Activation of LTBP3 Gene by a Long Noncoding RNA (lncRNA) MALAT1 Transcript in Mesenchymal Stem Cells from Multiple Myeloma*

Received for publication, April 9, 2014, and in revised form, August 27, 2014. Published, JBC Papers in Press, September 3, 2014, DOI 10.1074/jbc.M114.572693

Bingzong Li†1, Ping Chen†1, Jing Qu†1, Lei Shi†, Wenyue Zhuang‡, Jinxiang Fu‡, Jun Li‡, Xiaohui Zhang‡, Yu Sun‡, and Wenzhuo Zhuang§2

From the †Department of Haematology, the Second Affiliated Hospital of Soochow University, Suzhou 215006, the ‡Department of Cell Biology, School of Biology and Basic of Medical Science, Soochow University, Suzhou 215123, and the §Department of Molecular Biology, Medical Ecsomatics College of Beihua University, Jinlin 132013, China

Background: Long noncoding RNAs (lncRNAs) are emerging as important regulators of gene expression and tumorigenesis.

Results: MALAT1 promoted the activation effect of Sp1 on LTBP3 promoter.

Conclusion: In mesenchymal stem cells (MSCs), MALAT1 was critical for Sp1 transcriptional activation of LTBP3 that regulated the bioavailability of TGF-β.

Significance: MALAT1 is a promising target for therapeutic intervention in multiple myeloma (MM).

Long noncoding RNAs (lncRNAs) are emerging as important regulatory molecules in tumor suppressor and oncogenic pathways. However, the magnitude of the contribution of lncRNA expression to normal human tissues and cancers has not been investigated in a comprehensive manner. Here we explored the biology of the lncRNA MALAT1 and considered the potential significance in mesenchymal stem cells from myeloma patients. By using assays such as RNA interference, luciferase, chromatin immunoprecipitation, and RNA immunoprecipitation, we showed that in mesenchymal stem cells MALAT1 promoted the activation effect of the key transcription factor Sp1 on LTBP3 promoter by modulating recruitment of Sp1 to the LTBP3 gene that regulated the bioavailability of TGF-β in particular. Our data suggested that lncRNA MALAT1 directly interacted with Sp1 and LTBP3 promoter to increase expression of LTBP3 gene. The specificity and efficiency of activation were ensured by the formation of a stable complex between MALAT1 and the LTBP3 promoter, direct interaction of MALAT1 with Sp1, and recruitment of Sp1 to the promoter. In this study, we showed that the mechanism of transcriptional activation of LTBP3 promoter depended on MALAT1 initiated from neighboring gene LTBP3 and involved both the direct interaction of the Sp1 and promoter-specific activation. Our knowledge of the role of MALAT1 in cellular transformation is pointing toward its potential use as a biomarker and a target for novel therapeutic approaches in multiple myeloma.

Recent studies have identified numerous lncRNAs3 that exhibit differential expression between normal and tumor states. lncRNAs regulate key cancer pathways at a transcriptional, post-transcriptional, and epigenetic level (1–5). A challenging task is to determine how these lncRNAs are able to modulate those pathways.

MALAT1 (metastasis-associated lung adenocarcinoma transcript 1) is among the most abundant and highly conserved lncRNAs (6–8). MALAT1 is overexpressed in many human cancers, suggesting that MALAT1 misregulation may play a role in the development of numerous cancers (6, 9–13).

Multiple myeloma (MM) expands almost exclusively in the bone marrow and generates devastating bone lesions (14). Abundant TGF-β within the bone marrow milieu surrounding myeloma cells plays an important role in the bone lesions (15). The TGF-β family consists of three isoforms, TGF-β1, -β2, and -β3, with high sequence homology. Among them, TGF-β1 is the most abundant isoform in bone (16). Latent TGF-β-binding proteins (LTBPs) are extracellular matrix glycoproteins, which are important for efficient secretion, folding, and activation of TGF-βs (17). LTBP3 regulates the bioavailability of TGF-β especially in the bone (18, 19). On the basis of the genomic sequence information corresponding to the human LTBP3, we found a long noncoding RNA MALAT1 that had a transcription orientation opposite to that of LTBP3 (Fig. 1). MALAT1 is specifically retained in nuclear speckles (7) associated with the modification or storage of the pre-mRNA processing machinery (20) and thus with potential impact on gene regulation (21, 22).

Several mechanisms underlying ncRNA transcription-mediated gene regulation have been recently identified. It has been demonstrated that transcription of ncRNA genes can directly interfere with downstream/overlapping gene transcription by...


**Intronic MALAT1 Transcript in Mesenchymal Stem Cells**

altering transcription factor binding at the yeast SER3, FLO11, and IME4 loci (23–25), and the *Drosophila Ubx* locus (26).

