miR-335 and miR-34a Promote Renal Senescence by Suppressing Mitochondrial Antioxidative Enzymes

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ABSTRACT
The molecular basis for aging of the kidney is not well understood. MicroRNAs (miRNAs) contribute to processes such as development, differentiation, and apoptosis, but their contribution to the aging process is unknown. Here, we analyzed the miRNA expression profile of young (3-month) and old (24-month) rat kidneys and identified the biologic pathways and genes regulated by differentially expressed miRNAs. We observed upregulation of 18 miRNAs with aging, mainly regulating the genes associated with energy metabolism, cell proliferation, antioxidative defense, and extracellular matrix degradation; in contrast, we observed downregulation of 7 miRNAs with aging, principally targeting the genes associated with the immune inflammatory response and cell-cycle arrest. Bioinformatics analysis suggested that superoxide dismutase 2 (SOD2) and thioredoxin reductase 2 (Txnrd2), located in the mitochondria, are potential targets of miR-335 and miR-34a, respectively. Aging mesangial cells exhibited significant upregulation of miR-335 and miR-34a and marked downregulation of SOD2 and Txnrd2. miR-335 and miR-34a inhibited expression of SOD2 and Txnrd2 by binding to the 3′-untranslated regions of each gene, respectively. Overexpression of miR-335 and miR-34a induced premature senescence of young mesangial cells via suppression of SOD2 and Txnrd2 with a concomitant increase in reactive oxygen species (ROS). Conversely, antisense miR-335 and miR-34a inhibited senescence of old mesangial cells via upregulation of SOD2 and Txnrd2 with a concomitant decrease in ROS. In conclusion, these results suggest that miRNAs may contribute to renal aging by inhibiting intracellular pathways such as those involving the mitochondrial antioxidative enzymes SOD2 and Txnrd2.

Kidney aging is an important clinical problem, not only because normal aging reduces renal function but also because of the high frequency of ESRD, renal cancer, and renal failure in elderly people. Renal aging is of interest as a general model for organ aging because renal function can be quantitatively assessed more readily than that of other organs in clinical practice.1 At the present time, the molecular basis of renal aging is not clearly known. For example, nothing is known of the role of microRNAs (miRNAs) in the aging process of organs.

miRNAs are a novel class of small, regulatory, noncoding RNA molecules that inhibit the expression of multiple genes at the post-transcriptional level. miRNAs have been found to play a crucial role in development, differentiation, apoptosis, and metabolism and are involved in the pathogenesis of many human diseases.2,3 Bioinformatics studies suggest that miRNAs may regulate >60% of all human genes.4,5 Studies have shown that overexpression of miRNA lin-4 increases longevity in Caenorhabditis elegans, whereas loss of lin-4 leads to a
reduced lifespan. However, it is currently unclear whether miRNAs play an important role during the aging process in higher organisms, and any miRNAs involved in mammalian aging have yet to be identified. In this study, alterations in the level of miRNA expression during kidney aging were investigated using a miRNA chip, the target genes of the differentially expressed miRNAs were predicted using bioinformatics, and a global analysis of the biologic pathways and genes regulated by miRNAs was performed. Furthermore, we investigated whether miR-335 and miR-34a induce renal mesangial cell senescence by inhibiting the functioning of their corresponding target genes, mitochondrial superoxide dismutase 2 (SOD2) and thioredoxin reductase 2 (Txnrd2).

RESULTS

Morphologic Changes in Aged Rat Kidney Tissues
Renal histologic changes in young (3-month) and old (24-month) male Wistar rats were evaluated by periodic acid–Schiff (PAS) staining. Compared with young renal tissues, old renal tissues showed marked pathologic features of aging, including occasional focal segmental glomerular sclerosis, interstitial fibrosis and atrophy of renal tubules, and some inflammatory cell infiltration. Semiquantitative scoring for renal structural changes showed that the lesions in the aged renal tissues were significantly increased compared with those in young kidneys (Figure 1).

