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Albumin mediates PPAR-γ or C/EBP-α-induced phenotypic changes in pancreatic stellate cells

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ABSTRACT

Activation of quiescent hepatic stellate cells (HSCs) into myofibroblast-like cells is a key event of liver fibrosis, and adipogenic transcription factors, PPAR-γ and C/EBP-α, reverse HSC activation. As albumin was reported to maintain the quiescent phenotype of stellate cells, we examined whether it plays a role in PPAR-γ and C/EBP-α-mediated effects. Pancreatic stellate cells (PSCs) were isolated from rat pancreas and used in their culture-activated phenotype. Forced expression of PPAR-γ or C/EBP-α in PSCs increased albumin mRNA and protein levels by >2.5-fold, which is accompanied with increased C/EBP-β binding to albumin promoter. PPAR-γ and C/EBP-α also induced a phenotypic switch from activated to quiescent cells and, interestingly, suppression of albumin using short-hairpin RNA (shRNA) blocked their effects. Therefore, our findings suggest that albumin may be a downstream effector of PPAR-γ and C/EBP-α in PSCs and that it can be an attractive molecular target for anti-fibrotic therapies.

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Introduction

Pathological features of hepatic or pancreatic fibrosis is the excessive production and deposition of extracellular matrix (ECM) components and there is overwhelming evidence that activated hepatic or pancreatic stellate cells are the major producers of the fibrotic neomatrix [1,2]. Studies showed that pancreatic stellate cells (PSCs) display similar cellular behavior to hepatic stellate cells (HSCs) [3]. PSCs, in their quiescent state, can be identified by their angular appearance and the presence of vitamin A-containing lipid droplets in their cytoplasm [4]. When activated by profibrogenic mediators, they transform into myofibroblast-like cells characterized by loss of vitamin A droplets and greatly increased synthesis of the extracellular matrix components [5].

It has been demonstrated that the activity of adipogenic transcription factors is required for the maintenance of HSC quiescence. Levels of peroxisome proliferators-activator receptor-γ (PPAR-γ) are markedly reduced during HSC activation and its expression reverses the morphological and biochemical characteristics of activated HSCs [6,7]. Treatment of thiazolidinedione, a PPAR-γ ligand, ameliorates liver fibrosis in both toxic and cholestatic models [8]. Expression level of adipogenic factor CCAAT/enhancer-binding protein-α (C/EBP-α) also declines in activated HSCs [9]. Overexpression of C/EBP-α inhibits HSC activation [9] and its *in vivo* expression lessens CCL_4-induced hepatic fibrosis in mice [10]. These results support the notion that both PPAR-γ and C/EBP-α serve as important therapeutic targets for liver fibrosis, but the molecular mechanisms responsible for their action remain less clear.

Albumin is the most abundant plasma protein synthesized primarily by liver cells. It is important in regulating blood volume by maintaining the oncotic pressure and also serves as carriers for various molecules of low water solubility [11]. Recently, albumin was found to be expressed in quiescent (hepatic and pancreatic) stellate cells and plays a direct role in the formation of cytoplasmic lipid droplets [12]. In this study, we examine whether albumin acts downstream of PPAR-γ and C/EBP-α in phenotypic switch of PSCs. Forced expression of PPAR-γ or C/EBP-α in activated PSCs induced albumin expression, thereby reverting cells to the quiescent phenotype.

Materials and methods

Materials. Fetal calf serum and Dulbecco's modified Eagle's medium (DMEM) were purchased from Invitrogen (Carlsbad, CA) and fetal bovine serum was obtained from HyClone Laboratories, Inc. (Logan, UT). Male Sprague–Dawley rats of 8–10 weeks of age were

Abbreviations: C/EBP, CCAAT/enhancer-binding protein; PPAR-γ, peroxisome proliferator-activated receptor-γ; shRNA, short-hairpin RNA; PSCs, pancreatic stellate cells; HSCs, hepatic stellate cells; α-SMA, α-smooth muscle actin.

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purchased from Orient (Charles River Korea, Seoul, Korea). All rats were maintained under temperature-, humidity-, and light-controlled conditions. The animals received humane care according to the institutional guidelines.

