LincRNA-p21 Inhibits the Wnt/β-Catenin Pathway in Activated Hepatic Stellate Cells via Sponging MicroRNA-17-5p

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Abstract

Background/Aims: It is known that the activation of hepatic stellate cells (HSCs) is a pivotal step in the initiation and progression of liver fibrosis. Aberrant activated Wnt/β-catenin pathway is known to accelerate the development of liver fibrosis. microRNAs (miRNAs)-mediated Wnt/β-catenin pathway has been reported to be involved in HSC activation during liver fibrosis. However, whether long noncoding RNAs (lncRNAs) regulate Wnt/β-catenin pathway during HSC activation still remains unclear. Methods: Long intergenic noncoding RNA-p21 (lincRNA-p21) expression was detected in Salvianolic acid B (Sal B)-treated cells. Effects of lincRNA-p21 knockdown on HSC activation and Wnt/β-catenin pathway activity were analyzed in Sal B-treated cells. In lincRNA-p21-overexpressing cells, effects of miR-17-5p on HSC activation and Wnt/β-catenin pathway activity were examined. Results: LincRNA-p21 expression was up-regulated in HSCs after Sal B treatment. In primary HSCs, lincRNA-p21 expression was down-regulated at Day 5 relative to Day 2. Sal B-inhibited HSC activation including the reduction of cell proliferation, α-smooth muscle actin (α-SMA) and type I collagen was inhibited by lincRNA-p21 knockdown. Sal B-induced Wnt/β-catenin pathway inactivation was blocked down by loss of lincRNA-p21. Notably, lincRNA-p21, confirmed as a target of miR-17-5p, suppresses miR-17-5p level. Lack of the miR-17-5p binding site in lincRNA-p21 prevents the suppression of miR-17-5p expression. In addition, the suppression of HSC activation and Wnt/β-catenin pathway induced by lincRNA-p21 overexpression was almost inhibited by miR-17-5p. Conclusion: We demonstrate that lincRNA-p21-inhibited
Wnt/β-catenin pathway is involved in the effects of Sal B on HSC activation and lincRNA-p21 suppresses HSC activation, at least in part, via miR-17-5p-mediated-Wnt/β-catenin pathway.

Introduction

Liver fibrosis, representing a wound-healing response of the liver to infectious, toxic or metabolic agents, is the final common pathway of chronic liver diseases [1]. In particular, liver fibrosis is a major cause of morbidity and mortality worldwide, leading to a big medical problem. Liver fibrosis is characterized by an abundant deposition of extracellular matrix (ECM) proteins such as collagens, associated with the distortion of normal liver architecture. Advanced liver fibrosis may finally result in cirrhosis and hepatocellular carcinoma [2]. In liver fibrosis, it is well known that activated hepatic stellate cells (HSCs) are considered as the principal cell type to promote the synthesis and deposition of ECM proteins [3]. Thus, effectively inhibiting the activation of HSC is a good therapeutic strategy for the treatment of liver fibrosis.

Regulatory noncoding RNAs (ncRNAs), such as microRNAs (miRNAs), have been reported to serve as negative regulators of gene expression by repressing protein translation or causing mRNA degradation [4]. It is well known that miRNAs take part in the control of diverse biological processes, including proliferation, differentiation, and apoptosis [5, 6]. Numerous studies have demonstrated that miRNAs are involved in the regulation of HSC activation[7-9]. For example, loss of miR-33a expression inhibits HSC activation and ECM protein production, at least in part, via the activation of PI3K/Akt pathway and PPAR-α [10]. Other ncRNAs, such as long intergenic noncoding RNAs (lincRNAs) and the heterogeneous group of long noncoding RNAs (lncRNAs), have been demonstrated to be deregulated in many human diseases including liver fibrosis [11, 12]. LncRNAs structurally resemble mRNAs but do not encode proteins, and is characterized by longer than 200 nucleotides in molecule length. Deregulated lncRNAs are reported to be involved in the regulation of HSC activation. For example, we previously found that long intergenic noncoding RNA-p21 (lincRNA-p21) suppresses activated HSCs through p21 [13]. However, the underlying mechanism of the role of lncRNA-p21 in liver fibrosis is still not completely understood.

