect biological pathway function [3]. miRNAs bind complementary seed-region sequences in the 3′ untranslated regions (UTRs) of specific target mRNAs leading to the repression of protein production. Each miRNA has the potential to repress many target mRNAs, often in the same molecular pathway, highlighting the sophistication of this epigenetic regulation [4].

MicroRNAs emerge from long primary transcripts (pri-miRNAs) transcribed from independent miRNA coding genes or from introns of protein-coding mRNAs [5]. After transcription, pri-miRNAs are capped, poly-adenylated, and then cleaved into ~70 nt hairpin structures (pre-miRNAs) by a nuclear microprocessor complex composed of RNase III-type endonuclease Drosha and the DiGeorge critical region 8 protein. DGCR8 is essential for Drosha activity and is capable of binding single-stranded fragments of the pri-miRNA that are required for proper processing.

Materials and Methods

Cloning 3'UTR of SOCS1 mRNA and reporter gene assay

The wild type SOCS1-3'UTR and mut-SOCS1-3'UTR dual luciferase reporter vector (Promega) were synthetized and tested by GenePharma Co., Ltd. and were then co-transfected with miR-155 mimic or inhibitor in oxLDL-stimulated macrophages. Cells were also transfected with the pmirGLO-control vector, which is useful for monitoring transfection efficiency. We used miR-155 negative control, a miRNA non-homologous to the human genome, as a control. After 24 h, we determined firefly luciferase activity using the dual luciferase reporter assay system (Promega) under a GloMAX 20/20 Luminometer (Promega). We obtained relative reporter activity through normalization to the Renilla control.

Small RNA transfection

We synthesized RNA oligonucleotides for small interference RNA (siRNA) (GenePharma) with the following: for negative control, 5'- UUC UCC GAA CGU GUC ACG UTT-3' (sense), 5'- ACG UGA CAC GU UCG GAG AAT T-3' (anti-sense); for SOCS1, 5'- GAA CCU UCC UCC UCU UCC UCC UCC T-3' (sense), 5'- AGG AGG AGG AAG AGG AGG AAG GUU CUG-3' (anti-sense). PMA-induced THP-1 macrophages were transfected with 100 nM siRNA for 24 h, followed by oxLDL (50ug/ml) for another 24 h.

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Western blot analysis

Protein extracts were denatured and the solubilized proteins (40μg) subjected to electrophoresis on 10% polyacryl amide SDS gels. We then probed with antibodies for SOCS1, STAT3, phospho-STAT3 (Abcam), I κBα, phosphor-I κBα, and β-actin (Santa Cruz) (diluted 1:500, β-actin diluted 1:2000 in TBST), and then we used goat anti-rabbit secondary antibody labeled with far-red-fluorescent Alexa Fluor 680 dye. We detected the immunofluorescence signal using a SuperSignal West Femto Maximum Sensitivity Substrate kit (ThermoPierce) and performed densitometric analysis using Image Lab (Bio-Rad).

ELISA assays

We analyzed cell or serum supernatants to determine the presence of TNF-α, IL-1β, CCL2, CCL4 and CCL7 using Enzyme Immunoassay kits (eBioscience or Uscn Life Science, Inc.) according to the manufacturer's instructions. The values were determined in more than three independent experiments and are represented as the means ±SD. We extracted nuclear proteins to assay NF-κB in each sample's nucleic protein using the Trans-AM NF-κB p50 Transcription Factor Assay Kit (Active Motif) according to the manufacturer's instructions.

In vivo assay

We used C57BL/6J mice and ApoE$^{-/-}$ mice in our study. The 6-week-old male ApoE$^{-/-}$ mice (n=16) were fed a high-cholesterol diet (1.25% of cholesterol) for 12 weeks to build an atherosclerosis mouse model, and 6-week-old male C57BL/6 mice (n=8) were fed a normal diet as a control. ApoE gene knockout mice showed symptoms of high blood lipid abnormalities and were then divided into two groups (n=8): the first group was injected with mismatched miR-155 and the second with antagomiR-155(chemically modified antisense oligonucleotides). Mismatched miR-155 and antagomiR-155 were synthesized by GenePharma Co., Ltd. We began treatment 12 weeks after feeding mice a high-cholesterol diet, and they then received 0.2 ml saline and antagomir-155 via tail vein injections. They received three injections in three consecutive days at 80 mg/kg body weight [11, 12]. All animal studies were approved by the Animal Care and Ethics Committee of the Third Military Medical University.

