THE JOURNAL OF BIOLOGICAL CHEMISTRY VOL. 289, NO. 42, pp. 29365–29375, October 17, 2014

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

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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 lncRNAs[3] 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

* This work was supported by grants from the National Natural Science Foundation of China (81172256, 81102466, and 81272631), China Postdoctoral Science Foundation (2014M551650 and 2014M550307), Postdoctoral Science Foundation of Jiangsu Province, China (1302028B), the Applied Basic Research Programs of Suzhou City (SYS201132), and Jiangsu Province's Key Medical Department in 2011.
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[3] The abbreviations used are: lncRNA, long noncoding RNA; ncRNA, noncoding RNA; MSC, mesenchymal stem cell; MM, multiple myeloma; LTBP, latent TGF-β-binding protein; HD, healthy donor; chr, chromosome(s).

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 lncRNA *MALAT1* directly interacted with Sp1 and *LTBP3* promoter to increase expression of *LTBP3* gene. lncRNAs 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 lncRNA *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.

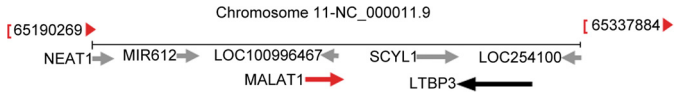

FIGURE 1. **Genomic organization of human *LTBP3* and *MALAT1*.** *MALAT1* and *LTBP3* genes are located at chromosome 11q13.1. They are separated by ~32.0 kb and are transcribed in opposite directions.

Highly specific and stable triplex structures formed by lncRNA with the transcription factor and the promoter may well contribute to promoter targeting and activation *in vivo*. These data provided an unexpected lncRNA/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), 10^{9} M dexamethasone (Sigma), 10^{4} 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-

TABLE 1
Characterization of myeloma patients
Clinical stage of patients was evaluated according to the Durie-Salmon Staging System. M, male; F, female.

Patient	Sex	Age (in years)	Clinical stage	Para protein	Percentage of monoclonal plasma cells	Bone lesions
1	F	59	IIIB	IgG-κ	79.0	Yes
2	F	65	IIIA	IgG-κ	44.0	Yes
3	M	69	IIA	IgG-λ	33.0	Yes
4	F	46	IIIB	IgG-λ	28.0	Yes
5	F	77	IIIA	IgG-λ	14.0	Yes
6	M	73	IIIB	IgG-κ	93.0	Yes
7	F	44	IIIA	IgA-κ	28.0	No
8	F	59	IIIA	IgG-κ	19.0	Yes
9	M	75	IIIA	IgG-κ	58.0	Yes
10	F	66	IIIA	Nonsecreted	90.0	Yes
11	F	46	IIIA	IgG-λ	21.0	No
12	M	59	IIIA	IgG-λ	31.0	Yes
13	F	59	IIIA	IgA-λ	25.0	Yes
14	F	73	IIIA	IgG-λ	57.0	Yes
15	M	50	IIIA	κ-Light chain	16.5	Yes
16	M	61	IIIB	IgG-κ	18.5	No
17	F	58	IIIA	IgG-κ	16.0	Yes
18	M	71	IIIA	IgG-λ	28.0	Yes
19	F	61	IIA	IgG-λ	42.0	Yes
20	F	62	IIIA	λ-Light chain	35.0	Yes
21	M	71	IIIA	IgA-κ	78.0	No
22	M	62	IIIA	IgG-κ	14.0	Yes
23	M	61	IIIA	Nonsecreted	67.0	Yes
24	M	67	IIIA	IgG-λ	5.0	Yes
25	M	64	IIIA	IgG-κ	16.0	Yes

nologies). Cells were cultured at 37 °C and 5% CO_2 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: si*MALAT1*-1, 5′-GAAUUCCGGUGAUGCGAGU-3′; si*MALAT1*-2, 5′-GGCAAUAGAGGCCCUCUAA-3′; siSp1-1, 5′-AGCCUUGAAGUGUAGCUAU-3′; siSp1-2, 5′-GGUAGCUCUAAGUUUUGAU-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/+200) 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-CTCCCCGCCCCGCCCCGCC-3′) or its mutation (5′-Biotin-CTCCCCTTCCCGCCCCGCC-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. 2*A*). The changes in protein levels of LTBP3 in cells as mentioned above were further confirmed by the immunoblotting analysis (Fig. 2*C*). To determine the biological source of TGF-β1 in MSCs, the synthesis and secretion