Salvianolic acid B (Sal B) (i.e., lithospermates B, C_{36}H_{30}O_{16}, molecular weight = 718 g) is one of water soluble component extracted from Radix Salviae miltiorrhizae, which is a traditional herb in China. Sal B has been demonstrated to suppress liver fibrosis in animal model as well as patients with chronic hepatitis B [14, 15]. But so far, the role of lncRNA in the anti-fibrotic effects of Sal B on liver fibrosis had never been studied.

Materials and Methods

Materials

Sal B (purity 99%) was extracted and identified by the Chinese National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). Antibodies against type I collagen, α-smooth muscle actin (α-SMA), phosphorylated β-catenin (Y86), β-catenin and β-actin were obtained from Abcam (Cambridge, MA, USA). Chemically synthesized RNAs including negative control (miR-NC) and miR-17-5p mimics were obtained from GenePharma (Shanghai, China). For transfection, the cells were transfected with 1 μg of the chemically synthesized RNA.

Plasmid transfection

LincRNA-p21 siRNA (si-lnc-p21, AAGAAGAACGAGCAATTATGA), glycogen synthase kinase 3β (GSK3β) siRNA (AAGCGCTTCTCAGATAATTGC) and adenoviral vectors expressing lincRNA-p21 (Ad-lnc-p21) were designed and synthesized by Gene Pharma. The scrambled siRNA (siCtrl) and adenoviral vectors expressing
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Isolation and culture of mouse HSCs

Primary HSCs were isolated from male C57BL/6J mice as described previously [16]. Mice were provided by the Experimental Animal Center of Wenzhou Medical University and the experimental protocol was approved by the Institutional Animal Committee of Wenzhou Medical College. The isolated cells were cultured in Dulbecco’s modified Eagle’s medium with 10% fetal bovine serum, 100 U/mL penicillin, and 100 μg/mL streptomycin. The purity of cultures was confirmed by immunocytochemical staining for α-SMA and the purity reached > 98%. The primary HSCs were studied at day 2 after isolation throughout all the studies. Cells were treated with 10 μmol/L Sal B for 48 h and then transfected with si-lnc-p21 for additional 48 h. In addition, HSCs were transduced with Ad-lnc-p21 for 48 h and transfected with miR-17-5p mimics for additional 48 h. Cells were also transfected with GSK3β siRNA for 48 h.

Quantitative real-time PCR

The mirNeasy Mini kit (Qiagen, Valencia, CA, USA) was used to extract total RNA in primary HSCs [6]. Fifty nanograms of total RNA was reverse-transcribed to cDNA using the RevertAce qPCR RT Kit (Toyobo, Osaka, Japan). Gene expression was measured by real-time PCR using SYBR Green real-time PCR Master Mix (Toyobo, Osaka, Japan). The primers of H19, lincRNA-p21, metastasis-associated lung adenocarcinoma transcript 1 (MALAT1), Alu-mediated p21 transcriptional regulator (APTR), growth arrest-specific transcript 5 (GASS), alpha-1 (I) collagen (Col1A1), α-SMA, GAPDH, and U6 were designed as described previously [17-22]. miR-17-5p expression was detected using TaqMan MicroRNA Assay (Applied Biosystems, Foster City, CA). The GAPDH (Applied Biosystems, Foster City, CA) level was used to normalize the relative abundance of lncRNAs and mRNAs. U6 snRNA (Applied Biosystems, Foster City, CA) was used to normalize the relative abundance of miRNAs.

Western blot analysis

To obtain total protein, HSCs were lysed with ice-cold lysis buffer (50 mM Tris-HCl, pH 7.4, 100 mM 2-Mercaptoethanol, 2% w/v SDS, 10% glycerol). Cytoplasmic and nuclear expression of β-catenin was also evaluated by western blot. Cytoplasmic and nuclear extracts were prepared according to the manufacturer’s instruction (Beyotime Biotechnology, Jiangsu, China) [23, 24]. Protein samples were quantified and separated by SDS-PAGE. Then, western blot assay was performed as described previously [25].