Analysis of atherosclerotic plaque size and composition

Three weeks after antagomir-155 treatment, we anesthetized mice with an intraperitoneal chloral hydrate injection (5%, 4μl/g), took blood from the apical, and then separated and collected the thoracic-abdominal aorta (spanning from the aortic arch to the iliac artery branch) after saline infusion. We performed morphometric and immunohistochemical studies in the thoracic-abdominal aorta and assessed lesion size and composition as previously described [13] .We took serial sections throughout the entire aortic valve area and routinely stained them with hematoxylin and eosin. To quantitate plaque size, we acquired the images of 3 sections with a DP70 digital camera connected to a microscope (Olympus) and determined lesion areas using Image-Pro plus v 6.0 software (Media Cybernetics, Inc.). We used the mean value of

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plaque cross-sectional areas from 3 sections to estimate the extent of atherosclerosis for each animal. We performed immunohistochemical analyses using the following primary antibodies: anti-mouse CD68 (Santa Cruz), anti-SOCS1 (Santa Cruz), and anti-SMA (Abcam). We analyzed at least 4 sections per animal for each immuno-staining.

Statistical analysis

We performed all statistical analyses using SPSS 19.0 software. Results are shown as the means ±SD. We determined statistical differences by Student's t-tests or one-way ANOVA. P-values< 0.05 were considered statistically significant.

Results

Inhibition of miR-155 attenuated atherosclerosis development and inflammation in an atherosclerosis mouse model

To test whether miR-155 inhibition was beneficial for atherosclerosis, we blocked miR-155 via tail-vein antagomir-155 injection in ApoE-/-mice with a high-fat diet. Real-time PCR analysis showed that antagomir-155 significantly inhibited miR-155 levels in the mice's plasma, vessel tissues (thoracic-abdominal aortas) and BMMCs (Fig. 1A, $P<0.05$). Meanwhile, we detected an obvious SOCS1 increase in both mRNA and protein levels in the antagomir-155 group (Fig. 5B, $P<0.05$). We separated the mice’s thoracic-abdominal aortas to observe the atherosclerotic lesions and analyzed the results using IPP 6.0 software. Consistent with the above results, the SOCS1-positive area was increased after antagomir-155 treatment (Fig. 1C, $P<0.05$). Although the lesion areas did not decrease after antagomiR-155 treatment, we observed a decrease in the positive area for atherosclerosis plaque macrophages (CD68), an increase in collagen deposition and the SMA-positive area in the antagomiR-155-treated group compared to the antagomir negative control (Fig. 1C, $P<0.01$).