Changes in the miRNA Expression Profile in Old Renal Tissues
To investigate whether miRNAs play a significant role in the aging process of organs, a miRNA microfluidic chip was used to analyze the miRNA expression profile in old renal tissues. The level of miRNA expression in old kidneys was compared with that in young kidneys. The results (Table 1) showed that 25 miRNAs were significantly differentially expressed during renal aging. Of these, 18 miRNAs exhibited increased expression (the log2 ratio of old/young signal intensity was >2). Among these miRNAs, rno-miR-184, rno-miR-335, and rno-miR-542–3p were upregulated by more than a 4-fold change in the log2 ratio. Only seven miRNAs were significantly downregulated (the log2 ratio was less than −2) in old kidneys. This shows that among these differentially expressed miRNAs, most miRNAs were upregulated during normal renal aging.

Confirmation of the Differentially Expressed miRNAs
To investigate the reliability of the miRNA microarray results, three miRNAs (rno-miR-184, rno-miR-335, and rno-miR-347) were selected for further verification using quantitative real-time PCR (qRT-PCR). The results showed that all of these miRNAs exhibited statistically significant differential expression between young and old rat renal tissues (Figure 2A). Some rat strains, such as Sprague–Dawley and Fisher 344, often develop chronic progressive nephrosis (CPN), a renal age-dependent phenotype, during the second year of life. To exclude the possibility that the above-mentioned changes in miRNA levels in old rat kidneys were caused by CPN rather than renal aging, we analyzed the changes in expression levels of miR-184, miR-335, and miR-347 in aged kidneys (24 months old) of mice, which do not develop CPN, using qRT-PCR. The results showed that these miRNAs were also significantly differentially expressed during mouse renal aging (Figure 2B).

Table 1. The significantly differentially expressed miRNAs in aging kidney

<table>
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<tr>
<th>miRNA Name</th>
<th>Fold Change (old/young)</th>
<th>miRNA Name</th>
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$^a$ represents *. 

Figure 1. The lesions in the aged renal tissues were significantly increased. (A) Semiquantitative analyses of the renal lesions in aged rats. Glomerular scoring: *P < 0.01 versus young. Interstitial scoring: #P < 0.01 versus young. (B) Histologic analysis of 3- and 24-month rat renal tissues by PAS staining. In some glomeruli, focal segmental glomerular sclerosis may exist occasionally.
Key Biologic Pathways and Genes Regulated by miRNAs in Aged Kidneys

To screen the key miRNAs implicated in the regulation of renal aging and to determine the probable functional roles of these miRNAs, three algorithms (TargetScan, PicTar, and miRanda) were used to further analyze the target genes of the above differentially expressed miRNAs. As expected, each of these miRNAs has a multitude of different target genes; some miRNAs share common mRNA targets, which then have a higher probability of being suppressed by the miRNAs. For example, rno-miR-184, rno-miR-335, and rno-miR-7a upregulated in old kidneys all target the antioxidative SODs.

We then collected these target genes and performed gene ontology (GO) term analysis, including biologic process and molecular function, and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis using the DAVID gene annotation tool to interpret the biologic functions, biologic processes, and biologic pathways of these miRNA targets. The results of GO and KEGG pathway analyses showed that the miRNAs upregulated in old kidneys mainly target the genes in biologic pathways involved in the antioxidative system (Table 2), energy metabolism (Supplemental Table 1), cell mitosis and proliferation (Supplemental Table 2), and extracellular matrix (ECM) degradation (Supplemental Table 3). Although some of these pathways and genes have been shown to participate in the modulation of the renal aging process, most have never been reported to play a role in renal aging. For example, it has been found that rno-miR-184 targets antioxidative genes such as [copper-zinc] superoxide dismutase and glutathione peroxidase (Gpx) 3, ECM-degradative gene membrane-type matrix metalloproteinase 3, and the longevity-related genes sirtuin 3 and 7. Rno-miR-335 targets the antioxidative genes Gpx 2, mitochondrial SOD2, and thioredoxin-like protein 1. In short, these miRNAs upregulated in old kidneys may play a critical regulatory role during the renal aging process by suppressing the expression of these target genes.

On the other hand, the results from the GO and KEGG pathway analyses indicated that the miRNAs downregulated in senescent kidneys principally target immune and inflammatory response genes (Supplemental Table 4), cell cycle arrest genes such as p21 and p16Ink4a (Supplemental Table 2), and ECM synthesis genes (Supplemental Table 3). Some of these targets have been found to be upregulated in aging renal tissues. Our analyses also revealed that many miRNAs target a common gene. For example, the upregulated miR-335, miR-224, and miR-21 all repress proliferating cell nuclear antigen.