Because *MALAT1* is highly conserved among mammals and predominantly localizes to nuclear speckles, we hypothesize that it plays a critical role in the *LTBP3* gene transcription. Our data suggested that IncRNA *MALAT1* directly interacted with Sp1 and *LTBP3* promoter to increase expression of *LTBP3* gene. IncRNAs can regulate the transcriptional process through a range of mechanisms. However, the pervasive transcription of promoters anticipates a core role for long ncRNA in regulating the process of transcription (27, 28). Here we showed that the IncRNA *MALAT1* was critical for Sp1 transcriptional activation of the *LTBP3*. *MALAT1* could recruit the transcription factor Sp1 on the *LTBP3* promoter. The specificity and efficiency of activation were ensured by the formation of a stable complex between *MALAT1* and the *LTBP3* promoter, direct interaction of *MALAT1* with Sp1, and recruitment of Sp1 to the promoter. Sp1 forms a complex with *MALAT1*, which can bridge Sp1 to the *LTBP3* promoter. Our results demonstrated that the transcription factor Sp1 could bind directly and specifically to *MALAT1*, which was expressed in the MSCs from myeloma patients at high levels. *MALAT1* specifically cooperated with Sp1 to increase the transcriptional activity of *LTBP3* in MSCs. A stable complex containing *MALAT1*, Sp1, and the Sp1 consensus site of *LTBP3* suggested that *MALAT1* activated transcriptional activity by directly influencing Sp1 activity. Our analysis revealed an RNA-dependent mechanism of transcriptional activation. It is a dynamic process dependent on the production of the regulatory transcript from the nearby gene. Highly specific and stable triplex structures formed by IncRNA with the transcription factor and the promoter may well contribute to promoter targeting and activation *in vivo*. These data provided an unexpected IncRNA/transcription factor-based strategy to integrate transcriptional programs. We also highlight the possibility of using this unique RNA as a promising target for therapeutic intervention in multiple myeloma.

**EXPERIMENTAL PROCEDURES**

**Patients and Subjects**—Twenty-five patients with newly diagnosed MM (14 males and 11 females, aged 48–83 years) and 5 normal subjects (2 males and 3 females, aged 47–63 years) were recruited in this study (Table 1). All samples were obtained at the Second Affiliated Hospital of Soochow University according to institutional guidelines. Informed consent was obtained according to procedures approved by the Ethics Committee of the Affiliated Hospital of Soochow University. All volunteers signed the informed consent form (ICF). Clinical and biologic information (sex, age at diagnosis, treatment, the level of *MALAT1*, *LTBP3* expression) was available for all patients.

**MSC Isolation and Culture**—Isolation and culture of bone marrow-derived MSCs were performed as described previously (29, 30). Briefly, mononuclear cells were separated by a Ficoll-Paque gradient centrifugation (specific gravity 1.077 g/ml; Nycomed Pharma AS, Oslo, Norway) and depleted of hematopoietic cells and myeloma cells using CD45, GlyA, CD34, and CD138 micromagnetic beads (Miltenyi Biotec Inc., Auburn, CA). Culture medium was Dulbecco’s modified Eagle’s medium/Ham’s F-12 medium (DF12; Gibco Life Technologies, Paisley, UK) containing 2% FCS (Life Technologies), 1 × insulin-transferrin-selenium (Life Technologies), 109 M dexamethasone (Sigma), 104 M ascorbic acid 2-phosphate (Sigma), 10 ng/ml EGF (R&D Systems, Minneapolis, MN), 10 ng/ml platelet-derived growth factor BB (PDGF-BB; R&D Systems), 100 units/ml penicillin, and 1000 units/ml streptomycin (Life Tech-
IncRNA MALAT1 Transcript in Mesenchymal Stem Cells

nologies). Cells were cultured at 37 °C and 5% CO₂ humidified atmosphere. Culture medium were changed every 3–4 days. Once adherent cells were more than 80% confluent, they were detached with 0.125% trypsin and 0.01% EDTA and replanted at a 1:3 dilution under the same culture conditions. Cells from the second and third passage were analyzed for further study. Cells were regular tested for mycoplasma contamination using MycoAlert mycoplasma detection kit (Lonza, Rockland, ME).

RNA Isolation and Quantitative Real-time Polymerase Chain Reaction—RNA was prepared using the EASYspin RNA extraction kit following the manufacturer’s protocol. mRNAs were assayed using the TaqMan microRNA and gene expression assays (Applied Biosystems, Foster City, CA), respectively. GAPDH was used as reference gene. The Ct value of each target gene was normalized against the Ct value of the reference gene (Ct_target – Ct_GAPDH).

ELISA—Levels of transforming growth factor-β1 (TGF-β1) were assessed by ELISA kits (R&D Systems) according to the manufacturer’s instructions. MSCs (4 × 10^5 cells/ml) were plated in complete medium, and the supernatants were harvested. Levels of TGF-β1 were determined by subtracting the value from medium alone and then normalized according to cell number.

Small Interfering RNAs—siRNAs targeting MALAT1 were obtained from GenePharma. Sequences of the siRNAs were as follows: siMALAT1-1, 5'-GAAUUCGGUGAUGCGAGU-3'; siMALAT1-2, 5'-GGCAUAAGGCGGGCCUCA-3'; siSp1-1, 5'-AGCCUUGAGUAGUGCCUAU-3'; siSp1-2, 5'-GGUAGGCUAAGUUUUGAU-3'. siRNA universal negative control (GenePharma) that does not target any human gene product was used as a negative control.

Antibodies—The following antibodies were used: LTBP3 (Santa Cruz Biotechnology, Santa Cruz, CA, sc-390913), Sp1 (Abcam, ab13370), and β-actin (Millipore, Temecula, CA).

Lentiviral Constructs—The sequence of MALAT1 was synthesized and subcloned into lentivector-transferred plasmid pCDH-CMV-MCS-EF1-cOGFP to generate pCDH-CMV-MALAT1-EF1-cOGFP. The recombinant vector pCDH-CMV-MALAT1-EF1-cOGFP or the control vector pCDH-CMV-MCS-EF1-cOGFP was triple-transfected with the packaging vectors psPAX2 and pMD2.G into 293T cells by calcium chloride to produce the lentivirus. MSCs were infected with control or MALAT1-expressing lentivirus. The expression of MALAT1 in cells was determined by quantitative PCR.