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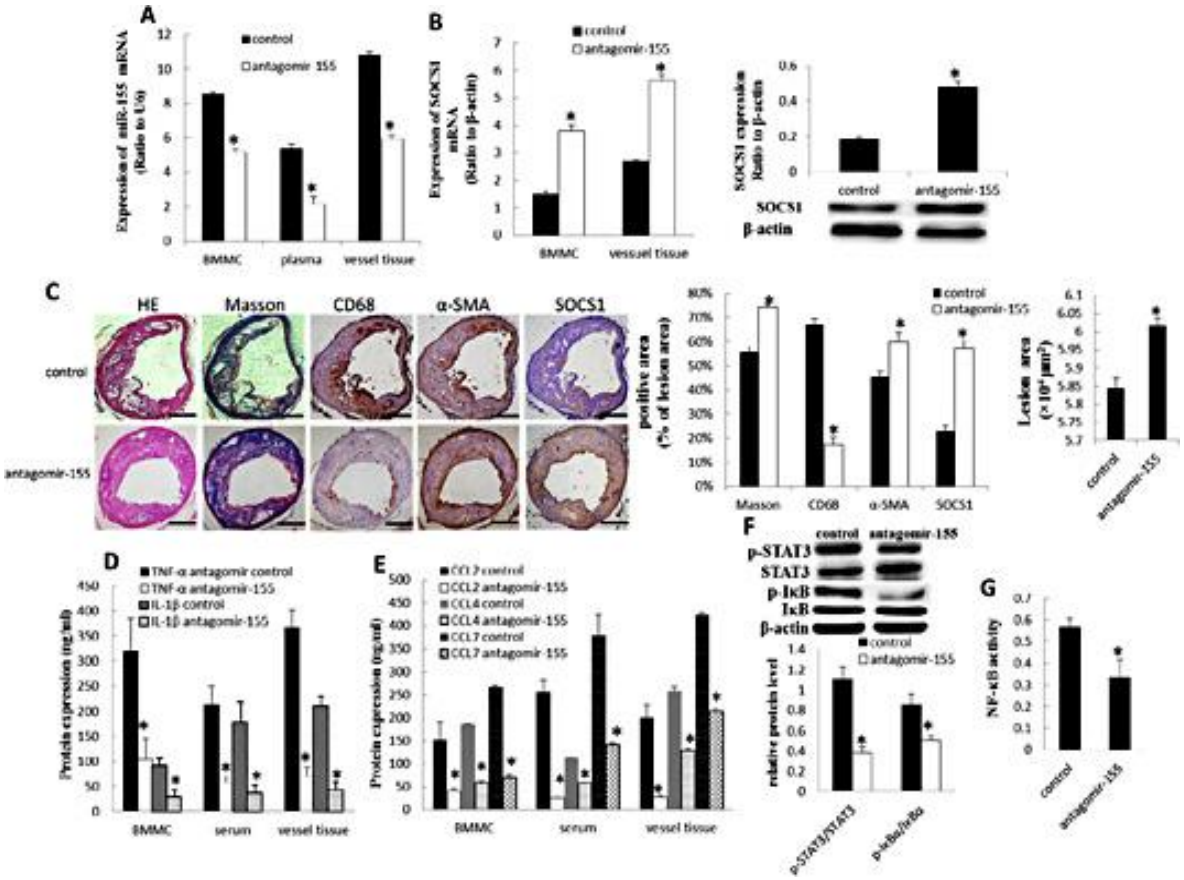

Figure 1.

Antagomir-155 attenuated atherosclerosis development and progression in an atherosclerosis mouse model. (A)Detection of miR-155 expression level in atherosclerosis models after treatment with antagomir-155 or antagomir negative control (control) by real-time PCR. *P < 0.01 relative to control. (B) Analysis of SOCS1 mRNA and protein levels in the antagomir-155 group by real-time PCR and western blot. Band densities were measured by Image Lab software. *P < 0.01 relative to control. (C) Paraffin-embedded cross sections from antagomir negative control or antagomiR-155-infused ApoE$^{-/-}$ mice were obtained throughout the thoracic-abdominal aorta area and stained with HE, Masson, CD68 antibody, α-SMA antibody, and SOCS1 antibody, as well as rat or rabbit nonspecific IgG, respectively. Images were quantified with Image-Pro Plus 6.0 at a 100× or 200×magnification. The scale is 100 μm. Data are represented as means ±SD (n=8). *P < 0.05 relative to control. (D) and (E) ELISA analysis of TNF-α, IL-1β, CCL2, CCL4, and CCL7 secretion in serum, vascular tissues, and BMMCs. (F) The phosphorylation and total protein levels of STAT3 and IκBα analyzed by western blot. Band densities measured by Image Lab software. (G) NF-κB transcript activation detected by ELISA. All data (means ±SD) were from three independent experiments. *P<0.05 versus the antagomir-155 negative control group.

miR-155 promoted inflammation and macrophage migration in oxLDL-stimulated macrophages

To further study miR-155's role in the atherosclerosis inflammatory response, we used miR-155 inhibitor and mimic in oxLDL stimulated macrophages. Results showed that secretions of inflammatory cytokines TNF-α and IL-1β, as well as chemokines CCL2, CCL4 and CCL7, were decreased after miR-155 inhibitor treatment, and this decrease could be counteracted by miR-155 mimic (Fig. 2A-B, *P<0.01). For better detection, we used siRNAs to knockdown SOCS1 in oxLDL-stimulated macrophages. The SOCS1 expression knockdown via siRNA treatment

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efficiently repressed SOCS1 mRNA and protein levels (Fig. 3C-D, *P<0.01). As expected, SOCS1 knockdown led to increased TNF-α, IL-1β, CCL2, CCL4 and CCL7, at both the mRNA and protein levels. These observations were similar to those results following miR-155 mimic treatment (Fig. 2E-F, *P<0.01). The results of transwell showed that miR-155 overexpression or SOCS1 knockdown with its specific siRNA led to increased macrophage migration, whereas when miR-155 was downregulated by inhibitor, the migrated macrophages decreased statistically (Fig. 2G, *P<0.01).