Proliferation assay

Cells were seeded in 96-well plates at a density of 1×10^3 cells per well and cultured for 24 h. Cells were ready for assessing cell proliferation after their relative treatment. Cell proliferation was assessed using CCK-8 (Dojindo, Kumamoto, Japan) according to manufacturer’s instructions. Absorbance was determined at 450 nm on a microplate reader (Molecular Devices, Sunnyvale, CA, USA).

TCF reporter activity assay

Cells were transiently transfected with TOPFLASH and FOPFLASH (Upstate Biotechnology Inc., Lake Placid, NY, USA) using Lipofectamine 2000. Twenty-four hours after transfection, the cells were harvested and luciferase and Renilla luminescence were measured using the Dual-Luciferase Reporter Assay System (Promega, Wisconsin, WI, USA) on a luminometer (BioTek Instruments, Winooski, VT, USA). TCF reporter activity was presented as the ratio of firefly-to-Renilla luciferase activity.

RNA binding protein immunoprecipitation (RIP) assay

RIP experiment was conducted using the EZ-Magna RIP Kit (Millipore) according to the manufacturer’s instructions. Briefly, primary HSCs at 80–90% confluency were lysed in complete RIP lysis buffer, following by incubation with RIP buffer including magnetic beads coupled with anti-Argonaute-2 (Ago2) antibody (Abcam). Isotype-matched immunoglobulin G (IgG) was used as a negative control. After samples were incubated with protease k, immuno precipitated RNA was isolated. qRT-PCR was performed to analyze the levels of lincRNA-p21 and miR-17-5p in the precipitates.
Luciferase activity assay

According to RNA22 analysis, oligonucleotides containing mouse lincRNA-p21 target sequence were annealed and cloned into the pmirGLO plasmids (Promega, Madison, WI, USA) to generate the pmioGLO-lincRNA-p21 vector: lincRNA-p21 for miR-17-5p (position of 89–113) forward, 5'-ATAGCCACAACTCTCTGCCG-3', and reverse, 5'-GGGATAGACCGACAGATACA-3'. Empty plasmid pmirGLO was used as a negative control. Luciferase reporter plasmids plus miR-17-5p mimics or miR-NC were co-transfected into HEK293T cells using Lipofectamine 2000. Forty-eight hours after transfection, relative luciferase activity was examined in a luminometer using a Dual-Luciferase Reporter Assay System (Promega).

Statistical analysis

Data from at least three independent experiments were expressed as the mean ± SD. Comparisons between two groups and multiple groups were made using Student's t-test and one-way analysis of variance, respectively. P<0.05 was considered significant. All statistical analyses were performed with SPSS software (version 13; SPSS, Chicago, IL).

Results

LincRNA-p21 is increased by Sal B and reduced in activated primary HSCs

Recent studies have shown that many lncRNAs including H19 [12], lincRNA-p21 [13], GAS5 [19], MALAT1 [20], and APTR [21], are involved in the progression of liver fibrosis. To investigate whether these lncRNAs play a role in the effects of Sal B on HSC activation, the expressions of H19, lincRNA-p21, MALAT1, APTR and GAS5 were examined in Sal B-treated primary HSCs. Our results showed that Sal B caused an increase in lincRNA-p21 and GAS5, with no effect on H19, MALAT1 and APTR (Fig.1A). Our data indicated that the expressions of lincRNA-p21 and GAS5 were increased by 12.6-fold and 1.4-fold, respectively, in Sal B group relative to the control group (Fig.1A). Expression of lincRNA-p21 enhanced by Sal B was far higher than that of GAS5, therefore, lincRNA-p21 was chosen for the next experiment. LincRNA-p21 was also examined in primary HSCs. Compared with Day 2, lincRNA-p21 was decreased by 52% at Day 5 (Fig.1B), suggesting that lincRNA-p21 was reduced during HSC activation. These data demonstrate that lincRNA-p21, down-regulated in activated HSCs, may be involved in the effects of Sal B on HSC activation.