Luciferase Assay—Genomic DNA from 293T was used to amplify the human LTBP3 promoter region that was cloned into the pGL3-basic luciferase vector (Promega) by using SacI and XhoI sites (primers listed in Table 1). IK-2, Sp1, GATA-2, and MZF1 binding site mutations were introduced into the (+2000/−2000) construct to disrupt the binding elements and to generate the mutIK-2 construct, Sp1mut construct, mutGATA-2 construct, and mutMZF1 construct using QuikChange site-directed mutagenesis kit (Stratagene), respectively (primers listed in Table 2).

MSCs were transiently transfected with different LTBP3 promoter reporter constructs and Renilla luciferase plasmid (Promega) as a normalization control using Lipofectamine 2000 (Invitrogen). Firefly and Renilla luciferase activities were measured consecutively by using the Dual-Luciferase assay (Promega) 48 h after transfection.

ChIP Assays—Cells were first cross-linked with 2% paraformaldehyde for 10 min at 37 °C and sonicated, and then the DNA-protein complexes were isolated with a ChIP assay kit (Millipore, catalog number 17-371) according to the manufacturer’s instructions with antibodies against Sp1 (Millipore). The precipitated DNA was quantified by real-time PCR with primers listed in Table 2. The results were normalized relative to the input control.

RNA Immunoprecipitation—RNA immunoprecipitation assay was performed with an RNA-binding protein immunoprecipitation kit (Millipore, catalog number 17-700) according to the manufacturer’s instructions with antibodies against Sp1 (Millipore). Quantitative PCR for RNA was performed using TaqMan universal PCR mix as described above. The results were normalized relative to the input control.

DNA Pulldown Assay—The pulldown assay, which was similar to RNA pulldown assay, was performed as described previously with modifications (31). Biotinylated oligonucleotides were synthesized that corresponded to LTBP3 promoter sequences that include putative Sp1 transcription factor binding sites (5’-Biotin-CTCCCGGCCTCCGCCCCC-3’) or its mutation (5’-Biotin-CTCCCCTTCCCGGCCCCC-3’). Double-stranded oligonucleotides were prepared by mixing equal amounts of forward (biotin-labeled) and reverse oligonucleotide at 100 °C for 1 h and then slow cooling to room temperature for 30 min. One milligram of whole-cell lysates from MM-MSCs was incubated with 3 μg of biotinylated oligonucleotides for 1 h at 25 °C; complexes were isolated with streptavidin-agarose beads (Invitrogen). The RNA present in the pulldown material was detected by quantitative RT-PCR analysis.

Statistical Analysis—All quantitative data presented are the mean ± S.D. from at least three samples per data point. The Pearson product-moment correlation coefficient (r) was used to establish the association of the two variables. The difference was considered statistically significant when p < 0.05. A p value of 0.05 was used as the boundary of statistical significance. All statistical tests were two-sided.

RESULTS

Expression of MALAT1 and LTBP3, as Well as TGF-β1 Secretion in MSCs from Bone Marrow of Myeloma Patients and Healthy Donors—To explore the biology of MALAT1 and consider the potential significance in MM, we examined the expression of MALAT1 in MSCs from MM patient samples and healthy donors. The expression of MALAT1 was assessed by quantitative real-time PCR. Consistently higher expression level of MALAT1 was found in MSCs from all 25 patient samples relative to that from healthy donors (p < 0.001). Under the same conditions, quantitative real-time PCR showed that LTBP3 was more highly expressed in MSCs from all 25 myeloma patients when compared with that from healthy donors (p < 0.001) (Fig. 2A). The changes in protein levels of LTBP3 in cells as mentioned above were further confirmed by the immunoblotting analysis (Fig. 2C). To determine the biological source of TGF-β1 in MSCs, the synthesis and secretion

OCTOBER 17, 2014 • VOLUME 289 • NUMBER 42

JOURNAL OF BIOLOGICAL CHEMISTRY 29367
of TGF-β1 by MSCs (Fig. 2B) were measured by ELISA. TGF-β1 was significantly higher in MSCs from myeloma patients than that from healthy donors (p < 0.01). Because both MALAT1 and LTBP3 were augmented in in MSCs from myeloma patients, to examine any correlation between MALAT1 and LTBP3, we performed Pearson correlation coefficient analysis in both healthy donors and myeloma patients. A positive correlation between MALAT1 and LTBP3 was observed in MSCs from healthy donors and myeloma patients (r = 0.7670, p < 0.0001, Fig. 2D).

**Knockdown of the MALAT1 Decreased LTBP3 Transcription and TGF-β1 Secretion in MM-MSCs**—To examine the functional significance of MALAT1 overexpression in MSCs from myeloma patients, we knocked down the MALAT1 using two independent siRNAs against MALAT1. MM-MSCs1, MM-MSCs2, MM-MSCs4, and MM-MSCs5, which correspond to patient 1, patient 2, patient 4, and patient 5, respectively, were used in the analyses. MM-MSCs1, MM-MSCs2, MM-MSCs4, and MM-MSCs5 were treated with control siRNA or with siRNA targeting MALAT1. As shown in Fig. 3, knockdown of the MALAT1 decreased LTBP3 transcription (p < 0.01). A dramatic reduction of TGF-β1 secretion was also observed in MALAT1 knockdown MM-MSCs1, MM-MSCs2, MM-MSCs4, and MM-MSCs5 by ELISA assays (p < 0.01). A similar result was obtained with another MALAT1 siRNA targeting a different sequence of MALAT1. Specific MALAT1 siRNA caused a