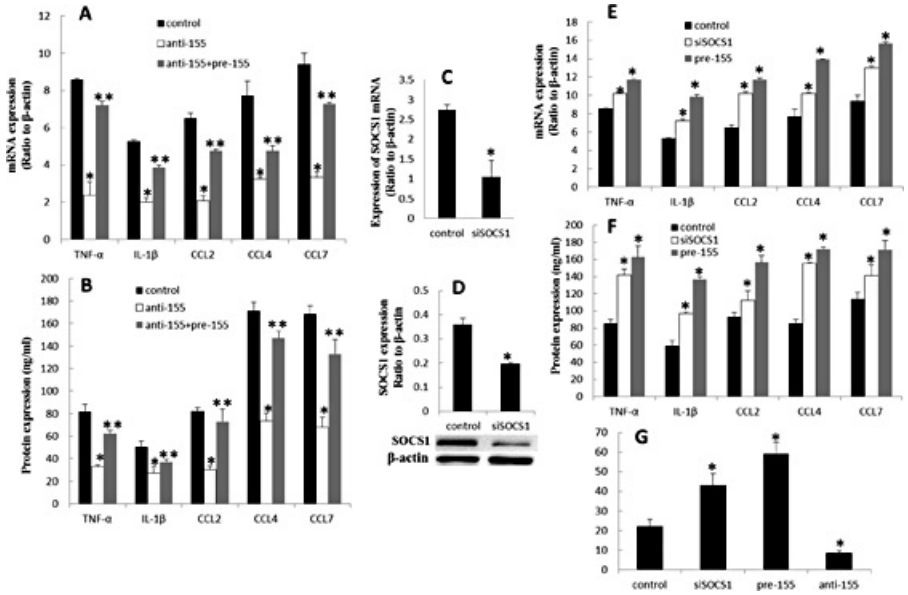

Figure 2.

miR-155 enhanced inflammatory cytokine and chemokine secretion and macrophage migration in oxLDL-stimulated macrophages. PMA-induced THP-1 cells were incubated with oxLDL(50μg/ml) for 24 h, followed by miRNA mimic/inhibitor were transfection for another 24 h. TNF-α, IL-1β, CCL2, CCL4, and CCL7 expression levels were regulated after miR-155 inhibitor and mimic incubation. (A) and (B) The mRNA and protein levels of TNF-α, IL-1β, CCL2, CCL4, and CCL7 were analyzed by real-time PCR and ELISA. Small interference RNAs for SOCS1 (siSOCS1) or a negative control sequence were transfected in oxLDL-stimulated macrophages. (C) and (D) SOCS1 mRNA and protein levels were detected by real-time PCR and western blot. (E) and (F) Detection of mRNA and protein levels of TNF-α, IL-1β, CCL2, CCL4, and CCL7. (G) Transwell analysis of macrophage migration. All data (means ±SD) were from three independent experiments. *P<0.01 versus the miR-155 control or siRNA control group (control), **P<0.01 versus the miR-155 inhibitor group (anti-155) group.

Discussion

Epigenetic modification represents an important mechanism of regulating gene expression that enables cells to respond appropriately to a changing environment. The mechanisms of epigenetic modification include, e.g., acetylation of histones, methylation of gene promoters, and silencing of mRNA transcripts by microRNAs (miRNA) [11]. miRNAs are a family of short, non-coding RNAs, acting at the posttranscriptional level [12-14], that fine-tune mRNA transcription [15]. More than 2,000 miRNAs have been identified in humans, and computational predictions show that these regulate the expression of approximately 60% of all human protein-

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coding genes [16]. miRNAs are pleotropic; a single miRNA can regulate a cellular response by targeting multiple components of a biological pathway [17]. More than 100 different miRNAs are expressed by cells of the immune system together influencing the pathways that control the development and function of cells of innate and adaptive immune responses [18]. Identifying disease-specific miRNAs improved the understanding of molecular pathways involved in diseases, and provided an evidence-base for new therapeutic strategies, e.g., in cancer and tendinopathy [19].