Isolation and Identification of Primary Renal Residential Cells

The primary renal cells were isolated, and their purity was identified by means of immunofluorescence staining with antibodies against the specific markers of the renal cells. The results showed that almost all mesangial cells showed positive reaction for desmin and vimentin, markers of the mesangial cells (Figure 3, A and B). The glomerular endothelial and epithelial cells stained positively for their corresponding specific markers, platelet/endothelial cell adhesion molecule-1 (CD31) (Figure 3C) and nephrin (Figure 3D), respectively.
mRNAs Modulate Renal Aging

To determine the target genes of miR-335 and miR-34a, we selected SOD2 and Txnrd2 localized in mitochondria as candidate target genes of miR-335 and miR-34a, respectively. The 3′-UTRs of SOD2 and Txnrd2, which respectively contain consensus-binding sequences of miR-335 and miR-34a, were PCR amplified and inserted downstream of the luciferase reporter cDNA gene in the pGL3 vector to construct Luc-SOD2-UTR and Luc-Txnrd2-UTR vectors (Figure 5A) so as to explore the effect of miR-335 and miR-34a on expression of SOD2 and Txnrd2 proteins. The results indicated that cotransfection of premiR-335 and premiR-34a (mimics, to mimic mature miR-335 and miR-34a) with Luc-SOD2-UTR and Luc-Txnrd2-UTR vectors resulted in 57% and 63% inhibition of luciferase activity, respectively, compared with samples with control miRNA (Figure 5, B and C). To further confirm that miR-335 and miR-34a acted via binding to the 3′-UTRs of SOD2 and Txnrd2, the binding elements in the 3′-UTRs of SOD2 and Txnrd2 were mutated. The results indicate that cotransfection of premiR-335 and premiR-34a with the mutated Luc-3′-UTR constructs (Luc-SOD2-mutant UTR and Luc-Txnrd2-mutant UTR) had no marked effect on luciferase activity (Figure 5, B and C).

miR-335 and miR-34a Mimics Downregulated Expressions of Endogenous SOD2 and Txnrd2 in Young Mesangial Cells
To further validate the hypothesis that miR-335 and miR-34a negatively regulate expression of SOD2 and Txnrd2, respectively, we transfected young mesangial cells with premiR-335 and premiR-34a or with corresponding control miRNAs. The West-
ern blot revealed that levels of SOD2 and Txnrd2 were significantly decreased in the young cells transfected with premiR-335 and premiR-34a compared with the controls (Figure 6A).

**miR-335 and miR-34a Mimics Induced Premature Senescent Phenotypes in Young Mesangial Cells**

To investigate the role of miR-335 and miR-34a in the senescence of renal mesangial cells, premiR-335 and premiR-34a were transfected into young mesangial cells, and senescence-associated β-galactosidase (SA-β-gal) activity (a biomarker of cellular senescence) and senescence-associated heterochromatic foci (SAHF) formation (a novel specific biomarker of senescent cells) were observed.11 SA-β-gal-staining results showed that in young cells, overexpression of miR-335 and miR-34a after transfection increased significantly the percentage of SA-β-gal-positive cells compared with the control (Figure 7A). As shown in Figure 7B, overexpression of miR-335 and miR-34a also led to pronounced DNA SAHF formation, which was visualized by 4′,6′-diamidino-2-phenylindole (DAPI) staining. By contrast, the cells in the control group displayed relatively uniform staining patterns.

**Antisense miR-335 and miR-34a Delayed the Senescence Process in Old Mesangial Cells**

Aging mesangial cells were transfected with antisense miR-335 and miR-34a inhibitors or corresponding control miRNAs. The SA-β-gal and DAPI staining results revealed that after transfection, the percentage of SA-β-gal-positive cells (Figure 7C) and the formation rate of SAHF (Figure 7D) decreased significantly compared with the controls.