TABLE 2
Primers used in this study

	Forward primers	Reverse primers
For Luciferase reporter vectors		
***LTBP3* promoter primers**		
1.(-2000-+200)	GGAGCTCAGATGCCCACAGGTG	CCCTCGAGTAGAGGGCCGGGAG
2. (-1800-+200)	GGAGCTCCAGGACTGGTTCTATT	CCCTCGAGTAGAGGGCCGGGAG
3. (-1600-+200)	GGAGCTCACTGTGCTGTGCTCC	CCCTCGAGTAGAGGGCCGGGAG
4.(-1400-+200)	GGAGCTCCGGCAGCAGGGATCCCT	CCCTCGAGTAGAGGGCCGGGAG
5.(-1200-+200)	GGAGCTCCCGGGAGAGGGTCTGGT	CCCTCGAGTAGAGGGCCGGGAG
6.(-1000-+200)	GGAGCTCCCTCTCCAGCCATGGCCA	CCCTCGAGTAGAGGGCCGGGAG
7.(-800-+200)	GGAGCTCGCCAGCACGGCCAGACG	CCCTCGAGTAGAGGGCCGGGAG
8.(-600-+200)	GGAGCTCTTGGACCAGCACTAACGA	CCCTCGAGTAGAGGGCCGGGAG
9.(-400-+200)	GGAGCTCTCCGCGGCCGGCCAGAC	CCCTCGAGTAGAGGGCCGGGAG
10.(-200-+200)	GGAGCTCCCCGGGGCCAGCGTCCC	CCCTCGAGTAGAGGGCCGGGAG
11.(0-+200)	GGAGCTCCTTGCTCCGGCCTCCTCG	CCCTCGAGTAGAGGGCCGGGAG
For mutagenesis		
IK-2-mut	CACGTTTGCAAGGGAGAGCTGGAAGGCC TG	CAGGCCTTCCAGCTCTCCCTTGCAAACGTG
Sp1-mut	CGCTCTACGCAGGCCGCCCCGCCCGGAGG	CCTCCGGGCGGGGCGGCCTGCGTAGAGCG
GATA-2-mut	CTCTCTCCCTCAGCCTCTCTACGCAGGCCT C	GAGGCCTGCGTAGAGAGGCTGAGGGAGAGA G
MZF1-mut	CGCTCTACGCAGGCCCCGCCCCGCCCGGA G	CTCCGGGCGGGGCGGGGCCTGCGTAGAGCG
For chip assay		
Primer1	CTGGGAAAGAGCTGGAAGGC	AGTCCCCACCCCAGATGCC
Primer2	CCGGCCTCCATCGTCAGTG	GCCTGGAGAAGCGAGGGTGT
Primer3	CGGCCTCCATCGTCAGTGT	CCTCACCTGGGACCGTCTG
Site1	AGTCCTGCCTCTTCCCTCCC	TGACAAACACTGGCCGCTCC
Site2	AAGTGCATCGCCTGTCAGC	TGTTTACAGACAGCGTGACCC
Site3	ACCCATGCCAAGCCCCTAG	TCCGTGGTCACCCCTAAAA
Site4	AGCACAGACACGCTCACGG	GCAGGCCCTACTTTAGACAT
Site5	CTGGGAAAGAGCTGGAAGGC	AGTCCCCACCCCAGATGCC

of TGF-β1 by MSCs (Fig. 2*B*) 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. 2*D*).