PSC isolation and culture. Rat PSCs were isolated as described previously [3]. Briefly, the pancreas was finely minced, placed in a solution of Hank's buffered salt solution with 0.05% collagenase, 0.02% pronase and 0.1% DNase, and shaken for 20 min at 37 °C. After filtration through 150 μm mesh, cells were centrifuged on a 13.2% Nycodenz gradient at 1400*g* for 20 min. PSCs were collected from the band just above the interface of the Nycodenz solution and the aqueous layer, suspended in DMEM supplemented with 10% fetal bovine serum, and plated on non-coated plastic dishes. After reaching confluence in the primary culture, serial passages were obtained always applying a 1:3 split.

Western blot analysis. Cells were rinsed in ice-cold phosphate-buffered saline (PBS) twice and harvested by scraping in the lysis buffer [13]. Equivalent amounts of protein were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE), followed by immunoblot detection using the primary antibody. Primary antibodies were as follows: albumin (Affinity Bioreagents, Rockford, IL), α-SMA (Sigma, St. Louis, MO), α-tubulin and PPAR-γ (Cell Signaling, Beverly, MA), C/EBP-α and C/EBP-β (Santa Cruz, Santa Cruz, CA).

Analysis of gene expression using real-time RT-PCR. Total RNA was isolated using an RNeasy kit (Qiagen, Valencia, CA). cDNA was synthesized from 1.0 μg total RNA with GeneAmp RNA PCR (Applied Biosystems, Foster City, CA) using random hexamers. Real-time PCR was performed using LightCycler-FastStart DNA Master SYBR Green 1 (Roche, Mannheim, Germany) according to the manufacturer's instructions. The reaction mixture (20 μl) contained LightCycler-FastStart DNA Master SYBR Green 1, 4 mM $MgCl_2$, 0.5 μM of the upstream and downstream PCR primers and 2 μl of the first strand cDNA as a template. To control for variations in the reactions, all PCRs were normalized against glyceraldehyde-3-phosphate dehydrogenase (GAPDH) or β-actin expression. The primers used were as follows: 5′-GGT GGT CTC CTC TGA CTT CAA CA-3′ (forward primer) and 5′-GTT GCT GTA GCC AAA TTC GTT GT-3′ (reverse primer) for rat GAPDH; 5′-CTG ATA TCT GCA CAC TCC CA-3′ (forward primer) and 5′-TCA GTG GCG AAG CAG TTA TC-3′ (reverse primer) for rat albumin.

Transfection and shRNA. The entire open reading frames of rat albumin, PPAR-γ, and C/EBP-α were amplified by PCR with the designed primers and inserted into mammalian expression vector pcDNA3.1+.

For the RNAi experiment, two target regions ((1) 675–693 bp and (2) 1881–1899 bp) for rat albumin were selected using the Qiagen siRNA online design tool and subcloned into pSuper vector (Oligoengine, Seattle, WA). As a negative control, the scrambled fragment 5′-CTAGCACCATAATCAACG-3′, which does not have similarity with any mRNA listed in GenBank, was generated. PSCs after

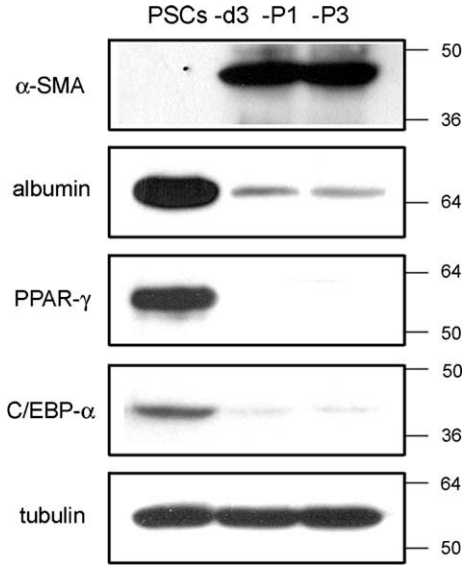

Fig. 1. Expression of PPAR-γ and C/EBP-α during the culture activation of rat pancreatic stellate cells (PSCs). Cell lysates were prepared from PSCs at 3 days (d3) after plating and from PSCs after passages 1 (P1) and 3 (P3), and analyzed by Western blotting. α-Smooth muscle actin (α-SMA) was used as a marker for PSC activation and β-actin was used as a loading control. This figure is representative of three independent experiments from separate cell preparations.

