LncRNA MALAT1 Inhibits Apoptosis and Promotes Invasion by Antagonizing miR-125b in Bladder Cancer Cells

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Abstract

Accumulating evidences suggest that long non-coding RNAs (lncRNAs) play functional roles in development of different cancers, including cancer initiation and progression. Metastasis associated lung adenocarcinoma transcript 1 (MALAT1) is a well-known lncRNA which was previously shown to be a direct target of miR-125b in bladder cancer (BCa) and to promote cancer progression and invasion. However, little is known whether MALAT1 can also target miR-125b. In the present study, using CRISPR-based technologies and qRT-PCR, we show that MALAT1 is capable of suppressing mature miR-125b and increasing the expression of its target genes (Bcl-2 and MMP-13), but has no effect on pri-miR-125b and pre-miR-125b. We observe that the biotin-labeled MALAT1–RNA probe is able to pull down Ago2 and miR-125b and that the negative regulation of miR-125b by MALAT1 is dependent on Ago2. Importantly, the results of flow cytometry assay and transwell assay reveal that the MALAT1-mediated cancer progression is in part due to specific suppression of miR-125b and activation of its two target genes. All together, these data suggest that the “MALAT1-miR-125b-Bcl-2 / MMP-13” axis plays an important role in the progression of BCa, thereby may provide a potential therapeutic strategy for the treatment of human BCa.

Key words: MALAT1; miR-125b; Ago-2; bladder cancer; CRISPR-based technology.

Introduction

Urothelial bladder cancer is one of the most common malignant tumors in the urinary system, and the prognosis is relatively poor[1]. There should be a multistep process that involves the activation of proto-oncogenes and the inactivation of tumor suppressor genes in the development and progression of bladder cancer[2, 3]. The abnormal activities of the downstream gene pathways caused by the expression changes of many non-coding RNAs have been reported to play a key role in this disease [4, 5].

Long non-coding RNAs (lncRNAs) are RNA molecules whose transcripts are more than 200nt in length [6]. They do not encode proteins, but play important roles in regulating gene expression through...
both epigenetic and posttranscriptional mechanisms [6]. Although little is known about the carcinogenic mechanisms of lncRNAs, many lncRNAs have been found to be abnormally expressed in bladder cancer [7], including UCA1 (urothelial carcinoma associated) [8], HOTAIR (HOX transcript antisense intergenic RNA) [9] and TINCR (tissue differentiation-inducing non-protein coding RNA) [10]. Yet, the biological roles of lncRNAs in bladder cancers are largely unknown. Metastasis associated lung adenocarcinoma transcript 1 (MALAT1) is a long non-coding RNA that is upregulated in many cancers, such as breast, pancreatic, lung, colon, prostate and liver cancer [4]. Our previous studies have shown that MALAT1 inhibits tumor cell apoptosis and promotes cell invasion in bladder cancer [11]. However, the underlying mechanism of MALAT1-mediated gene expression having an impact on tumorigenesis is still unclear.

As another type of non-coding RNA, miRNAs (micro RNAs) have also been revealed to play a similar role in tumors [12]. Accumulating evidences demonstrated that abnormal expression of miRNAs plays a critical role in regulating the biological behaviors of tumor cells by modulating the mRNA levels of its downstream target genes [12]. For example, miR-125b is a very active member of the miRNA family and upregulated in various tumors including leukemia [13], colorectal cancer [14], bladder cancer [15] and lung cancer [16]. Previous studies have revealed that miR-125b suppresses cancer cell proliferation and promotes apoptosis by inhibiting the expression of the anti-apoptotic proteins, such as B cell leukemia 2 (Bcl-2) [17] and matrix metallopeptidase 13 (MMP13) [18]. Likewise, our group has indicated that miR-125b functions as a tumor suppressor in bladder cancer by down-regulating MALAT1 [15]. We showed that the MALAT1 is a direct target of miR-125b through interaction with the putative miR-125b-binding site at MALAT1 RNA.

Intriguingly, some recent studies showed that whereas miRNAs can degrade lncRNAs, at the same time, lncRNAs can also repress miRNA expression[19-21]. miR-125b negatively regulates MALAT1 possibly through the RNA-induced silencing complex (RISC), suggesting that there may be a reciprocal repression feedback loop between miR-125b and MALAT1. We hypothesized that MALAT1 can also suppress miR-125b by acting as a molecular sponge in bladder cancer, which may in part account for MALAT1-mediated cancer progression.

