Inhibition of pro-collagen I expression by oxymatrine in hepatic stellate cells is mediated via nuclear translocation of Y-box binding protein 1

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Abstract. Accumulating evidence indicated that oxymatrine (OMT), an alkaloid compound from the Chinese medicinal herb *Sophora flavescens*, exhibits activity against hepatic fibrosis. The present study attempted to explore the underlying mechanisms of OMT-mediated inhibition of collagen production. For this, the LX-2 human hepatic stellate cell line was treated with OMT (240, 480 or 960 mg/l) for 3-5 days. The endogenic expression of pro-collagen I was decreased by OMT in a dose- and time-dependent manner, accompanied with the downregulation of Y-box binding protein 1 (YB-1), a vital transcription factor, particularly on the fourth day of incubation with a high concentration of OMT. To further explore the intracellular changes in YB-1 levels, nuclear/cytoplasmic proteins were extracted separately, and subsequent western blot analysis revealed a significant upregulation of YB-1 in the nucleus in parallel with its downregulation in the cytoplasm, indicating the nuclear translocation of YB-1 induced by OMT treatment. In another experiment, knockdown of YB-1 using small interfering RNA led to elevated mRNA levels of collagen I, thereby reversing the effects of OMT treatment. In conclusion, these present study suggested that the attenuation of pro-collagen I expression caused by OMT was, to a certain extent, mediated via nuclear translocation of YB-1.

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Introduction

Y-box binding protein 1 (YB-1), a member of the highly evolutionarily conserved Y-box family, has an important role in cell physiological activities, including gene transcription (1-3), translation (4,5), damage repair and cancer drug resistance (6,7). It consists of three principal domains (Fig. 1): i) The N-terminal domain, also termed the A/P domain, is rich in alanine and proline and involved in transcriptional regulation. ii) The middle part is the cold shock domain (CSD), a highly conserved sequence, which performs the core function, i.e., it binds to the Y-box, an inverted CCAAT box in the promoter of numerous specific genes, including major histocompatibility complex class II, multi-drug resistance gene 1 as well as cyclin A and B1 (8-10). iii) An elongated C-terminal domain, containing alternating clusters of positively and negatively charged amino-acid residues, which is implicated in protein-protein interactions and serves as a pivotal signaling peptide during nuclear translocation (11).

Several studies have reported that CSD is implicated in the regulation of the transcription of the α1- and α2-strands of collagen I [COLα1(I) and COLα2(I)], which are intrinsically linked to the development of hepatic fibrosis (12). Type I collagen is a heterotrimer composed of two α1-strands and one α2-strand. YB-1 was reported to suppress the transcription of the COLα1(I) gene via binding to collagen Y-box element or transforming growth factor (TGF)-β response element in the promoter of COLα1(I) (13,14). Furthermore, YB-1 was confirmed to be involved in interferon-γ (IFN-γ)-induced downregulation of the COLα2(I) gene (15) through binding to the IFN-γ-responsive element in the promoter of COLα2(I) (16).

YB-1 regulates pro-collagen I expression in the nucleus only; therefore, nuclear translocation of YB-1 is essential for it to exert its function. Agents with the ability to promote the nuclear translocation of YB-1 may have beneficial effects on hepatic fibrosis. In previous studies, p53 (17), ultraviolet irradiation (18) and the small molecule HSc025 (19) were demonstrated to enhance the nuclear translocation of YB-1.

Oxymatrine (OMT) is the major active component of the Chinese medicinal herb Kushen, which is the dried root of *Sophora flavescens*. The anti-viral (20), anti-oxidative (21,22), anti-fibrotic (23) and immunoregulatory

effects (24,25) of OMT have been demonstrated in numerous studies (20,21,23-25). At present, OMT is widely applied in the clinic to protect the liver from fibrosis.

The present study assessed whether OMT may exert its long-known effects against hepatic fibrosis via promoting the nuclear translocation of YB-1 to suppress the production of pro-collagen I. The present study considerably enhanced the current understanding of the underlying mechanism of the anti-fibrotic effects of OMT.