**miR-335 and miR-34a Modulated Oxidative Stress Level in Mesangial Cells**

To confirm that miR-335 and miR-34a induced premature senescence in young mesangial cells by inhibiting the antioxidative function of their target genes, SOD2 and Txnrd2, respectively, thereby augmenting the oxidative stress level in the cells, we next determined the levels of reactive oxygen species (ROS) in the young mesangial cells transfected with premiR-335 and premiR-34a. The results revealed that both genes were downregulated in the aged kidneys in vivo (Figure 6C).

**DISCUSSION**

The mechanisms of renal organ aging are currently unknown. Studies have found that in the aged kidney and brain, expres-
The downregulation of oxidative defense proteins such as SOD, catalase, Gpx, and peroxiredoxins is associated with reduced antioxidant capacity. This could be the result of post-transcriptional suppression by miRNAs. Therefore, we postulate that miRNA may play a very important role in the regulation of renal aging.

In this study, the role of miRNAs during renal aging in rats was investigated using a miRNA chip. We have observed significant age-related changes in miRNA expression in old kidneys. Among these differentially expressed miRNAs, most miRNAs were upregulated, and a few miRNAs were downregulated. To gain further insights into the role of miRNAs in renal aging, the target genes of the differentially expressed miRNAs in aging kidneys were determined. The biologic function and biologic pathways of these targets were analyzed. The results revealed that the miRNAs upregulated in aging kidneys mainly regulate the pathways or genes associated with energy metabolism, cell proliferation, antioxidative defense, and ECM degradation, whereas the downregulated miRNAs principally target the pathways or genes related to immune-inflammatory response and cell-cycle arrest. For example, rno-miR-184, rno-miR-335, rno-miR-34a, rno-miR-224, and rno-miR-7a upregulated in old kidneys were found to target antioxidative genes such as Gpx, SOD, catalase, and Txnrd. This suggests that these miRNAs may be implicated in an aging mechanism related to oxidative stress. Under physiologic conditions, antioxidative gene products play major roles in the detoxification of ROS. Decreased expression and activity of these antioxidative genes induced by high expression of miRNAs in the aging kidney expose the organism to higher oxidative stress and cellular injury. A previous study found that the expression and activity of SOD and Gpx decrease with age in rat kidneys. Therefore, the increase in expression level of these miRNAs in old kidneys may contribute to the oxidative damage associated...
with aging by inhibiting expression of the antioxidative target genes and increasing the oxidative stress level. The antioxidative enzymes SOD2 and Txnrd2 are located in the mitochondria and may play a key role in modulating cellular aging by detoxifying ROS generated in the mitochondria. Because miR-335 and miR-34a/708 were predicted to target SOD2 and Txnrd2, respectively, we selected two highly expressed miRNAs (miR-335 and miR-34a) in the aging renal tissues to further investigate whether both play a modulating role in renal mesangial cell senescence. Here, we have shown that in the aging mesangial cells, the expression levels of miR-335 and miR-34a were significantly upregulated, whereas the predicted target genes (SOD2 and Txnrd2) of miR-335 and miR-34a were markedly downregulated. miR-335 and miR-34a could inhibit SOD2 and Txnrd2 expression through binding to the corresponding binding sites in the 3′-UTRs of SOD2 and Txnrd2 genes. In young mesangial cells, miR-335 and miR-34a induced premature senescent phenotypes via suppression of SOD2 and Txnrd2 expression with a concomitant increase in ROS levels. Conversely, antisense miR-335 and miR-34a reduced senescent phenotypes by upregulating SOD2 and Txnrd2 expression and lowering ROS levels in old mesangial cells. miR-34a has also been found to be upregulated in aging mouse brains and human diploid fibroblasts.12,15 These results suggest that miRNAs may regulate the aging process by modulating genes involved in the scavenging of ROS in mitochondria because mitochondria are major sites of intracellular ROS production. The free-radical theory of aging suggests that if production of ROS exceeds the capacity of the antioxidative system to remove the ROS, oxidative damage occurs to intracellular biologic macromolecules such as DNA, protein, and lipids, leading to aging.16 In conclusion, this study demonstrates that miRNAs may play a crucial role during renal aging by regulating the expression of some key target genes in multiple pathways. miR-335 and miR-34a can modulate mesangial cell senescence via inhibition of the expression and function of the mitochondrial antioxidative enzymes SOD2 and Txnrd2.