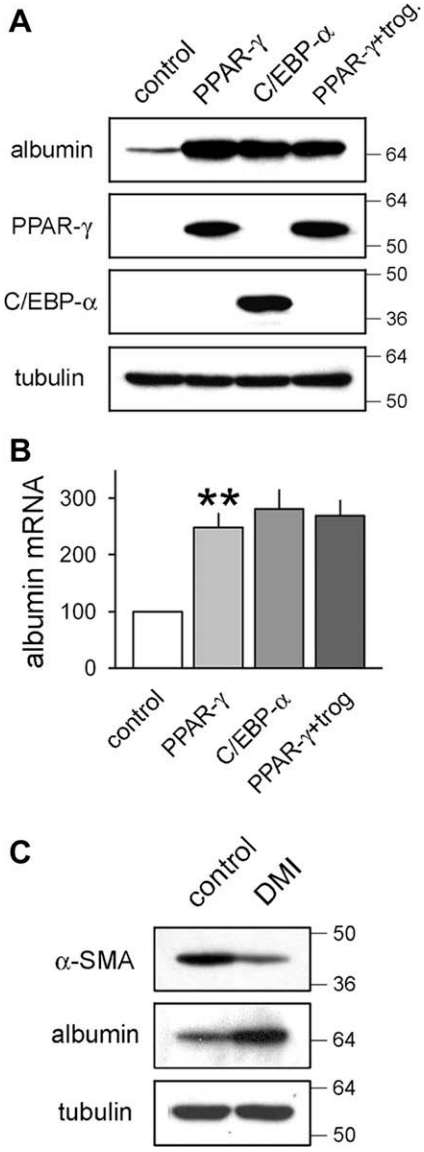

Fig. 2. PPAR-γ and C/EBP-α induce albumin expression. (A) PSCs after passage 2 were transiently transfected with empty vector, pcDNA3.1-PPAR-γ or pcDNA3.1 -C/EBP-α and incubated in the presence or absence of troglitazone (10 μM) for 36 h as indicated, and cell lysates were analyzed by Western blotting. (B) Following the same treatment as above, total RNAs were prepared from PSCs and analyzed for albumin by real-time PCR. The data are expressed as the percent of control cells and represent means (SD) for three independent experiments. $^{**}P < 0.01$ compared with the untreated control. (C) PSCs after passage 2 were treated with adipogenic differentiation mixture (DMI) for 4 days and analyzed by Western blotting.

passage 2 were transiently transfected with shRNA-expressing plasmids and/or expression vectors using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) and subject to analysis after 36 h.

Magnetic DNA affinity purification. Separation of sequence specific DNA binding proteins was performed as described with minor modification [14]. Briefly, cell lysates were mixed with poly(dI–dC) and biotinylated promoter DNA fragments in 1× binding buffer (20 mM Hepes, pH 7.9, 50 mM KCl, 1 mM DTT, 1 mM EDTA, 0.05% NP-40, 10% glycerol) at 4 °C overnight. The promoter fragment of the rat albumin gene between nt −386 and +16 was obtained by PCR with the designed primers. Streptavidin-coated magnetic particles (Roche, Mannheim, Germany) were added into binding mixture, washed, and collected using magnetic separation rack (New England Biolabs, Ipswich, MA). Proteins binding to DNA-magnetic particles were analyzed by Western blotting.

Immunofluorescence. PSCs were plated onto glass coverslips in 12-well plates coated with gelatin. Samples were fixed in paraformaldehyde and incubated with anti-albumin antibody (Affinity Bioreagents #PA1-25673) overnight at 4 °C in a moist chamber, followed by fluorescein isothiocyanate-conjugated goat anti-chicken IgY (Santa Cruz #sc-2431). Cells were washed with PBS and mounted onto slides. Stained cells were visualized on a Zeiss AXIO Imager M1 microscopy.

Oil red O staining. PSCs were stained with Oil red O to visualize lipid droplets, essentially as described by Koopman et al. [15]. Oil red O were diluted in triethyl phosphate instead of isopropanol.