### TABLE 2
Primers used in this study

| For Luciferase reporter primers | Reverse primers |
|-------------------------------|-----------------|
| **LTBP3 promoter primers**    |                 |
| 1. (-2000+200) GGAGCTCAGATGCCACAGGGGT | CCCTCGAGTGGGCGGAGG |
| 2. (-1800+200) GGAGCTCAGAGCTGAGCTTAT | CCCTCGAGTGGGCGGAGG |
| 3. (-1600+200) GGAGCTCAGAGCTGAGCTTGT | CCCTCGAGTGGGCGGAGG |
| 4. (-1400+200) GGAGCTCAGAGCTGAGCTTCTT | CCCTCGAGTGGGCGGAGG |
| 5. (-1200+200) GGAGCTCAGAGCTGAGCTTCC | CCCTCGAGTGGGCGGAGG |
| 6. (-1000+200) GGAGCTCAGAGCTGAGCTCAGA | CCCTCGAGTGGGCGGAGG |
| 7. (-800+200) GGAGCTCAGAGCTGAGCTCAGG | CCCTCGAGTGGGCGGAGG |
| 8. (-600+200) GGAGCTCAGAGCTGAGCTCACC | CCCTCGAGTGGGCGGAGG |
| 9. (-400+200) GGAGCTCAGAGCTGAGCTGAC | CCCTCGAGTGGGCGGAGG |
| 10. (-200+200) GGAGCTCAGAGCTGAGCTGCC | CCCTCGAGTGGGCGGAGG |
| 11. (0+200) GGAGCTCAGAGCTGAGCTGCG | CCCTCGAGTGGGCGGAGG |

| For mutagenesis | For chip assay |
|----------------|---------------|
| IK-2-mut CACCTTTGCAAGGAGCTGGAAGGCG | AGTCCGACCCAGATGCC |
| Sp1-mut CGCTCTACGAGGCGGCCCGCCCGGAGGG | GCGTGGAGAAGCGGAGGTTG |
| GATA-2-mut CTCTCTCTCTAGCTGAGCTGAGG | GAGCCGGAGGAGGTAGAGGAG |
| MZF1-mut CGTCTACGAGGCGGCCCGCCCGGAGG | CTCCGGGCGGGCCGGGTCCTGAGAG |

**Primer1** CTTGGCAAGAGAAGCTGGAAGG |
**Primer2** CGCCTCCATCATCGTAAG |
**Primer3** CGCCTCCATCATCGTAAG |
**Site1** AGTCTTGGCCTGCTCTTCTTCC |
**Site2** AAGTGCACTGGTCTGCAAGC |
**Site3** ACCCATGCAAGGCCCTTAG |
**Site4** AGCAGACAGACGATCTCAGG |
**Site5** CTGGCAAGAGAAGCTGGAAGG |
marked decrease in LTBP3 mRNA levels, suggesting that LTBP3 expression is dependent on MALAT1 function.

Overexpression of MALAT1 Increased LTBP3 Transcription and TGF-β1 Secretion in HD-MSCs—To further elucidate the functional connection between MALAT1 and LTBP3, we used a lentiviral vector system to efficiently overexpress MALAT1 in MSCs from healthy donors (HD-MSCs). HD-MSCs1 and HD-MSCs2, which correspond to healthy donor 1 and healthy donor 2, respectively, were used in the analyses. MALAT1 expression was quantitated by real-time PCR 48 h after infection. Levels of MALAT1 were determined by subtracting the value from medium alone and then normalized according to cell number. The error bars represent three independent ELISA readings. C, LTBP3 was determined by immunoblotting in MSCs from bone marrow of myeloma patients and healthy donors; r = 0.7670, p < 0.0001.
In lncRNA MALAT1 Transcript in Mesenchymal Stem Cells

sion of MALAT1 increased LTBP3 transcription (p < 0.01). An increase of TGF-β1 secretion was also observed in MALAT1-overexpressing HD-MSCs1 (HD-MSCs1-OM) and HD-MSCs2 (HD-MSCs2-OM) by ELISA assays (p < 0.01) (Fig. 4D). These data suggested that MALAT1 may play a specific role in LTBP3 transcriptional activation in MSCs.

MALAT1 Cooperates with Sp1 to Increase LTBP3 Transcription—The close relationship between MALAT1 and the LTBP3 led us to ask whether MALAT1 may function by regulating the transcriptional activity of the LTBP3. To test this hypothesis, we constructed reporter plasmids carrying various genomic sequences around the transcription start site of the LTBP3 and subjected them to luciferase assay. A significant reduction in promoter activity was detected when the region from −600 to −400 was deleted, indicating that it contains important regulatory elements for the basal expression of LTBP3 (p < 0.01) (Fig. 5A). Further definition of this region is necessary to determine the precise transcription factor that is involved in the MALAT1-mediated interaction. Because conserved regions in a given gene promoter are expected to contain regulatory elements, we focused on the highly conserved region (Fig. 5B). A bioinformatics search identified a previously reported high score Sp1 consensus site (32). In addition, conserved IK-2, GATA-2, and MZF1 elements were identified in this region. To define the MALAT1 active region, we used the different mutation reporters to assay for the effect of MALAT1 knockdown on the activity of different transcription factors. Luciferase assay using a construct mutated for IK-2, Sp1, GATA-2, and MZF1 consensus sequence revealed that Sp1 region was both necessary and sufficient for the activity. These findings also suggested that Sp1 was a key transcription factor of LTBP3 through directly binding to the core promoter element. MALAT1 knockdown had a repressive effect on the LTBP3 promoter. This effect was observed only when IK-2, GATA-2, and MZF1 consensus sequences were mutated, respectively. The construct mutated for Sp1 did not have the same effect (Fig. 5C) (p < 0.01). To provide further confirmation, we examined luciferase activity in MSCs cotransfected with two independent siRNA against Sp1 or with control siRNA and empty luciferase reporter, LP8 reporter construct, or LP8 reporter construct with mutated Sp1 binding site. We found that mutating Sp1 binding sites or knocking down Sp1 reduced the luciferase activity of the LP8 reporter vector. Knocking down Sp1 did not influence the luciferase activity of LP8 reporter with mutated Sp1 binding site (Fig. 5D) (p < 0.01). These data demonstrated that MALAT1 cooperated with Sp1 but not with IK-2, GATA-2, or MZF1 to increase the LTBP3 transcription in MSCs. These results strongly suggested that MALAT1-mediated activation was Sp1-dependent.