Materials and methods

Cell culture. The LX-2 human hepatic stellate cell (HSC) line (Shanghai Fuxiang Biotechnology Co., Ltd, Shanghai, China), was cultured in Dulbecco's modified Eagle's medium (DMEM; Invitrogen; Thermo Fisher Scientific, Waltham, MA, USA) supplemented with 10% fetal bovine serum (FBS; Invitrogen) and kept in an incubator containing 5% CO_2 at 37°C. The cells were sub-cultured every 3-4 days.

Drug treatment. OMT (BOC Sciences, New York, NY, USA) was dissolved in complete medium to produce a stock solution. Prior to use in the assays, cells were synchronized by serum-starvation for 24 h. Cells ($5x10^5$) were then seeded into 60 mm dishes. Subsequently, OMT solution was added to generate a series concentrations of OMT (240, 480 and 960 mg/l). Furthermore, a control group without OMT treatment was established. The cells were incubated for 3-5 days prior to analysis.

Reverse-transcription quantitative polymerase chain reaction (RT-qPCR) analysis. RNA from LX2 cells was extracted by using TRI Reagent® (Sigma-Aldrich, St. Louis, MO, USA) according to the manufacturer's instructions. cDNA was synthesized from 1 μg RNA using the FastQuant RT kit (Tiangen, Beijing, China) according to the manufacturer's protocol. Real Time PCR mixtures (10 μl) were then prepared in triplicate, each containing 1 μl cDNA template, 5 μl of SYBR® Premix Ex *Taq* (TaKara, Japan), 0.2 ul of ROX Reference Dye II (Takara Bio, Inc., Otsu, Japan), 0.2 μl primer (10 mM; Genewiz, Inc., Suzhou, China), and 3.6 ml DEPC-treated water. Primers were as follows: COLα1(I), 5'-CATGTTCAGCTTTGTGGACC-3' (forward) and 5'-TTCTGTACGCAGGTGATTGG-3' (reverse); COLα2(I), 5'-TGCTGCTCAGTATGATGGAAA-3' (forward) and 5'-CAGGTCCTTGGAAACCTTGA-3' (reverse); YB-1, 5'-TCGCCAAAGACAGCCTAGAGA-3' (forward) and 5'-TCT GCGTCGGTAATTGAAGTTG-3' (reverse); α-SMA, 5'-AGC AGTTGTAGCTACCCGCC-3' (forward) and 5'-CACGAT GGACGGGAACAC-3' (reverse). GAPDH, 5'-GAAGGTGAA GGTCGGAGTC-3' (forward) and 5'-GAAGATGGTGAT GGGATTTC-3' (reverse). cDNA samples were amplified in an Applied Biosystems 7500 Real-Time PCR system (Thermo Fisher Scientific). The thermocycling conditions included initial denaturation at 95°C for 30 sec, followed by 40 cycles of 95°C for 5 sec and 60°C for 34 sec. Finally all quantified values were normalized to the endogenous GAPDH control. Gene expression levels were analyzed using the ΔΔCt method (26).

Transient transfection. LX2 cells in 60 mm cell culture dishes without serum were cultured to ~50% confluence on the second day. The cells were transiently transfected with 300 pmol small interfering (si)RNA (Gene Pharma, Shanghai, China) targeting YB-1 (siYB-1) or negative control siRNA (NC) mixed with 15 μl Lipofectamine 2000 (Invitrogen) at 6 h prior to treatment with 960 mg/l OMT. Cells were harvested after incubation for four days. The siYB-1 sequence was UGA CACCAAGGAAGAUGUAUU and the NC sequence was UUCUCCGAACGUGUCACGUTT.

Nuclear/cytoplasmic extraction and western blot analysis. Nuclear and cytoplasmic proteins were separated using the Nuclear and Cytoplasmic Protein Extraction kit (Beyotime Institute of Biotechnology, Shanghai, China). The separation procedure was performed with caution to avoid contamination of the nuclear protein in the pellet with cytoplasmic extract.