**CONCISE METHODS**

**Preparation of miRNA Microarray**
The miRNA probe sets used in this study were composed of 711 human miRNAs, 568 mouse miRNAs, 348 rat miRNAs, and positive and negative controls, which included all miRNA bases (on the basis of Sanger miRBase version 10.0, http://microrna.sanger.ac.uk/). LC Sciences (Houston, TX) provided the miRNA microarray chip (Paraflo microfluidic chip). The detection probes were made by in situ synthesis using photogenerated reagent chemistry. On the microfluidic chip, each detection probe consisted of two parts: a chemically modified antisense oligonucleotide fragment, which is complimentary to the corresponding miRNA or control RNA, and a spacer segment of polyethylene glycol, which extended the antisense fragment away from the chip substrate and decreased space hindrance in the hybridization process. Every miRNA probe on each chip was repeated 3 times.

**Tissue Sample and Histology**
Male Wistar rats and male C57BL/6 mice were purchased from the Vital River Laboratory Animal Technology, Co., Ltd. (Beijing, China). The kidney tissues from 3- (young) and 24-month (old) rats and mice were collected and placed immediately into either liquid nitrogen or 10% neutral buffered formalin. Formalin-fixed kidney tissues were paraffin embedded, and 4-μm sections were cut. Sections were stained with PAS and examined under a light microscope at ×200 magnification for age-associated changes. Semiquantitative scoring was used to evaluate the lesions in the aged kidneys. The
severity of glomerular lesions was graded from 0 to 4 as follows: 0 represents no lesion; 1 represents lesions of <25% of the glomerulus; and 2, 3, and 4 represent lesions of 25 to 50, 50 to 75, and >75% of the glomerulus, respectively. The degree of interstitial lesions was graded from 0 to 3 on the basis of cast, tubular atrophy, interstitial inflammation, and fibrosis.

**miRNA Labeling and Microarray Hybridization**

Small RNAs were isolated from total RNAs using an YM-100 Microcon centrifuge filter column (Millipore). The enriched small RNAs were 3′-extended with a poly (A) tail using poly (A) polymerase. An oligonucleotide tag was then ligated to the poly (A) tail for subsequent fluorescent dye labeling. The small RNA samples from young and old rat renal tissues were labeled with Cy3 or Cy5 fluorescence dyes (Amersham Pharmacia, Piscataway, NJ), respectively. Hybridization on a Paraflo microfluidic chip was performed overnight at 34°C in 100 μl 6× SSPE buffer (0.9 M sodium chloride, 60 mM sodium hydrogen phosphate, 6 mM EDTA, pH 6.8) containing 25% formamide using a microcirculation pump (Atactic Technologies, Houston, TX). After hybridization, the hybridized chips were washed and then scanned using a GenePix 4000B laser scanner (Molecular Devices, Sunnyvale, CA) to collect and quantify hybridization images.

**Data Analysis**

Fluorescence intensities for each spot were scanned and calculated using Array-Pro image analysis software (Media Cybernetics, Bethesda, MD) after subtracting the local background (on the basis of the median intensity of the area surrounding each spot) from the total intensities. A miRNA was considered detectable on an array if its fluorescence was significantly above negative controls. Median values of the nine spots (three replicate probes in every chip, three chips used for hybridization) for each probe were calculated, and the signal from each spot was then normalized to the average signal of the whole block using a LOWESS filter (Locally Weighted Regression). A miRNA was considered to be differentially expressed between old and young rat kidneys if the P value of the t test was <0.01. The ratio of old sample signal versus young sample signal was log2 transformed. If the log2 ratio is positive, a miRNA is upregulated; if the log2 ratio is negative, a miRNA is downregulated.

**Validation of Individual miRNA Expression Using qRT-PCR**

Expression levels of individual miRNA were quantified using qRT-PCR with TaqMan. The methods were similar to conventional real-time PCR, with modifications that allowed detection of the short, mature miRNAs. To avoid any bias introduced during the miRNA enrichment procedure, total RNA samples were used in these analyses, and 5S ribosomal RNA was used as an internal normalizer. The kidney tissues were collected from 3- (young) and 24-month (old) male Wistar rats and male C57BL/6 mice from the Vital River Laboratory Animal Technology Co., Ltd. (Beijing, China).