Four Sp1 binding sites were detected at the LTBP3 locus in ChIP-Seq experiments from UCSC Genome Browser (Table 3). To validate the binding between these Sp1 binding sites of LTBP3 and Sp1, we performed ChIP to pull down endogenous Sp1 associated with the Sp1 binding site at LTBP3 locus and demonstrated by quantitative PCR analysis with primers specific to Site1, Site2, Site3, and Site4 (binding regions indicated in ChIP-Seq experiments from UCSC Genome Browser) and Site5 (binding region indicated in Fig. 5C). As shown in Fig. 5E, the endogenous Sp1 binding sites of LTBP3 pulldown by Sp1 was specifically enriched in Site5 but not in Site1, Site2, Site3, or Site4 in MSCs.

The Interactions of Sp1 with Sp1 Consensus Site at the LTBP3 Promoter Were Controlled by lncRNA MALAT1—To prove that MALAT1 directly regulates LTBP3 transcription by recruiting Sp1 to the LTBP3 promoter, we performed ChIP assay. MM-MSCs7, MM-MSCs8, MM-MSCs10, and MM-MSCs12, which correspond to patient 7, patient 8, patient 10, and patient 12, respectively, were used in the analyses. MM-MSCs7, MM-MSCs8, MM-MSCs10, and MM-MSCs12 were treated with control siRNA or with siRNA targeting MALAT1. Indeed, we found that endogenous Sp1 directly interacts with the core element of the LTBP3 promoter in MSCs, as demonstrated by ChIP assay. Moreover, we observed a marked decrease in the occupancy of the LTBP3 promoter by Sp1 in MALAT1 knockdown cells when compared with control knockdown cells and untreated cells (Fig. 6, A–C) (p < 0.01). These data suggested that recruitment of Sp1 to the LTBP3 promoter to cause gene-specific activation was directed by lncRNA MALAT1 tethered
FIGURE 5. Identification of MALAT1 core element in the LTBP3 promoter. A, luciferase reporter activity of promoter constructs of LTBP3 on chromosome 11q13.1 in MSCs2 cells. Schematic presentation of LTBP3 promoter deletion constructs is shown on the left. The arrow above construct LP1 indicates the position of the transcription start site. Transcriptional activity of the corresponding constructs is indicated on the right. Luciferase activities were normalized by Renilla luciferase activities. Values represent mean ± S.D. from three experiments. B, conservation track in UCSC Genome Browser. The LTBP3 promoter locus from chr 65326100 to 65326300 contains regions of conservation subtracks. The subtracks display phyloP scores (in blue and red). The phyloP tracks measure conservation (with positive scores, shown in blue), whereas the phyloP’ tracks also indicate accelerated evolution (with negative scores, shown in red). C, relative luciferase activity of LP8 reporter construct. Transcription factor binding sites are indicated (blue boxes: 1, IK-2; 2, Sp1; 3, GATA-2; and 4, MZF1). Deletions introduced into the LP8 construct are shown as yellow X showing abolition of the promoter activity. EV, empty vector. D, luciferase activity in MSCs2 cotransfected with two independent siRNA against Sp1 or with control siRNA and empty luciferase reporter, LP8 reporter construct, or LP8 reporter construct with mutated Sp1 binding site. Data are presented as the relative ratio of firefly luciferase activity to Renilla luciferase activity.
IncRNA MALAT1 Transcript in Mesenchymal Stem Cells

TABLE 3
Sp1 binding sites detected at the LTBP3 locus in ChIP-Seq experiments from UCSC Genome Browser

| Site  | Chromosome | Chromosome start | Chromosome end | Name | Score |
|-------|------------|------------------|----------------|------|-------|
| Site1 | chr11      | 65308130         | 65308450       | Sp1  | 161   |
| Site2 | chr11      | 65313921         | 65314257       | Sp1  | 126   |
| Site3 | chr11      | 65319045         | 65319381       | Sp1  | 187   |
| Site4 | chr11      | 65324717         | 65325053       | Sp1  | 293   |

A, schematic representation of the primer sets specific for LTBP3 promoter. B, MSCs were untreated or treated with control siRNA or with siRNA targeting MALAT1, and ChIP assays were performed with the Sp1 antibody and the indicated PCR primers. The binding of Sp1 to the LTBP3 promoter was detected by quantitative real-time PCR. The results were normalized to the input control. All data are shown as mean ± S.D. C, representative analysis of PCR products derived by ChIPs, which were separated on an agarose gel. D, RNA immunoprecipitation using Sp1 antibody followed by quantitative real-time PCR for MALAT1 in MSCs transfected with two independent MALAT1 siRNA or control siRNA revealed specific interaction between Sp1 and MALAT1. E, MM-MSCs lysates were incubated with biotinylated Sp1 consensus site DNA probe; after pulldown, MALAT1 was extracted and assessed by quantitative RT-PCR. The error bars represent mean ± S.D. of three independent experiments.