Statistical analysis. Results are expressed as mean ± standard deviation (SD). Paired statistical analysis was done using *t*-tests. Comparisons were considered significant at $P < 0.05$ and P values were two tailed.

Results

Levels of PPAR-γ and C/EBP-α are reduced during PSC activation

As levels of PPAR-γ and C/EBP-α were reported to decline during the activation of HSCs [6,9], we first confirmed their expression pattern in PSCs. PSCs were isolated from rat pancreas, and whole-cell lysates were prepared from PSCs at different stage of activation in culture and analyzed by Western blotting. The activation status of the PSC cultures was assessed by the expression of α-smooth muscle actin (α-SMA), a marker for the activated PSC phenotype [16] (Fig. 1). PPAR-γ and C/EBP-α proteins were detected in PSCs at day 3 after plating (PSCs-d3; preactivated PSCs), but their levels were markedly reduced in PSCs after passages 1, 3 (PSCs-P1, -P3; activated PSCs). It is interesting to note that PPAR-γ, C/EBP-α, and albumin, all of which were reported to cause phenotypic rever-

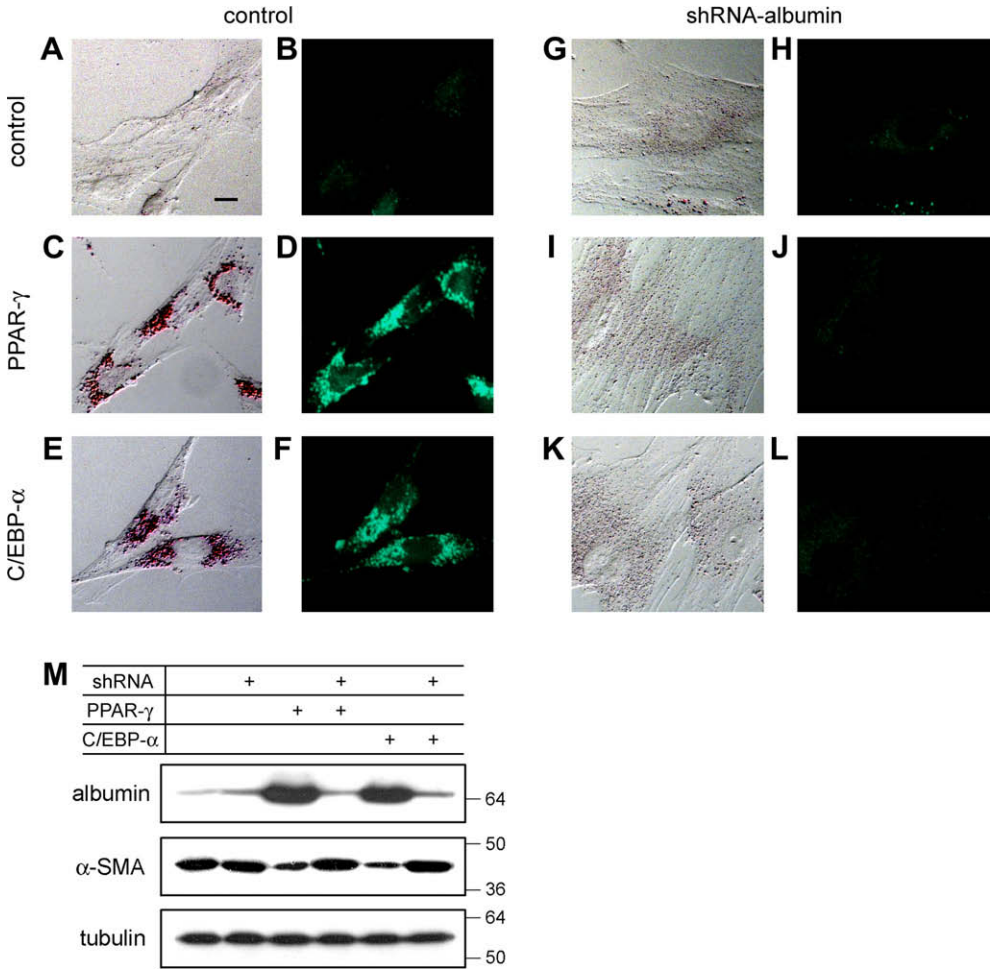

Fig. 3. Albumin acts downstream of PPAR-γ and C/EBP-α in PSCs. (A–L) PSCs-P2 were transfected with either the scrambled shRNA (A–F) or shRNA-albumin (G–L). The following day, shRNA-transfected cells were transfected with empty vector (1st row), pcDNA3.1-PPAR-γ (2nd row) or pcDNA3.1-C/EBP-α (3rd row), respectively, and examined for phenotypic changes. DIC images of Oil red O staining (A, C, E, G, I, and K) and immunofluorescence images (B, D, F, H, J, and L) using anti-albumin antibody were seen for the transfectants. Scale bar = 10 μm. (M) Levels of albumin and α-SMA in transient transfectants were analyzed by Western blotting.

sal into quiescent cells when expressed in activated stellate cells [7,9,12], exhibit similar expression patterns during stellate cell activation.

PPAR-γ and C/EBP-α induce albumin expression in PSCs

We then tested the possibility that PPAR-γ and C/EBP-α may modulate albumin gene expression. PSCs after passage 2 (PSCs-P2; activated PSCs) were transiently transfected with empty vector or expression vector for PPAR-γ or C/EBP-α and analyzed by Western blotting and real-time PCR. Expression of these adipogenic factors led to a >2.5-fold increase in both the protein and mRNA levels of albumin, and addition