The inhibitory effects of Sal B on HSC activation are suppressed by the loss of lincRNA-p21

The effects of Sal B on cell proliferation, collagen expression and HSC transdifferentiation were examined in primary HSCs. As shown by CCK-8 assays, the growth rate was reduced to...
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63% in Sal B-treated cells compared to untreated cells (Fig. 2A). The results of real-time PCR indicated that the mRNA expressions of α-SMA and Col1A1 were decreased by 76% and 84%, respectively, in Sal B-treated cells relative to untreated cells (Fig. 2B). Of note, si-Ctrl had no effect on the results caused by Sal B (Fig. 2A and Fig. 2B). Consistent with the mRNA results, Sal B resulted in a significant reduction in α-SMA and type I collagen (Fig. 2C and Fig. 2D). Our results suggest an inhibitory role of Sal B in the activation of HSC. Next, the role of lincRNA-p21 in anti-fibrotic effects Sal B on HSC activation was examined. Interestingly, Sal B-suppressed cell proliferation was restored by lincRNA-p21 knockdown (Fig. 2A). Moreover, the reduced mRNA and protein expressions of α-SMA and Col1A1 induced by Sal B were blocked down by loss of lincRNA-p21 (Fig. 2B-D). These data suggest that lincRNA-p21 plays a vital role in the effects of Sal B on HSC activation.

Sal B-inhibited Wnt/β-catenin pathway was re-activated by loss of lincRNA-p21

Aberrant activated Wnt/β-catenin pathway has been reported to contribute to the progression of liver fibrosis [25]. As shown in Fig. 3A, there was a significant increase in β-catenin in cytoplasm and nucleus during culture days. In addition, c-myc, one of the downstream target genes of Wnt/β-catenin pathway, was found to be increased during culture days (Fig. 3A). Then, we examined whether Sal B had an effect on the activity of Wnt/β-catenin pathway. Sal B treatment led to a significant reduction in TCF activity (Fig. 3B). As shown by the results of western blot, the protein level of P-β-catenin was increased by 4.5-fold in Sal B-treated cells compared with untreated cells (Fig. 3C). In line with it, β-catenin in cytoplasm and nucleus was significant reduced by Sal B (Fig. 3C). Next, we examined the
activity of Wnt/β-catenin pathway in Sal B-treated cells transfected with si-lnc-p21. Of note, lincRNA-p21 knockdown led to the restoration of Sal B-inhibited TCF activity (Fig.3B). Loss of lincRNA-p21 inhibited Sal B-induced P-β-catenin (Fig.3C). Meanwhile, β-catenin protein reduced by Sal B in cytoplasm and nucleus was restored by loss of lincRNA-p21 (Fig.3C). Our data demonstrate that Sal B inhibited Wnt/β-catenin pathway was through lincRNA-p21. To exclude nuclear contamination in the cytoplasmatic fraction, LaminA (a marker for the nuclear fraction) was detected in the cytoplasmatic fraction. Also, HSP90 (a marker for the cytoplasmatic fraction) was detected in the nuclear fraction. As shown

Fig. 3. Sal B-inhibited Wnt/β-catenin pathway was blocked down by loss of lincRNA-p21. Primary 2-day-old HSCs were treated with Sal B for 48 h and then transfected with si-lnc-p21 for 48 h. (A) β-catenin in cytoplasm and nuclear, and c-myc were examined in primary HSCs at Day 1, Day 2 and Day5. (B) TCF activity. (C) P-β-catenin and β-catenin in cytoplasm and nuclear. Each value is the mean ± SD of three experiments. *P<0.05 compared with the control and #P<0.05 compared with the Sal B group.

Fig. 4. Interaction of miR-17-5p with lincRNA-p21. (A) miR-17-5p was detected in cells transduced with Ad-lnc-p21 or Ad-lnc-p21-mut. (B) Schematic diagram of the miR-17-5p binding site in the lincRNA-p21 based on RNA22 software. (C) Relative luciferase activities of luciferase reporters bearing wild-type or mutant lincRNA-p21 were analyzed 48 h following transfection with the indicated miR-17-5p mimics or miR-NC in HEK293T cells. *P<0.05 compared with the control.
LincRNA-p21 inactivates Wnt/β-catenin pathway was through miR-17-5p