Rheumatoid arthritis (RA) is an autoimmune disease that affects approximately 1% of the global population and leads to progressive loss of joint function [20]. Currently, more than half of RA patients do not achieve sustained drug-induced disease remission [21], which constitutes an important clinical unmet need. A better understanding of the disease process is required to improve the treatment options for patients resistant to current therapeutics and to provide an evidence base for personalized medicine.

Accumulating evidence suggests that inflammation drives the formation, progression, and rupture of atherosclerotic plaques [7]. Therefore, focusing on the potential targets of inflammatory processes in atherosclerosis would provide novel treatment strategies [22].

Many miRNAs play an important role in atherogenesis [23]. For example, miR-126 is reportedly an anti-atherosclerosis microRNA since it regulates angiogenesis and vascular inflammation [24] and miR-150 promotes atherosclerosis via enhancing the targeted endothelial cell migration [25]. miR-155 may be one miRNA which plays an important role in atherosclerosis. It reportedly increases in ACS patients and could promote atherosclerosis by repressing Bcl6 in macrophages [14]. Wei Y et al. reported that the microRNA-342-5p could foster inflammatory macrophage activation through an Akt1- and microRNA-155-dependent pathway during atherosclerosis [17]. Qing Jing *et al.* found that elevated microRNA-155 promotes foam cell formation by targeting HBP1 in atherogenesis [26]. Among miR-155's targets, SOCS1 attracted our attention. SOCS1 is a powerful attenuator of JAK/STAT signaling [27] and has more recently been shown to disrupt NF-κB and the JNK and p38 pathways [28]. Activation of NF-κB is reported to be higher in patients with ACS and in oxLDL-induced mast cells [29]. Also, STAT3 signal pathway is reported to be involved in atherosclerosis inflammation [30]. Thus, we wished to investigate the role of SOCS1-miR-155 in regulating the atherosclerotic vascular inflammation response to find new therapeutic strategies for treating atherosclerosis and other vascular diseases.

First, we confirmed SOCS1 as a target of miR-155 in oxLDL-induced macrophages. We then observed miR-155 and SOCS1 levels in oxLDL-stimulated macrophages and in atherosclerosis model mice. We noticed a statistically significant inverse correlation between SOCS1 and miR-155expressions, suggesting a significant biological function of SOCS1-miR-155 in atherosclerosis. Since miR-155 mimic led to a significant decrease, while miR-155 inhibitor led to an obvious increase of SOCS1 mRNA and protein expressions, we proposed that high miR-

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155 levels are likely responsible for low SOCS1expression levels in oxLDL-stimulated macrophages.

Considering miR-155 is involved in some aspects of the atherosclerotic pathological process and inflammation [31], we hypothesized that SOCS1-miR-155 was deeply involved in regulating atherosclerosis inflammation. The present results showed that ectopic high levels of miR-155 as well as siSOCS1 could increase, and blocking miR-155 could decrease the production and secretion of some pro-inflammatory cytokines and chemokine in oxLDL-stimulated macrophages, which could lead to increased monocyte recruitment to plaque and promote atherosclerosis progression. These results confirmed our hypothesis that miR-155 acts as a promoter of inflammation in oxLDL-stimulated macrophages partly via targeting SOCS1. miR-155 greatly influenced the production and secretion of inflammatory cytokines and chemokines [32].

SOCS1 functions most classically to limit IFN activation of STATs (namely STAT1, STAT2, and STAT3) [5]. Others gave evidence indicating that STAT3 collaborates with NF-κB to regulate certain gene expressions [33], and other researchers reported IKBKE as a direct target of STAT3 [34], which could activate NF-κB and STAT signaling [35].

Conclusion

Our results also showed that miR-155 could enhance STAT3 and NF-κB signaling in oxLDL-stimulated macrophages. Furthermore, we noticed that siSOCS1 was less efficient than miR-155 mimic at inducing atherosclerosis inflammation, suggesting that other targets might be involved in miR-155's promotable function in oxLDL-stimulated macrophages. Upon using antagomir-155 to block miR-155 expression in ApoE$^{-/-}$mice with high fat diets, the positive area for atherosclerosis plaque macrophages decreased, the collagen deposition and the SMA-positive area increased, suggesting that the plague may be stable, although the lesion area was increased slightly, the continuous high-fat diet could be one of the important reasons. Besides, we noticed that inhibiting miR-155 alleviated inflammation, as well as STAT3 and NF-κB signaling *in vivo*.

Competing interests

The authors declare that they have no competing interests.

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