of a PPAR-γ ligand, troglitazone (10 μM), to PPAR-γ transfectants had no further effect on albumin levels (Fig. 2A and B). As it was reported that a treatment of activated HSCs with the adipocyte differentiation mixture (containing dexamethasone, isobutylmethyl-xanthine, and insulin; DMI) induces a panel of adipogenic transcription factors and causes the phenotypic reversal to quiescent HSCs [17], we also examined how adipocyte differentiation mixture affects albumin expression. Western blot analysis revealed that DMI treatment of activated PSCs also increases albumin expression by >2-fold (Fig. 2C).

Albumin mediates the effects of PPAR-γ and C/EBP-α on PSC phenotype

To examine the possibility that albumin acts downstream of PPAR-γ and C/EBP-α in phenotypic switch of PSCs, we blocked albumin expression using RNAi and measured its effects on PPAR-γ or C/EBP-α-induced phenotypic changes. Among two shRNA expression plasmids for albumin with different target regions (1 and 2), region 1 gave greater reduction (data not shown) and thus the plasmid including region 1 was used for the knockdown of albumin.

PSCs-P2 were transiently transfected with albumin-shRNA or the scrambled shRNA expression plasmids in combination with expression vector for PPAR-γ or C/EBP-α. When grown in standard culture conditions, control PSCs-P2 had elongate fibroblastoid morphology (Fig. 3A). Expression of PPAR-γ or C/EBP-α induced the formation of lipid droplets occupying the major part of the cell volume, as assessed by Oil red O staining, and caused the phenotypic reversal to the quiescent cells (Fig. 3C and E). Such a phenotypic change is accompanied by an increase in albumin levels and a decrease in α-SMA levels (Fig. 3M). Immunofluorescence study revealed that an intense signal for albumin is localized at the lipid droplets in PPAR-γ or C/EBP-α-transfected PSCs (Fig. 3D and F), whereas only a weak signal could be detected in the cytoplasm of control PSCs-P2 (Fig. 3B). Suppression of albumin expression, however, abrogated the effects of PPAR-γ or C/EBP-α expression; cells exhibited a flattened morphology with a failure of lipid droplet formation (Fig. 3I and K) and α-SMA levels increased (Fig. 3M). Thus, these findings suggest that albumin may be a downstream effector of PPAR-γ and C/EBP-α and can serve as a molecular target for anti-fibrotic therapy.