Our previous study has demonstrated that miR-17-5p overexpression contributes to the activation of Wnt/β-catenin pathway and Sal B inhibited Wnt/β-catenin pathway via miR-17-5p [25]. To determine whether miR-17-5p is involved in lincRNA-p21-inhibited Wnt/β-catenin pathway, miR-17-5p was detected in lincRNA-p21-overexpressing HSCs. There was a significant reduction in miR-17-5p in lincRNA-p21-overexpressing HSCs (Fig.4A). Bioinformatic analysis (RNA22) for miRNA recognition sequences on lincRNA-p21 revealed the presence of a putative miR-17-5p site (Fig.4B). To investigate whether lincRNA-p21 is a target of miR-17-5p, we used pmirGLO construct to generate a lincRNA-p21 luciferase reporter containing the miR-17-5p-binding sites (pmirGLO-lnc-p21-wt) or mutated sites (pmirGLO-lnc-p21-mut). miR-17-5p caused a reduction in luciferase activity of pmirGLO-lnc-p21-wt (Fig.4C). Moreover, miR-17-5p had no effect on luciferase activity of pmirGLO-lnc-p21-mut (Fig.4C). In line with it, lincRNA-p21 overexpression with the mutation of the miR-17-5p binding site did not suppress miR-17-5p expression, indicating that lack of the miR-17-5p binding site in lincRNA-p21 prevents the suppression of miR-17-5p expression (Fig.4A). Taken together, lincRNA-p21 is a target of miR-17-5p.

miR-17-5p is involved in the effects of lincRNA-p21 on HSC activation

To investigate the role of miR-17-5p in the effects of lincRNA-p21 on HSC activation, cell proliferation, collagen expression and HSC transdifferentiation were examined in lincRNA-p21-overexpressing cells transfected with miR-17-5p mimics. Our results showed...
that lincRNA-p21 overexpression led to a reduction in cell growth rate (Fig. 5A). Also, the expressions of type I collagen and α-SMA were down-regulated by lincRNA-p21 (Fig. 5B-D). These results confirm the inhibitory role of lincRNA-p21 in HSC activation. However, these effects induced by lincRNA-p21 were almost blocked down by miR-17-5p, suggesting that lincRNA-p21 inhibited HSC activation, at least in part, via miR-17-5p (Fig. 5).

miR-17-5p inhibited lincRNA-p21-induced Wnt/β-catenin pathway inactivation

To further confirm whether lincRNA-p21 inhibits miR-17-5p-mediated Wnt/β-catenin pathway, we examined P-β-catenin, β-catenin and TCF activity in lincRNA-p21-overexpression cells transfected with miR-17-5p. As expected, increased P-β-catenin and reduced TCF activity induced by lincRNA-p21 were inhibited by miR-17-5p (Fig. 6A and Fig. 6B). In line with it, miR-17-5p restored lincRNA-p21-suppressed β-catenin in cytoplasm and nucleus (Fig. 6A). Notably, as shown in Fig. 6A, there was no LaminA in the cytoplasmatic fraction and no HSP90 in the nuclear fraction. In addition, the protein expression of GSK3β, a key element of β-catenin/Wnt pathway, was examined in lincRNA-p21-overexpression cells transfected with miR-17-5p. It was found that GSK3β was increased by lincRNA-p21, which was blocked down by miR-17-5p (Fig. 6A). Interestingly, the loss of GSK3β led to an increase in miR-17-5p and a reduction in lincRNA-p21 (Fig. 6C), suggesting that activated Wnt/β-catenin pathway inhibited lincRNA-p21 and contributed to enhance miR-17-5p expression. Of note, RIP experiment showed that compared with IgG pellets, lincRNA-p21 and miR-17-5p were enriched by 10.5-fold and 16.1-fold in the Ago2 pellet, respectively (Fig. 6D). In line with luciferase assays, our data confirmed that lincRNA-p21 is recruited to Ago2-related RNA-induced silencing complexes and functionally interacts with miR-17-5p. Our results demonstrate that lincRNA-p21 induces the inactivation of Wnt/β-catenin pathway is through miR-17-5p.
Discussion

It is known that there is an increase in cell proliferation, ECM production and α-SMA synthesis in activated HSCs compared with quiescent HSCs [26]. In our study, Sal B treatment caused the suppression of activated HSCs including the reduction of cell growth rate, type I collagen and α-SMA. Interestingly, it was found that lincRNA-p21 was increased by Sal B. Loss of lincRNA-p21 resulted in the suppression of Sal B-inhibited HSC activation, indicating that lincRNA-p21 may be responsible for the effects of Sal B on HSC activation. Further studies showed that the inactivation of Wnt/β-catenin pathway induced by Sal B was re-activated by lincRNA-p21 knockdown. Our data demonstrate that Sal B inhibits HSC activation, at least in part, through lincRNA-p21-mediated Wnt/β-catenin pathway and this is a first report.