Cytoplasmic and nuclear extracts and total protein samples were quantified using a Bichinchoninic Acid Protein assay kit (Beyotime Institute of Biotechnology, Shanghai, China) and then mixed with 4X Protein SDS-PAGE loading buffer (Takara Bio, Inc.) at 100°C for 20 min. Proteins were separated on 10-15% SDS-PAGE (Invitrogen; Thermo Fisher Scientific) and transferred onto polyvinylidene Fluoride membranes (EMD Millipore, Billerica, MA, USA). Following blocking with Tris-Buffered Saline and Tween 20 (TBST) with 5% fat-free milk, the membranes were incubated with the following primary antibodies overnight at 4°C: Anti-α-smooth muscle actin (α-SMA; cat. no. ab32575), anti-extracellular signal-regulated kinase 1 and 2 (ERK1/2; cat. no. ab17942), anti-YB-1 (cat. no. ab12148; all 1:1,000; Epitomics, Burlingame, CA, USA), as well as anti-GAPDH (cat. no. ab2302) and anti-histone3 (cat. no. 2514256; both 1:1,000; Millipore, Billerica, MA, USA). The membranes were then washed with TBST for 30 min and incubated with secondary antibodies conjugated with horseradish peroxidase for 2 h at room temperature. Finally the images were captured using a gel imaging analysis system (Tanon 4100, Tanon Science and Technology Co., Ltd., Shanghai, China).

Statistical analysis. Blots were representative of three independent experiments performed in triplicate. Statistical analysis of the data was performed using Student's t-test and analyzed using SPSS software version 13.0 (SPSS, Inc., Chicago, IL, USA). Data were expressed as the mean ± standard deviation. $P<0.05$ was considered to indicate a statistically significant difference.

Results

OMT reduces the expression of endogenic type I pro-collagen. RT-qPCR analysis indicated a marked decrease in COLα1(I) and COLα2(I) gene expression following incubation with OMT (240, 480 and 960 mg/l for 3-5 days) in a dose- and time-dependent manner (Fig. 2). The high concentration of OMT (960 mg/l) was then used in the subsequent experiments.

OMT accelerates nuclear translocation of YB-1. Transcription factor YB-1 is known to inhibit pro-collagen I production (13-16). However, factors modulating the activity of YB-1 have remained to be elucidated.

The present study hypothesized that OMT inhibits the expression of pro-collagen I via facilitating the nuclear translocation of YB-1. In order to test this hypothesis, LX-2 cells were
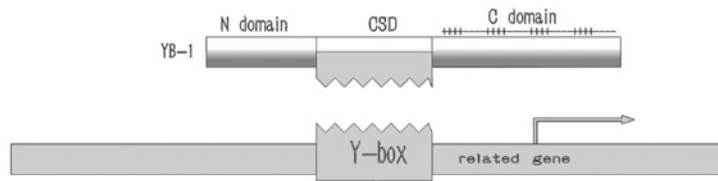
Figure 1. YB-1 is composed of three principle domains: N/C domain and CSD. The CSD, the functional part of YB-1, is able to bind to the Y-box in the promoter of specific genes, which regulates their transcription. YB-1, Y-box binding protein 1; CSD, cold shock domain.
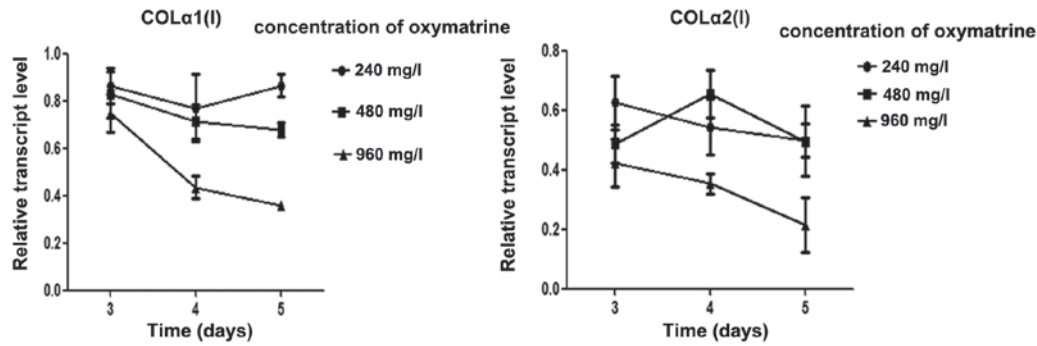