Results

MALAT1 negatively regulates miR-125b

To determine whether MALAT1 can suppress miR-125b expression, we have used the newly-developed technologies "CRISPR Activation" (CRISPRa)[22] and "CRISPR Interference" (CRISPRi)[23] to up- and down-regulate MALAT1 expression respectively causing for its higher stability and lower off-target effect than RNAi [24, 25].

We targeted the dCas9-VP64 protein to the promoter regions of MALAT1 using designed sgRNAs (sgRNA1~3). The expression of these sgRNAs induced various significant increases in MALAT1 expression, and the strongest activation was achieved with sgRNA-3 (Figure 1A).

We then targeted the dCas9 protein to the promoter regions of MALAT1 using another group of designed sgRNAs (sgRNA4~6). Both sgRNA-5 and sgRNA-6 repressed MALAT1 expression to some extent (Figure 1B), with highest repression observed with sgRNA-6. Sequences of the first 20 nucleotides of these engineered sgRNAs were shown in Figure 1C. We chose sgRNA-3 and sgRNA-6 to do the following experiments.

Firstly, we introduced dCas9-VP64 and sgRNA-3 into T24 cells and found that activation of MALAT1 reduced mature miR-125b level (Figure 1D). Then, we introduced dCas9 and sgRNA-6 into T24 cells and observed that inactivation of MALAT1 increased mature miR-125b level (Figure 1E). To dissect the underlying mechanism of this negative regulation of miR-125b by MALAT1, we examined the effect of activation or inactivation of MALAT1 on the levels of pri-miR-125b and pre-miR-125b. As shown in Figure 1D and E, MALAT1 has no effect on pri-miR-125b or pre-miR-125b, suggesting a possible post-transcriptional regulation involved. Moreover, our previous study found that MALAT1 contains two direct binding sites for mature miR-125b, 5'-ttuggcagaaacctTCAGGGa-3' (position 3386, R_002819) and 5'-tttatttccagaaagTCAGGGg-3' (position 6183, NR_002819), in its 3'UTR [20]. Taken together, these findings implicate that MALAT1 may suppress miR-125b by acting as a molecular sponge in bladder cancer.

MALAT1 affects Expression of Endogenous miR-125b Targets

To determine whether MALAT1 affects the expression of endogenous miR-125b targets, we also analysed the impact of MALAT1 on the expression levels of two well known miR-125b targets, “Bcl-2”[17] and “MMP13”[23]. They were detected by western-blot after transfection of CRISPR-encoding plasmid or the
negative control, and the results showed that MALAT1 inhibits endogenous miR-125b function, promoting Bcl-2 and MMP13 protein expression (Figure 1F; Supplementary Figure 1).

**Association of both miR-125b and MALAT1 with the Ago2-complex**

Argonaute 2 (AGO2, EIF2C2) is the only member in AGO family with catalytic activity during the silencing processes of RNA-induced silencing complex (RISC) [26]. AGO2 has been demonstrated involved in tumorgenesis through miRNAs-dependent or independent ways [27]. Since MALAT1-mediated regulation of miR-125b is likely through a post-transcriptional mechanism, we reasoned that MALAT1 may degrade miR-125b through the Ago2-complex. We synthesized a RNA probe containing partial MALAT1 sequence, labeled it with biotin and then mixed it with T24 cellular extract. After precipitation with streptavidin beads, Ago2 was detected by western blot (Figure 2A; Supplementary Figure 1). The results showed that MALAT1 interacts with Ago2. Interestingly, we also detected a significant amount of miR-125b in this MALAT1-pulled down pellet (Figure 2A). There was no obvious increase in miR-125b when TUG1 (another lncRNA studied by our group) RNA probe was used as a negative control.