FIGURE 6. IncRNA MALAT1 in recruitment of the Sp1 on LTBP3 promoter in vivo. A, schematic representation of the primer sets specific for LTBP3 promoter. B, MSCs were untreated or treated with control siRNA or with siRNA targeting MALAT1, and ChIP assays were performed with the Sp1 antibody and the indicated PCR primers. The binding of Sp1 to the LTBP3 promoter was detected by quantitative real-time PCR. The results were normalized to the input control. All data are shown as mean ± S.D. C, representative analysis of PCR products derived by ChIPs, which were separated on an agarose gel. D, RNA immunoprecipitation using Sp1 antibody followed by quantitative real-time PCR for MALAT1 in MSCs transfected with two independent MALAT1 siRNA or control siRNA revealed specific interaction between Sp1 and MALAT1. E, MM-MSCs lysates were incubated with biotinylated Sp1 consensus site DNA probe; after pulldown, MALAT1 was extracted and assessed by quantitative RT-PCR. The error bars represent mean ± S.D. of three independent experiments.
to the Sp1 consensus site of LTBp3. MALAT1 acted as a co-factor to modulate transcription factor Sp1 activity.

To strengthen the evidence for RNA-dependent activation of transcription factor Sp1 in vivo, we then performed an RNA immunoprecipitation assay and showed that immunoprecipitation of Sp1 from MM-MSCs7, MM-MSCs8, MM-MSCs10, or MM-MSCs12 contained MALAT1 transcript. Knockdown of MALAT1 caused weak interaction between Sp1 and MALAT1 (Fig. 6D). The specific association between MALAT1 and the Sp1 consensus site of LTBp3 was further validated by pulldown of endogenous MALAT1 using in vitro biotinylated Sp1 consensus site DNA probe (Fig. 6E) (p < 0.01). The data strengthen the evidence for the interaction of the Sp1 with MALAT1. These results clearly implicated that IncRNA MALAT1 positively regulates LTBp3 transcription by recruiting Sp1 to the LTBp3 promoter. Recent studies of transcription factors that bind DNA and RNA with distinct roles have been reported (33, 34). In our studies, we showed that Sp1, an important transcription factor, known to bind and activate promoters, cooperated with the IncRNA MALAT1, resulting in an increase of LTBp3 transcription.

DISCUSSION

The notion that ncRNAs can have numerous molecular functions is rapidly evolving (35, 36). Although long ncRNAs represent a large class of transcriptional units and appear to be evolutionarily conserved, their specific roles in critically modulating regulated gene expression remain poorly understood. With the recent recognition of IncRNAs flanking many genes (4, 37–39), a central issue is to fully understand their potential roles in regulating the process of transcription, possibly through different mechanisms (26, 28, 33, 40–43).

Among a small number of genes that were dysregulated in adult MALAT1 knock-out mice, many were MALAT1 neighboring genes, including LTBp3, thus indicating a potential regulatory role of MALAT1 gene transcription (44). Our data showed that in MSCs, IncRNA MALAT1 regulated transcription of nearby protein-coding gene LTBp3 that regulates the bioavailability of TGF-β in particular. MALAT1 affected transcriptional activity by cooperation and transcription factor complex formation. MALAT1 bound with the Sp1 transcription factor and enhanced Sp1 activity in the LTBp3 target. Knockdown of MALAT1 using specific siRNAs significantly decreased LTBp3 transcription, indicating required functions of this coactivator on this gene. The interaction of transcription factors, in particular the bicoid homeodomain protein with both DNA and RNA, has been reported previously (33, 34, 45), raising the possibility of a direct interaction between the Sp1 protein and the MALAT1 ncRNA. By using assays such as luciferase, chromatin immunoprecipitation, and RNA immunoprecipitation, we showed that MALAT1 had a critical function in a mechanism of promoter-specific transcriptional activation. MALAT1 functioned as an Sp1 transcriptional coactivator. Our data suggested that the Sp1-MALAT1 complex stabilized the interaction between Sp1 and Sp1 consensus sequences to increase transcriptional activity. A model of one possible mechanism is shown in Fig. 7. In this model, MALAT1 bound to Sp1 in a DNA-independent manner, and the entire complex bound to Sp1 consensus sequences. We propose that the IncRNA MALAT1 acted as specific transcriptional coactivators. Transcriptional coregulators, including coactivators and corepressors, are required for regulating programs of gene expression in a transcription factor- and gene-specific manner (46, 47). MALAT1 recruited the binding and action of the transcription factor Sp1 to the promoter to increase expression of the adjacent gene. Sp1 bound both DNA and RNA during the cooperative interaction. Here we suggested the model in which IncRNA MALAT1 serves as a molecular “ligand” for a specific transcription factor, Sp1. This in turn permitted gene-specific Sp1-MALAT1 interactions resulting in increase of LTBp3 transcription. It is tempting to speculate that other transcription factors exert functional roles on gene transcription by being analogously recruited to the transcription units through gene-specific IncRNAs.