Sustained activated Wnt/β-catenin pathway is involved in the activation of HSCs [27, 28]. In this study, as indicated by the results of western blot, β-catenin in cytoplasm and nucleus was increased during HSC activation. By contrast, inactivated Wnt/β-catenin pathway contributes to the suppression of liver fibrosis. For example, the activation of HSCs could be inhibited by Wnt antagonist such as Dickkopf-1 (Dkk-1) in vitro [27, 29]. miR-17-5p, often deregulated in various cancers, has been reported to act as an oncogenic miRNA [30-32]. Previously, we demonstrated that miRNA-17-5p-activated Wnt/β-catenin pathway contributes to the progression of liver fibrosis and is involved in the anti-fibrotic effects of Sal B on HSC activation [25]. In this study, we found that miR-17-5p was reduced by lincRNA-p21. Luciferase activity assays revealed that lincRNA-p21 is a target of miR-17-5p. Further studies showed that the effects of lincRNA-p21 on HSC activation and Wnt/β-catenin pathway were blocked down by miR-17-5p. All these data suggest that miR-17-5p-mediated Wnt/β-catenin pathway is involved in the effects of lincRNA-p21 on HSC activation. A recent study has been reported that non-canonical Wnt is involved in activated rat hepatic stellate cells through influencing HSC survival and paracrine stimulation of Kupffer cells [33]. It is not clear whether non-canonical Wnt is involved in the effects of lincRNA-p21 on HSC activation and further studies are warranted to prove it.

In past decades, lncRNAs are generally considered as simply transcriptional “noise” or cloning artifacts. Emerging evidence has demonstrated that lncRNAs can act as vital regulators in the control of fundamental biological processes at multiple levels [20]. LncRNAs have been reported to regulate various cellular functions through diverse molecular mechanisms such as chromatin modification, transcriptional regulation and post-transcriptional regulation [11, 34, 35]. Recently, lncRNAs have been shown to serve as competing endogenous RNAs (ceRNAs) to sponge miRNAs, consequently modulating the de-repression of miRNA targets [36, 37]. For example, Wang et al. found that up-regulated lncRNA-UCA1 contributes to progression of hepatocellular carcinoma through inhibition of miR-216b [37]. LincRNA-p21 has been reported to be deregulated in various human diseases and act as a tumor suppressor in cancers. For instance, Wang et al. found that lincRNA-p21 suppresses β-catenin signaling and tumorigenicity of colorectal cancer stem cells [38]. In line with it, we found that lincRNA-p21 suppresses Wnt/β-catenin signal through sponging miR-17-5p. In vitro, lincRNA-p21 contributes to a reduction in miR-17-5p. With the restoration of miR-17-5p, the anti-fibrotic effects of lincRNA-p21 were almost inhibited. Moreover, lincRNA-p21-inhibited Wnt/β-catenin pathway was also blocked down by miR-17-5p overexpression. Notably, lincRNA-p21 is a target of miR-17-5p and lack of the miR-17-5p binding site in lincRNA-p21 prevents the suppression of miR-17-5p expression. Consistent with luciferase assays, RIP assays further demonstrated that lincRNA-p21 is recruited to Ago2-related RNA-induced silencing complexes and functionally interacts with miR-17-5p. Our data reveal that lincRNA-p21 inhibits HSC activation, at least in part, through inhibiting miR-17-5p-mediated Wnt/β-catenin pathway. However, the mechanism of the direct regulation of lincRNA-p21 by Sal B still remains largely unknown and further studies are warranted.

In conclusion, our results reveal that lincRNA-p21-inhibited Wnt/β-catenin pathway is involved in the effects of Sal B on HSC activation and lincRNA-p21 suppresses HSC activation,
at least in part, via miR-17-5p-mediated-Wnt/β-catenin pathway. Our results provide a new insight of the role of lincRNA-p21-inhibited Wnt/β-catenin signaling in liver fibrosis.

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Disclosure Statement

None.

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