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**Figure 1.** MALAT1 negatively regulates miR-125b expression. A) Activation of MALAT1 expression using the designed sgRNAs. The error bars indicate the s.d. from five different experiments. NC, negative control. *P < 0.05, relative to the negative control by paired, one-sided t-test. **P < 0.01, relative to the negative control by paired, one-sided t-test. B) Repression of MALAT1 expression using the designed sgRNAs. The error bars indicate the s.d. from five different experiments. NC, negative control. *P < 0.05, relative to the negative control by paired, one-sided t-test. **P < 0.01, relative to the negative control by paired, one-sided t-test. C) cDNA sequences of the first 20nt of the sgRNAs. D) Effect of activation of MALAT1 on pri-miR-125b, pre-miR-125b and mature miR-125b in T24 cells. **P < 0.01, relative to the negative control by paired, one-sided t-test. E) Effect of repression of MALAT1 on pri-miR-125b, pre-miR-125b and mature miR-125b in T24 cells. The error bars indicate the s.d. from five different experiments. **P < 0.01, relative to the negative control by paired, one-sided t-test. F) After activation or repression of MALAT1, the protein expression changes of Bcl-2 and MMP-13 were detected by western-blot. Uncropped blots were presented in Supplementary Figure 1.
To further determine whether MALAT1 degrades miR-125b through Ago2-mediated RNAi pathway, we used Ago2 siRNA to downregulate Ago2 expression at protein level (Figure 2B; Supplementary Figure 1). Compared to the negative control siRNA, the downregulation effect of miR-125b by MALAT1 was markedly eliminated with the presence of Ago2 siRNA (Figure 2C). All these data implied that MALAT1 can function as a natural miRNA sponge and reduce miR-125b expression through the Ago2-mediated post-transcriptional regulation mechanism.

**miR-125b inhibitor partly reversed the apoptotic changes caused by CRISPRi-MALAT1**

The increased apoptotic rate was observed in CRISPRi (sgRNA-6)-transfected T24 cells by flow cytometry assay (Figure 3A). To determine whether this phenomenon was mediated by up-regulation of miR-125b, we used the miRNA inhibitor and found that inhibitor for miR-125b partly reversed T24 apoptosis induced by CRISPRi (sgRNA-6) (Figure 3A and B). Interestingly, further addition of Bcl-2 siRNA partly recovered T24 apoptosis induced by CRISPRi (sgRNA-6), suggesting that there may be a “MALAT1-miR-125b-Bcl-2” axis in T24 cells. We have also analyzed and confirmed these effects on apoptosis in another bladder cancer cell line 5637 (Supplementary Figure 2).

**miR-125b inhibitor partly reversed the motility changes caused by CRISPRi-MALAT1**

The decreased invasion ability was observed in CRISPRi (sgRNA-6)-transfected T24 cells by transwell assay (Figure 4A). To determine whether this phenomenon was also mediated by up-regulation of miR-125b, we used the miRNA inhibitor again and found that inhibitor for miR-125b partly reversed T24 invasion inhibition induced by CRISPRi (sgRNA-6) (Figure 4A and B). Interestingly, further addition of MMP13 siRNA partly recovered T24 quiescence induced by CRISPRi (sgRNA-6), suggesting that there may be a “MALAT1-miR-125b-MMP13” axis in T24 cells. We have also analyzed and confirmed these effects on invasion in another bladder cancer cell line 5637 (Supplementary Figure 3).
Figure 3. miR-125b inhibitor partly reversed the apoptotic changes caused by CRISPRi-MALAT1. A) Cell apoptosis changes were determined using flow cytometry analysis and representative images in T24 cells were shown. B) Cell apoptosis induction observed in T24 cells. The error bars indicate the s.d. from three different experiments. NC, negative control. **P < 0.01, relative to the negative control group by paired, one-sided t-test.

Figure 4. miR-125b inhibitor partly reversed the motility changes caused by CRISPRi-MALAT1. A) Cell motility changes were determined using transwell assay and representative images in T24 cells were shown. Scale bar, 100μm in each figure. B) Inhibition of cell invasion observed in T24 cells. The error bars indicate the s.d. from three different experiments. NC, negative control. **P < 0.01, relative to the negative control group by paired, one-sided t-test.
All together, the above results show that MALAT1 “sponges” miR-125b in bladder cancer to regulate the expression of Bcl-2 and MMP-13, functional genes that inhibit apoptosis and promote invasion (Figure 5).