Myeloma cells enhance bone resorption and suppress bone formation (14). TGF-β plays a role in the suppression of bone formation in MM bone lesions. The inhibition of TGF-β signaling causes not only an enhancement of bone formation but also a suppression of MM cell growth (15). Here we showed that in MSCs, MALAT1 promoted the activation effect of the key transcription factor Sp1 on LTBp3 promoter by modulating recruitment of Sp1 to the LTBp3 gene that regulates the bioavailability of TGF-β in particular. Our growing knowledge of the role of MALAT1 is pointing toward their potential use as biomarkers and targets for novel therapeutic approaches in MM. Broadly, our data highlight combinational actions of transcription factor and IncRNA. An ultimate goal should be the use of cis- and trans-acting IncRNAs as both diagnostic markers and RNA-based targets for the treatment of human diseases. To reach this end, a fuller understanding of the functional versatility of IncRNAs as key regulators of genome expression in physiological conditions and diseases will be required.

REFERENCES

1. Khaitan, D., Dinger, M. E., Mazar, J., Crawford, J., Smith, M. A., Mattick, J. S., and Perera, R. J. (2011) The melanoma-upregulated long noncoding RNA SPRY4-IT1 modulates apoptosis and invasion. Cancer Res. 71, 3822–3862.
2. Kotake, Y., Nakagawa, T., Kitagawa, K., Suzuki, S., Liu, N., Kitagawa, M., and Xiong, Y. (2011) Long non-coding RNA ANRIL is required for the PRC2 recruitment to and silencing of p15INK4B tumor suppressor gene. Oncogene 30, 1956–1962.
3. Gupta, R. A., Shah, N., Wang, K. C., Kim, J., Horlings, H. M., Wong, D. J., Tsi, M. C., Hung, T., Argani, P., Rinn, J. L., Wang, Y., Brzoska, P., Kong, B., Li, R., West, R. B., van de Vijver, M. J., Sukumar, S., and Chang, H. Y. (2010) Long non-coding RNA HOTAIR reprograms chromatin state to promote cancer metastasis. Nature 464, 1071–1076.
4. Carninci, P., Kasukawa, T., Katayama, S., Gough, J., Frith, M. C., Maeda,
Intronic long non-coding MALAT-1 RNA indicates a poor prognosis in non-small cell lung cancer and induces migration and tumor growth. *Thorac. Oncol.* 6, 1984–1992

13. Xu, C., Yang, M., Tian, J., Wang, X., and Li, Z. (2011) MALAT-1: a long non-coding RNA and its important 3’ end functional motif in colorectal cancer metastasis. *Int. J. Oncol.* 39, 169–175

14. Barillé-Nion, S., and Bataille, R. (2003) New insights in myeloma-induced osteolysis. *Leuk. Lymphoma* 44, 1463–1467

15. Matsumoto, T., and Abe, M. (2011) TGF-β-related mechanisms of bone destruction in multiple myeloma. *Bone* 48, 129–134

16. Seyed, M. S., Thomas, T. C., Thompson, A. Y., Rosen, D. M., and Piez, K. A. (1985) Purification and characterization of two cartilage-inducing factors from bovine demineralized bone. *Proc. Natl. Acad. Sci. U.S.A.* 82, 2267–2271

17. Miyazono, K., Olofsson, A., Colosetti, P., and Heldin, C. H. (1991) A role of the latent TGF-β1-binding protein in the assembly and secretion of TGF-β1. *EMBO J.* 10, 1091–1101

18. Dabovic, B., Chen, Y., Colarossi, C., Ohata, H., Zambuto, B., Perle, M. A., and Rifkin, D. B. (2002) Bone abnormalities in latent TGF-β-binding protein (Ltpb)-3 null mice indicate a role for Ltbp-3 in modulating TGF-β bioavailability. *J. Cell Biol.* 156, 227–232

19. Dabovic, B., Chen, Y., Colarossi, C., Zambuto, B., Ohata, H., and Rifkin, D. B. (2002) Bone defects in latent TGF-β binding protein (Ltbp)-3 null mice; a role for Ltbp in TGF-β presentation. *Endocrinol.* 175, 129–141

20. Lamond, A. I., and Spector, D. L. (2003) Nuclear speckles: a model for nuclear organelles. *Nat. Rev. Mol. Cell Biol.* 4, 605–612

21. Calin, G. A., and Croce, C. M. (2006) MicroRNA signatures in human cancers. *Nat. Rev. Cancer* 6, 857–866

22. Yang, L., Lin, C., Liu, W., Zhang, J., Ohgi, K. A., Grinstein, J. D., Dorrestein, P. C., and Rosenfeld, M. G. (2011) ncRNA- and Pc2 methylation-dependent gene relocation between nuclear structures mediates gene activation programs. *Cell* 147, 773–788

23. Bumgarner, S. L., Neurett, G., Voigt, B. F., Symbor-Nagrabska, A., Grisafi, P., van Oudenaarden, A., and Fink, G. R. (2012) Single-cell analysis reveals that noncoding RNAs contribute to clonal heterogeneity by modulating transcription factor recruitment. *Mol. Cell* 45, 470–482

24. Hongay, C. F., Grisafi, P. L., Galitski, T., and Fink, G. R. (2006) Antisense transcription controls cell fate in *Saccharomyces cerevisiae*. *Cell* 127, 735–745

25. Mattens, J. A., Laprade, L., and Winston, F. (2004) Intergenic transcription is required to sequester the *Saccharomyces cerevisiae SRE3* gene. *Nature* 429, 571–574

26. Petruk, S., Sedkov, Y., Riley, K. M., Hodgson, J., Schweiguth, F., Hirose, J., Jaynes, J. B., Brock, H. W., and Mazo, A. (2006) Transcription of non-coding *bxd* RNAs promoted by the Trithorax TAC1 complex represses *Ubx* by a transcriptional interference mechanism. *Cell* 127, 1209–1221