Figure 2. Reverse-transcription quantitative polymerase chain reaction analysis of COLα1(I) and COLα2(I) expression in LX-2 cells treated with oxymatrine (240, 480 or 960 mg/l) for 3-5 days. Transcription of COLα1(I) and COLα2(I) relative to GAPDH was decreased in a dose- and time-dependent manner. COLα1(I), α1-strand of collagen I.
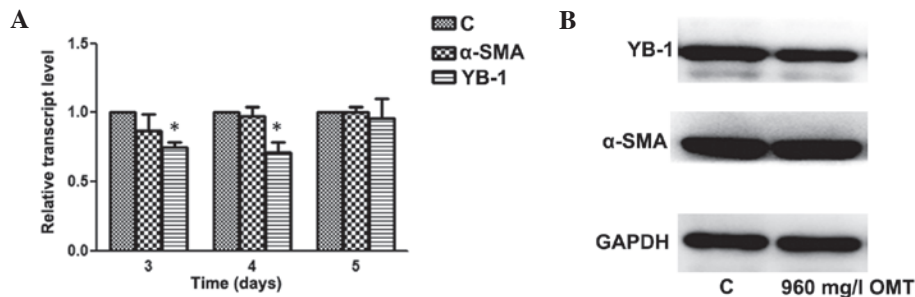

Figure 3. OMT effectively inhibits the expression of YB-1 in LX-2 cells. (A) LX-2 cells treated with OMT (960 mg/l) showed lower levels of YB-1 mRNA on the third and fourth day (*P<0.05) compared to those in the control group. However, α-SMA was not significantly affected. (B) On the fourth day, total YB-1 was markedly suppressed at the protein level, while α-SMA expression was not obviously affected. OMT, oxymatrine; SMA, smooth muscle actin; YB-1, Y-box binding protein 1; C, control.
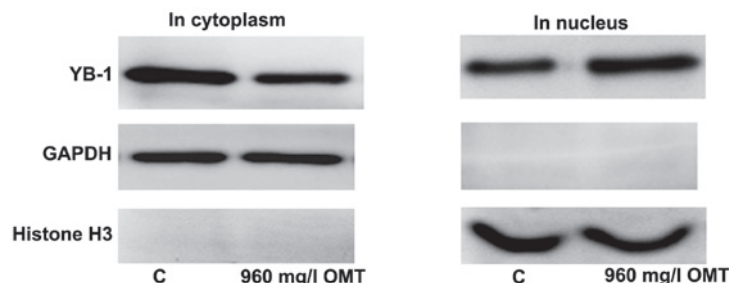

Figure 4. LX-2 cells treated with 960 mg/l OMT for four days showed a decrease of YB-1 in the cytoplasm and an increase of YB-1 in the nucleus, as compared with that in the control group. OMT, oxymatrine; YB-1, Y-box binding protein 1; C, control.

treated with OMT (960 mg/l), which resulted in a reduction in YB-1 expression at the transcriptional and translational level, particularly on the fourth day of incubation, while α-SMA expression was not affected (Fig. 3). As it is well known that YB-1 regulates gene transcription in the nucleus only, the present study examined the expression of YB-1 in the cytoplasm and nucleus separately. The results showed that LX-2 cells treated with 960 mg/l OMT for four days exhibited a decrease of YB-1 in the cytoplasm and an increase of YB-1 in the nucleus as compared with that in the control group (Fig. 4).
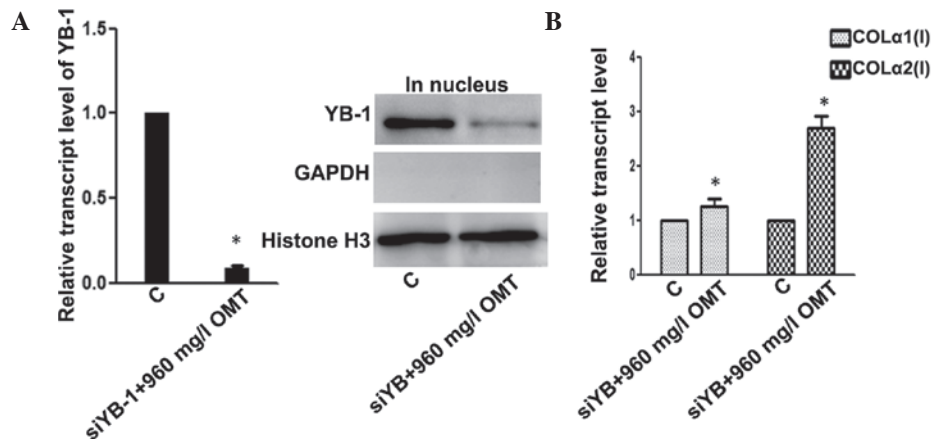
Figure 5. Depletion of YB-1 reverses the inhibitory effect of OMT on pro-collagen. (A) When LX-2 cells were treated with siYB-1 and 960 mg/l OMT for 4 days, the expression of YB-1 was significantly reduced at the mRNA and nuclear protein level. (B) mRNA levels of COLα1(I) and COLα2(I) were elevated following OMT treatment. *P<0.05 vs. C. siYB-1, small interfering RNA targeting YB-1; COLα1(I), α1-strand of collagen I; OMT, oxymatrine; YB-1, Y-box binding protein 1; C, control.
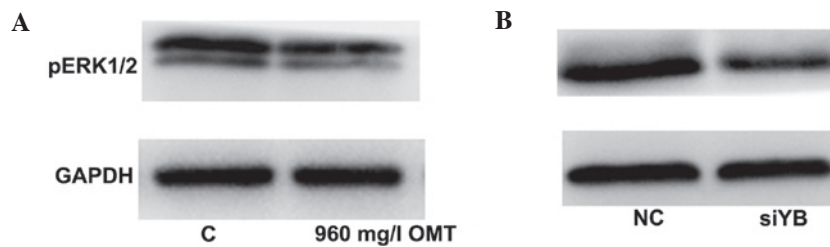