Figure 5. Schematic representation of the circuitry linking MALAT1, miR-125b, Bcl-2, MMP13 and cell apoptosis or invasion. “MALAT1-miR-125b-Bcl-2/MMP13” axis plays a critical role in the complex network of regulatory interactions governing progression of bladder cancer.

Discussion

In our present study, we investigated whether MALAT1 can also suppress miR-125b in bladder cancer. We have used the newly-developed technologies CRISPRa and CRISPRi. The results implicated that MALAT1 may suppress miR-125b by acting as a molecular sponge in bladder cancer, through a post-transcriptional mechanism. To further determine whether MALAT1 degrades miR-125b through Ago2-mediated RNAi pathway, we used Ago2 siRNA to downregulate Ago2 expression at protein level. All the data implied that MALAT1 can function as a natural miRNA sponge and reduce miR-125b expression through the Ago2-mediated post-transcriptional regulation mechanism. Furthermore, we clearly show that MALAT1 promotes bladder cancer cell invasion and inhibits its apoptosis in part through the suppression of miR-125b. Collectively, these results suggest that miR-125b-MALAT1 interaction plays an important role in the progression of bladder cancer.

MiRNAs and lncRNAs are two new classes of RNAs and constitute an important component of the repertoire of non-coding RNAs[5]. The miRNAs were initially shown to inhibit expression of specific target mRNA using miRNA binding sites. After that, some lncRNAs were also reported to be the direct targets of various miRNAs. Further evidences reveal that miRNAs target lncRNA in the nucleus in an Ago2-dependent manner [27]. Intriguingly, accumulating evidences have shown that lncRNAs can antagonize miRNA function [19-21, 28-30].

During the last few years, many researchers have constructed artificial miRNA sponges [31], which sequester and degrade miRNAs. Interestingly, emerging evidences indicated that lncRNAs can modulate the role and functions of miRNAs, acting as a molecular sponge, such as HOTAIR [32], H19 [20], ATB [19], ANRIL [33] and so on. Among them, previous studies have demonstrated MALAT1 promotes gallbladder cancer development by acting as a molecular sponge to regulate miR-206 [34]. Likewise, our data also found the similar result in bladder cancer cells, suggesting that MALAT1 could function as an effective miRNAs sponge to suppress miR-125b. Furthermore, we observe that the biotin-labeled MALAT1–RNA probe is able to pull down Ago2 and miR-125b and that the negative regulation of miR-125b by MALAT1 is dependent on Ago2, which revealed MALAT1 can function as a natural miRNA sponge through the Ago2-mediated post-transcriptional regulation mechanism.

miR-125b, as a member of miR-125 family, has been reported to be involved in various cancers and other diseases as either repressors or promoters [35]. Although dysregulation of miR-125b has been verified in various cancer types, its role in disease is not completely understood [36]. Thus, to understand the role of miR-125b in tumorigensis, we should identify various miR-125b target genes. A number of studies demonstrated miR-125b plays crucial roles in many different cellular processes like cell differentiation, apoptosis and invasion by targeting several genes [37], MMP13 [18], Bcl-2 [17], BAK1 [37] and so on. In bladder cancer, it was reported that MMP13 was over-expressed in tumor cells, particularly at the invading edges. Similarly, Bcl-2, an anti-apoptotic protein, plays an important role in apoptosis which is largely regulated by the Bcl-2 family mediated pathway [17]. In our experiments, further addition of Bcl-2/MMP13 siRNA partly recovered cell apoptosis and quiescence respectively induced by CRISPRi. These data suggest that the “MALAT1-miR-125b-Bcl-2 / MMP-13” axis plays an important role in the progression of bladder cancer, which can explain the interaction between Bcl-2 and MALAT-1, thus providing a new therapeutic strategy for patients.

The present study is the first to demonstrate that there is reciprocal repression between miR-125b and MALAT1, and to verify that “MALAT1-miR-125b-Bcl-2 / MMP-13” axis exerts function in bladder cancer. Additionally, to ensure the precision of this study, we used the CRISPR technology which can achieve up to 100 fold transcriptional repression or activation [38]. Taken together, our present study
provides a new strategy for cancer research. However, it should be emphasized that there are several limitations in our study. Firstly, our conclusions were only based on the responses of bladder cancer cell lines, which might not reflect processes in the intact organism and should be confirmed in animal models. Secondly, previous studies have demonstrated that MALAT1 can function as a competing endogenous RNA and impact myoblast differentiation through inhibiting miR-133 [39]. Thus, apart from cancer research, our findings about miR-125b–MALAT1 interaction still needs to be confirmed in other biological processes, such as myogenesis, metabolism regulation, embryonic development and so on. Thirdly, MALAT1 contains binding sites for additional miRNAs, suggesting that MALAT1 may also regulate other miRNAs, such as miR-133, miR-206, etc. Whether there is any crosstalk between the pathways regulated by these miRNAs remains to be investigated.