C/EBP-β binding to the albumin promoter is increased

To elucidate the molecular mechanism for the PPAR-γ- and C/EBP-α-induced albumin expression, we examined DNA–protein interaction with albumin promoter. Cell lysates were prepared from PSCs-P2 transiently transfected with empty vector or expression vector for PPAR-γ or C/EBP-α and mixed with biotinylated albumin promoter sequence containing the region between nt −386 and +16 from the transcription start site [18]. Sequence specific binding proteins after magnetic separation were analyzed by Western blotting. Interestingly, C/EBP-β binding to the albumin promoter was significantly increased by PPAR-γ and C/EBP-α, whereas C/EBP-α binding was not affected (Fig. 4A). No significant binding of PPAR-γ to the albumin promoter was detected (data not shown). Furthermore, PPAR-γ and C/EBP-α expression increased C/EBP-β protein levels but did not affect the phosphorylation levels of C/EBP-β at Ser-105, a positive regulatory site within its transactivation domain [19] (Fig. 4B).

Discussion

It has been demonstrated that PPAR-γ and C/EBP-α, master adipogenic transcription factors, induce a phenotypic switch from activated to lipid droplet-storing quiescent HSCs [7,9]. Our data suggest that albumin is a downstream effector of PPAR-γ and C/EBP-α in phenotypic modulation.

To elucidate the mechanism by which PPAR-γ and C/EBP-α induce albumin gene expression in PSCs, we performed DNA–protein interaction study and found that C/EBP-β binding to the albumin promoter (nt −386 and +16) is significantly increased by PPAR-γ and C/EBP-α (Fig. 4A). The albumin promoter has six closely spaced binding sites for nuclear proteins (A–F), among which the D-site contains high affinity binding sites for C/EBP-α and C/EBP-β [18]. C/EBP-α binding to albumin promoter was not affected (Fig. 4A) and binding of other transcription factors, PPAR-γ and HNF-1, was not detected (data not shown). Western blotting also revealed that PPAR-γ and C/EBP-α increase C/EBP-β expression levels but not the phosphorylation levels of C/EBP-β at Ser-105, a positive regulatory site (Fig. 4B). Although further study should be needed to elucidate detailed molecular mechanisms, our findings suggest that C/EBP-β may act as a positive regulator in PPAR-γ or C/EBP-α-induced albumin expression.

Numerous efforts have been made to provide therapeutic treatment for patients with liver fibrosis, but there are currently no effective therapeutic options except liver transplantation. Significant progress has been, however, made during the last decade in our understanding of the pathogenesis of hepatic and pancreatic fibrosis. Activation of fat-storing, quiescent stellate cells to myofib-

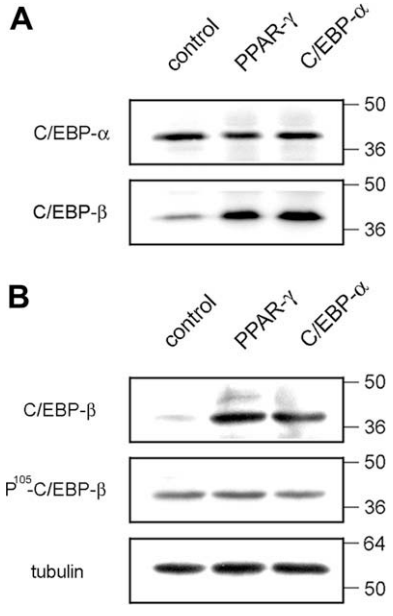

Fig. 4. PPAR-γ and C/EBP-α increase C/EBP-β binding to albumin promoter. (A) Cell lysates were prepared from PSCs-P2 overexpressing PPAR-γ or C/EBP-α and mixed with biotinylated albumin promoter sequence (nt −386 to +16), and DNA binding proteins were analyzed by Western blotting. (B) Cell lysates prepared above were analyzed for C/EBP-β by Western blotting. The Western blots are representative of at least two independent experiments.

roblastic cells is a key cellular event in the fibrogenesis, and understanding the molecular mechanism that underlie this cellular event provides novel therapeutic targets for anti-fibrotic drug development. HSCs have been a target in the treatment of hepatic fibrosis [20], and the analogy between the adipocyte differentiation and HSC transdifferentiation [21] supports the molecular basis to test adipogenic transcription factors as therapeutic targets [8,10]. Likewise, PSCs have been a new target in the treatment of chronic pancreatitis [22]. Since albumin was found to act downstream of PPAR-γ and C/EBP-α and induce phenotypic changes in both PSCs and HSCs [12], our findings raise a possibility that albumin can be an attractive molecular target for anti-fibrotic therapies.

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