27. Guenther, M. G., Levine, S. S., Boyer, L. A., Jaenisch, R., and Young, R. A. (2007) A chromatin landmark and transcription initiation at most promoters in human cells. *Cell* 130, 77–88

28. Martinez, I., Ramadass, A., Serra Barros, A., Chow, N., and Akoulitch, A. (2007) Repression of the human dihydrofolate reductase gene by a non-coding interfering transcript. *Nature* 445, 666–670

29. Li, B., Fu, J., Chen, P., and Zhuang, W. (2010) Impairment in immune-modulatory function of mesenchymal stem cells from multiple myeloma patients. *Arch. Med. Res.* 41, 623–633

30. Li, B., Shi, M., Li, J., Zhang, H., Chen, B., Chen, L., Gao, W., Giuliani, N., and Zhao, R. C. (2007) Elevated tumor necrosis factor-α suppresses TAZ expression and impairs osteogenic potential of Fk-1+ mesenchymal stem cells in patients with multiple myeloma. *Stem Cells Dev.* 16, 921–930

31. Yuan, J. H., Yang, F., Wang, F., Ma, J. Z., Guo, Y. J., Tao, Q. F., Liu, F., Pan, W., Wang, T. T., Zhou, C. C., Wang, S. B., Wang, Y. Z., Yang, Y., Yang, N., Zhou, W. P., Yang, G. S., and Sun, S. H. (2014) A long non-coding RNA activated by TGF-β promotes the invasion-metastasis cascade in hepatocellular carcinoma. *Cancer Cell* 25, 666–681

32. Kantola, A. K., Keski-Oja, J., and Koli, K. (2005) Induction of human LTBp-3 promoter activity by TGF-β is mediated by Smad3/4 and AP-1 binding elements. *Gene* 363, 142–150

33. Feng, J., Bi, C., Clark, B. S., Mady, R., Shah, P., and Kohtz, J. D. (2006) The long non-coding MALAT-1 RNA
Evf-2 noncoding RNA is transcribed from the Dlx-5/6 ultraconserved region and functions as a Dlx-2 transcriptional coactivator. *Genes Dev.* **20**, 1470–1484

34. Cassiday, L. A., and Maher, L. J., 3rd. (2002) Having it both ways: transcription factors that bind DNA and RNA. *Nucl. Acids Res.* **30**, 4118–4126

35. Eddy, S. R. (2001) Non-coding RNA genes and the modern RNA world. *Nat. Rev. Genet.* **2**, 919–929

36. Ponting, C. P., Oliver, P. L., and Reik, W. (2009) Evolution and functions of long noncoding RNAs. *Cell* **136**, 629–641

37. Kapranov, P., Willingham, A. T., and Gingeras, T. R. (2007) Genome-wide transcription and the implications for genomic organization. *Nat. Rev. Genet.* **8**, 413–423

38. Bernstein, E., and Allis, C. D. (2005) RNA meets chromatin. *Genes Dev.* **19**, 1635–1655

39. Bertone, P., Stolc, V., Royce, T. E., Rozowsky, J. S., Urban, A. E., Zhu, X., Rinn, J. L., Tongprasit, W., Samanta, M., Weissman, S., Gerstein, M., and Snyder, M. (2004) Global identification of human transcribed sequences with genome tiling arrays. *Science* **306**, 2242–2246

40. Rinn, J. L., Kertesz, M., Wang, J. K., Squazzo, S. L., Xu, X., Brugmann, S. A., Goodnough, L. H., Helms, J. A., Farnham, P. J., Segal, E., and Chang, H. Y. (2007) Functional demarcation of active and silent chromatin domains in human HOX loci by noncoding RNAs. *Cell* **129**, 1311–1323

41. Zhou, Y., Zhong, Y., Wang, Y., Zhang, X., Batista, D. L., Gejman, R., Ansell, P. J., Zhao, J., Weng, C., and Klibanski, A. (2007) Activation of p53 by MEG3 non-coding RNA. *J. Biol. Chem.* **282**, 24731–24742

42. Lanz, R. B., McKenna, N. J., Onate, S. A., Albrecht, U., Wong, J., Tsai, S. Y., Tsai, M. J., and O’Malley, B. W. (1999) A steroid receptor coactivator, SRA, functions as an RNA and is present in an SRC-1 complex. *Cell* **97**, 17–27

43. O’Neill, M. J. (2005) The influence of non-coding RNAs on allele-specific gene expression in mammals. *Hum. Mol. Genet.* **14**, Spec. No. 1, R113–R120

44. Zhang, B., Arun, G., Mao, Y. S., Lazar, Z., Hung, G., Bhattacharjee, G., Xiao, X., Booth, C. J., Wu, J., Zhang, C., and Spector, D. L. (2012) The lncRNA Malat1 is dispensable for mouse development but its transcription plays a cis-regulatory role in the adult. *Cell Rep.* **2**, 111–123

45. Dubnau, J., and Struhl, G. (1996) RNA recognition and translational regulation by a homeodomain protein. *Nature* **379**, 694–699

46. Rosenfeld, M. G., Lunyak, V. V., and Glass, C. K. (2006) Sensors and signals: a coactivator/corepressor/epigenetic code for integrating signal-dependent programs of transcriptional response. *Genes Dev.* **20**, 1405–1428

47. McKenna, N. J., and O’Malley, B. W. (2002) Combinatorial control of gene expression by nuclear receptors and coregulators. *Cell* **108**, 465–474