In summary, our study reveals that there is reciprocal repression between miR-125b and MALAT1 in bladder cancer cell, suggesting that this reciprocal repression is related to the pathway involving the Ago2-mediated post-transcriptional regulation. Furthermore, our work highlights the significance of “MALAT1-miR-125b-Bcl-2 / MMP-13” axis in tumorigenesis including tumor apoptosis and invasion, which adds a new piece of puzzle to the non-coding RNA regulatory networks.

Materials and Methods

Cell lines and cell culture

The bladder cancer cell line T24 and 5637 were purchased from the Institute of Cell Research, Chinese Academy of Sciences, Shanghai, China. Cells were cultured in DMEM medium containing 10% fetal bovine serum and incubated at 37 °C in 5% CO2.

Plasmids construction

The plasmid vectors pcDNA-dCas9-HA (Plasmid #61355, Addgene) and M-SFn-VP64 (Plasmid #48674, Addgene) were used to transiently express in active Cas9 protein (for CRISPR-based interference) and Cas9-VP64 fusion protein (for CRISPR-based activation) respectively in mammalian cells. The designed cDNA sequence for each sgRNA was designed (http://crispr-era.stanford.edu/), synthesized and inserted into pGPU6/GFP/Neo vector digested with BamHI / BbsI.

Cell transfection

T24 or 5637 cells were incubated for 24 h prior to transfection. According to the manufacturer’s instructions, the plasmids were transiently transfected into T24 or 5637 cells by using Lipofectamine 2000 Transfection Reagent (Invitrogen, Carlsbad, CA, USA). siRNAs were transfected using RNAfectin reagent (Applied Biological Materials) following the manufacturer’s protocol.

RNA extraction and qRT-PCR

At 48 hours after transfection, 1 × 10^6 cells of T24 were collected for extracting total RNA. Total cellular RNA was extracted from different groups with TRIzol reagent (Invitrogen, USA) following the manufacturer’s procedures. The concentration and purity of total RNA were measured by using UV spectrophotometer analysis at 260 nm.10mg of total RNA was converted to cDNA using the All-in-One™ miRNA qRT-PCR Detection Kit (Gene-Copoiea Inc, Rockville, MD, USA). To specifically detect the expression levels of MALAT1, pri-miR-125b and pre-miR-125b, we used the SYBR Green method with the designed primers. GAPDH or U6 was used as an internal control. The primer sequences were as follows: MALAT1 forward: 5’-AAAGCAAGGTCTCCACAAAG-3’, reverse: 5’-GGTCTGTGCTAGTCAAAAAGGCA-3′; GAPDH forward: 5’-CGCTCTCTGCTCCTTGTCCT-3′, reverse: 5’-ATCCGTTGACTCCGACCCTCAC-3′; pri-miR-125b-1 forward: 5’-CGAACAGAATTGTGCCTGTA-3′, reverse: 5’-ACCAAATTTCAGGTGCAAA-3′; pri-miR-125b-2 forward: 5’-GAATTCCTACCCGATCAAAACCA-3′, reverse: 5’-TCTGGTGATCTCAGGACACAA-3′; pri-miR-125b-3 forward: 5’-ACCAGACTTTTCTAGTCCCT-3′, reverse: 5’-AGTTCCCGAGGACTGACCT-3′; pre-miR-125b-1 forward: 5’-TGCGCTCCTCTCAGTCCCTGAGA-3′, reverse: 5’-GACTCGCAGCTCCCAAGA-3′; pre-miR-125b-2 forward: 5’-GCTGGCGCCGAGGAGCTCT-3′, reverse: 5’-GGTCCCAAGAGCCTGACTT-3′. Homo sapiens RNAU6 was purchased from GeneCopoiea Inc, Rockville, MD, USA. To detect the relative expression level of mature miR-125b, we use the poly A polymerase-based SYBR Green method (SBI). Expression fold changes were calculated using 2^{-ΔΔCt} methods.