Figure 6. The ERK1/2 signaling pathway is involved in the anti-fibrotic effects of OMT-induced YB-1. (A) When LX-2 cells were only treated with 960 mg/l OMT for four days, the phosphorylation level of ERK1/2 protein declined. (B) With siYB-1 and 960 mg/L OMT treatment for four days, LX-2 cells showed lower expression of pERK2 (44 KDa) in comparion with the NC group (NC + 960 mg/l OMT). NC, scrambled siRNA-transfected negative control; siYB-1, siRNA targeting YB-1; YB-1, Y-box binding protein 1; C, control; OMT, oxymatrine; pERK, phosphorylated extracellular signal-regulated kinase; siRNA, small interfering RNA.

Depletion of YB-1 reverses the inhibitory effects of OMT on pro-collagen I production. In the abovementioned experiments, OMT restrained the expression of pro-collagen I, at least in part, by enhancing the nuclear translocation of YB-1. In order to further verify this mechanism, the effects of YB-1 knockdown on the expression levels of pro-collagen I were assessed. As shown in Fig. 5A, transient transfection with siYB-1 successfully knocked down YB-1. As expected, siYB-1 abrogated the inhibitory effects of OMT (960 mg/l) on pro-collagen I synthesis, as indicated by significantly elevated mRNA levels of COLα1(I) and an even greater elevation of COLα2(I) mRNA expression (Fig. 5B).

The ERK1/2 signaling pathway is involved in the anti-fibrotic effects of YB-1. Since YB-1 participates in the negative regulation of fibrosis, the present study investigated the associated signaling pathways. It was found that the phosphorylation level of ERK1/2 proteins was positively associated with the changes in YB-1 expression. In the cells treated with OMT (960 mg/l), phosphorylated ERK1/2 proteins and YB-1 were declined compared to those in the control group. Furthermore, the levels of phosphorylated ERK1/2 proteins were also decreased as a result of a knockdown of YB-1 in LX-2 cells, indicating that the ERK1/2 signaling pathway have a significant role in OMT-mediated inhibition of pro-collagen I expression (Fig. 6).
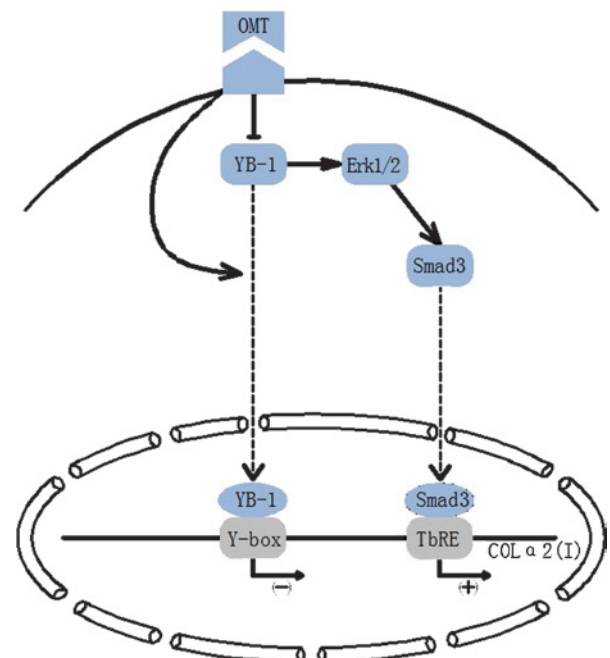