**Prediction and GO and Pathway Analyses of the miRNA Target Genes**

Three bioinformatics algorithms were used to computationally predict the target genes of the differentially expressed miRNAs in the aged kidneys, including miRanda (http://microrna.sanger.ac.uk/targets/v4.0), PicTar (http://pictar.bio.nyu.edu/), and TargetScan 3.1 (http://www.targetscan.org/). To fully inspect the function of the differentially expressed miRNAs, we collected the reported and predicted miRNA targets from the Sanger database (http://microrna.sanger.ac.uk/) and performed GO term analysis. GO analysis was applied to organize genes into hierarchical categories and uncover the miRNA gene regulatory network on the basis of biologic process and molecular function; the network of miRNA-mRNA interaction, representing the critical miRNAs and their targets, was established according to the miRNA degree. Meanwhile, the miRNA targets were subjected to KEGG pathway annotation (http://www.genome.jp/kegg/) using the DAVID gene annotation tool (http://david.abcc.ncifcrf.gov/).

**Isolation and Identification of Primary Renal Residential Cells**

Primary mesangial cells of renal glomeruli from male Wistar rats at the ages of 2 (young) and 24 (old) months were isolated and cultured as described previously. Briefly, cortices were separated from medullas, cut into small pieces, incubated with 0.1% collagenase for 20 minutes, passed serially through 200- and 90-mm stainless steel...
fibroblasts were isolated and identified as reported.28 FSP-1 was used as marker for renal fibroblasts.29 To perform immunofluorescence (AmiR34a), and control miRNA (Thermo Fisher, Waltham, MA). miR-34a mimic (premiR-34a) or antisense miR-34a inhibitor mimic (premiR-335) or antisense miR-335 inhibitor (AmiR335), electrotransfection solution, to which was added 40 nmol/L miR-335 natural L-valine. Because of a deficiency of D-amino acid oxidase in the cells were plated and incubated at 37°C for 3 days.

Western blot
Western blots were performed using rabbit anti-SOD2 and anti-Txnrd2 antibodies and β-actin antibody (Abcam). The blots were then incubated with the corresponding secondary antibodies conjugated with horseradish peroxidase for 1 hour. After washing with Tris-Tween Buffered Saline, the membranes were incubated with electrochemiluminescence reagent (Amersham Life Science, Inc., Buckinghamshire, United Kingdom), exposed to x-ray film, and developed. β-actin was used as a normalization control.

SA-β-gal Staining
Cells were washed twice in PBS, fixed to plates using 3% formaldehyde for 3–5 minutes, and washed with PBS again. These were then incubated overnight at 37°C without carbon dioxide in a freshly prepared staining buffer (1 mg/ml X-gal, 40 mM citric acid/sodium phosphate, pH 6.0, 5 mM potassium ferrocyanide, 5 mM potassium ferricyanide, 150 mM sodium chloride, 2 mM magnesium chloride). Stained cells were examined using a Zeiss Axiosvert 200 inverted microscope (Carl Zeiss, Inc., Germany) under bright-field illumination.

SAFH Analysis
SAFH formation is a novel specific biomarker of cellular senescence because there is marked focal heterochromatin in the aged cells. To determine SAHF formation, cells were cultured directly on glass coverslips and then fixed with 4% paraformaldehyde. After washing with PBS, cells were permeabilized with 0.2% Triton X-100/PBS for 10 minutes. DNA was visualized by DAPI (1 mg/ml) for 1 minute and then washed with PBS twice. Cover slips were mounted in a 90% glycerol PBS solution and examined under a laser confocal microscope.

Determination of Intracellular ROS Level
Intracellular ROS generation was measured using the oxidant-sensitive fluorescence probe carboxy-dichlorodihydrofluorescein diacetate. Briefly, suspensions of cells (1 × 10^6) were loaded with 5 µM carboxy-dichlorodihydrofluorescein diacetate (Molecular Probes, Eugene, OR) for 10 minutes at 37°C. After centrifugation and washing to remove unincorporated probe, measurements of cellular fluorescence were performed by flow cytometry (Becton-Dickinson, San Jose, CA) using excitation at 488 nm and observing emission at 515 nm.
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DISCLOSURES

None.

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