Western blot analysis

Cells were grown in 10-cm dishes and homogenized in buffer (10 mMTris-HCl, 5 mM EDTA, 50 mMNaCl, 50 mM sodium fluoride, 30 mM sodium pyrophosphate, 1% Triton-X, 200 µM sodium orthovanadate, 1 mM phenyl methyl sulfonyl fluoride, 1µg/mL pepstatin, 2µg/mL leupeptin and 5µg/mL aprotinin). The protein concentration was determined using the Pierce (Rockford, IL) BCA (bicinchoninic acid) protein assay. Equal amounts of whole protein extract were electrophoresed onto 12% or 15% sodium dodecyl sulfate polyacrylamide gel.
electrophoresis gels and then transferred to nitrocellulose membranes (Millipore, Billerica, MA) using a semi-dry transfer cell (Bio-Rad Laboratories, Hercules, CA) at 1 mA/cm² for 2 h. After transferred to polyvinyl difluoride membranes, samples were blocked in 1% dry milk and incubated over-night with the primary antibodies. Antibodies specific to Bcl-2, MMP13, Ago2 and GAPDH were purchased from Santa Cruz, Dallas, Texas, USA. The levels of proteins were detected with horseradish peroxidase-linked secondary antibodies and revealed using the ECL® System (GE Healthcare, Milan, Italy).

**RNA precipitation**

To determine whether MALAT1 is associated with the RISC complex, we carried out an RNA precipitation assay using synthesized partialMALAT1 sequence containing miR-125b binding site as a probe and then detected Ago2 from the pellet using western blot and miR-125b using qRT-PCR as described elsewhere.

**Flow cytometry assay**

At 48 hours post-transfection, T24 or 5637cells were harvested and resuspended in fixation fluid. 5µl Annexin V - FIFC and 2µl propidium iodide were added to 500 µl cell suspension. Cell apoptosis was then determined by using flow cytometry (EPICS, XL-4, Beckman, CA, USA). In the graphs, the quadrant respectively stands for dead cells, living cells, early apoptotic cells and late apoptotic cells.

**Transwell assay**

After 24 hours post-transfection, about 1×10⁵ cells with 100 µl serum-free medium were plated into the upper chambers (24-well insert, pore size 8 µm, Corning) which were added with Matrigel (1:8, 50µl/well, BD Bioscience, San Jose, CA, USA). Concurrently, the lower chambers were filled with 500 µl medium containing 10 % fetal bovine serum. Cells were cultured at 37°C in a 5% CO₂ atmosphere for 48 hours. Cells under the surface of the lower chamber were washed with 1×PBS, fixed with 4% paraformaldehyde for 20 min, stained with 0.1 % crystal violet for 25 min, and then washed 3 times. Invaded cells were observed under the inverted microscope and imaged. Afterwards, each chamber were washed with 1×PBS, fixed with 4% paraformaldehyde for 20 min, stained with 0.1 % crystal violet for 25 min, and then washed 3 times. Invaded cells were observed under the inverted microscope and imaged. Afterwards, each chamber was soaked into 1ml 33% acetic acid for 10 min to wash out the crystal violet. Then 100µl of 33% acetic acid was added into per well of 96-well plates, and the absorbance was measured at a wavelength of 570nm using a microplate reader (Bio-Rad, Hercules, CA, USA). Experiments were performed in triplicate.

**Statistical analysis**

All experimental assays were performed in triplicate. All data were presented as mean ± standard deviation (SD). All statistical analyses were executed by using SPSS 20.0 software (IBM, Chicago, IL, USA). P value of less than 0.05 was considered to be statistically significant.

**Supplementary Material**

Supplementary figures.
http://www.jcancer.org/v08p3803s1.pdf

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**Author contribution**

H.X., X.L., Z.C., Y.F. and A.H. performed experiments and data analysis. Y.L. and H.X. designed the project and wrote the paper. W.H., J.L. and Y.L. supervised the project. W.H. and Y.L. provided financial support for the project.

**Competing Interests**

The authors have declared that no competing interest exists.

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