Figure 7. Attenuation of COLα2(I) expression by OMT was, to a certain extent, mediated via nuclear translocation of YB-1. In parallel, ERK1/2 was inhibited, which indirectly affected the TGF-β signaling pathway. YB-1, Y-box binding protein 1; OMT, oxymatrine; ERK, extracellular signal-regulated kinase; COLα1(I), α1-strand of collagen I; TbRE, TGF-β-responsive element; TGF, transforming growth factor.

Discussion

It is thought that patients with hepatocellular carcinoma (HCC) present with hepatitis or cirrhosis prior to developing carcinoma, with fibrosis having a central role in the pathology of all of these conditions (27). Furthermore, overexpression of YB-1, a multifunctional transcription factor, was observed in HCC, which made it a promising prognostic biomarker used in the clinic (28,29).

However, excess YB-1 can repress type I pro-collagen expression, resulting in a reduction of fibrosis. It is well known that the TGF-β/Smad signaling pathway is implicated in pro-collagen I transcription. Furthermore, YB-1 was confirmed to be an antagonist of TGF-β, not only by inducing overexpression of Smad7, but also by inhibiting the p300-mediated activation of Smad3 and its binding to TGF-β-responsive element in the promoter of COLα2(I) (16).

Although the association between OMT and downregulation of type I collagen is well established (30-32), the precise mechanism has largely remained elusive. The findings of the present study suggested that OMT stimulated the nuclear translocation of YB-1, thereby inhibiting the endogenic expression of type I pro-collagen.

It was effectively verified that OMT suppressed the expression of COLα1(I) and COLα2(I) genes in a dose- nd time-dependent manner. Furthermore, α-SMA, which is expressed in activated HSCs only, was not significantly affected, indicating that OMT did not cause the recovery of the HSCs to reach the quiescent state (LX-2 is an active cell line).

Of note, the phosphorylation level of ERK1/2 proteins was positively correlated with the changes in YB-1 levels following OMT treatment. ERK1/2, members of the mitogen-activated protein kinase family, regulate diverse cellular functions, including proliferation, differentiation and cell cycle progression (33,34). Studies have indicated that YB-1 activates the physiological features of certain types of cancer cell via the ERK1/2 pathway (35-37); however, its roles in fibrotic cells, such as HSCs, have remained elusive.

In addition, ERK1/2 may have interacted with the TGF-β signaling pathway as an alternative mechanism. The functions of Smad3, the key protein in the TGF-β signaling pathway, were shown to be ERK-dependent in certain cell types (38). Therefore, the present study hypothesized that a link exists between the YB-1-mediated regulation of the TGF-β signaling pathway and ERK1/2.

In the present study, the total protein levels of YB-1 were decreased after OMT treatment, which was in parallel to the phosphorylation of ERK1/2. A similar result was observed after YB-1 knockdown in LX-2 cells. The appearance of only the pERK2 (44 KDa) band in the western blot may be due to the cell status during transfection.

The molecular mechanisms of the anti-fibrotic effects of OMT by facilitating the nuclear translocation of YB-1 are illustrated in Fig. 7 and can be summarized as follows: Besides directly regulating the promoter of COLα1(I) and COLα2(I) genes, YB-1 decreases the production of pro-collagen I through inhibiting the TGF-β signaling pathway by ERK1/2.

In conclusion, OMT attenuates pro-collagen I expression, to a certain extent by increasing the levels of YB-1 in the nucleus. Furthermore, OMT is capable of mediating the inhibition of the TGF-β signaling pathway in the presence of ERK1/2. Further study is required to completely elucidate the mechanism of action of OMT against liver fibrosis.

Acknowledgements

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