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

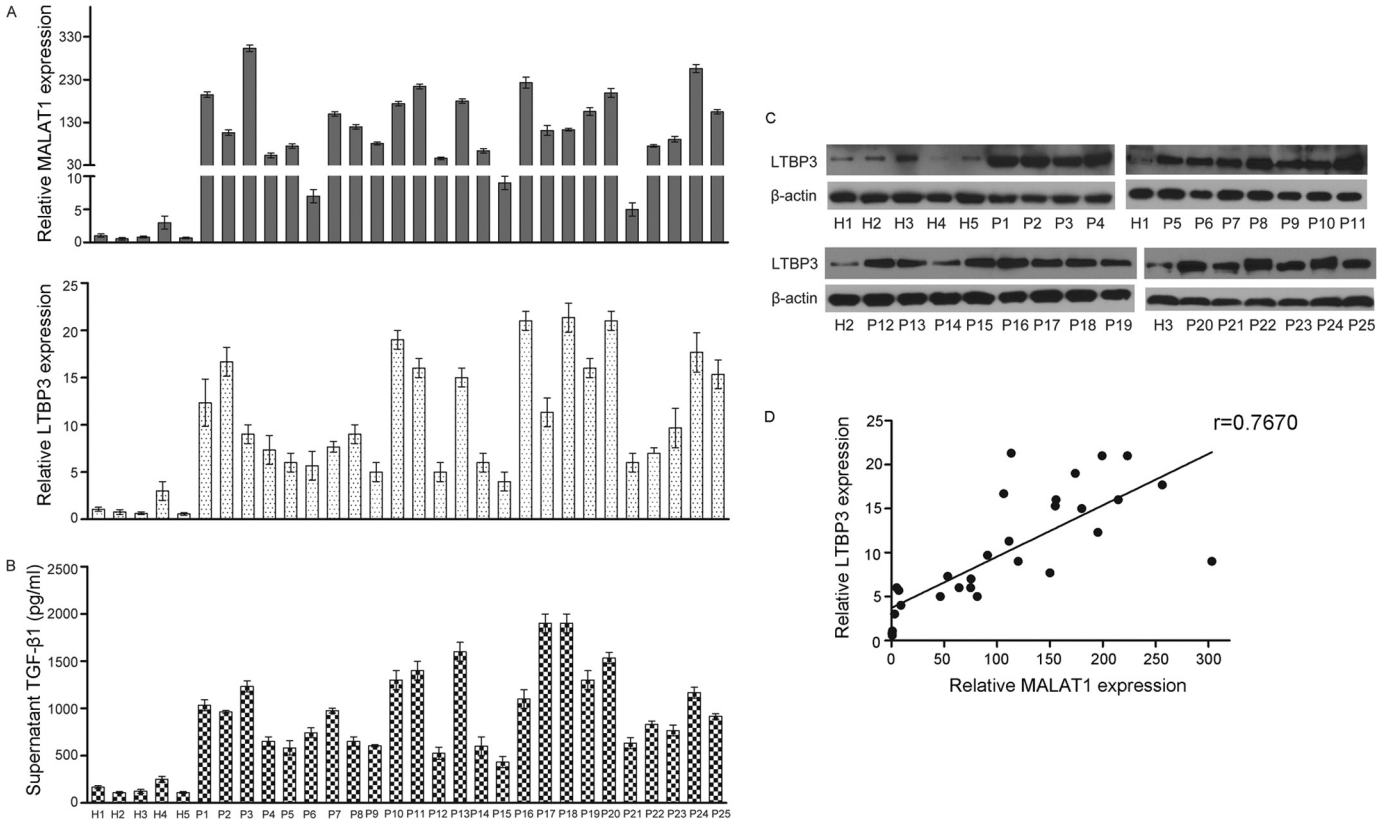

FIGURE 2. **Different expression of *MALAT1* and *LTBP3*, as well as different TGF-β1 secretion between MSCs from bone marrow of myeloma patients and those from healthy donors.** *A*, *MALAT1* and *LTBP3* transcript levels were determined by quantitative real-time PCR analysis of MSCs from bone marrow of myeloma patients and healthy donors. GAPDH was measured for an internal control and used to normalize the data. *B*, supernatant TGF-β1 secreted by the cultured MSCs was determined by ELISA assay as described under "Experimental Procedures." Levels of TGF-β1 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. *D*, correlation between *MALAT1* and *LTBP3* in MSCs from healthy donors and myeloma patients; $r = 0.7670$, $p < 0.0001$.

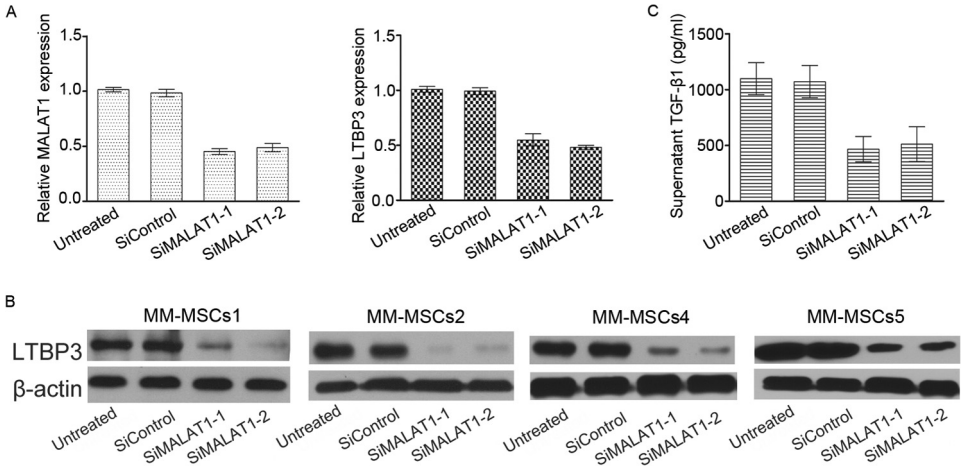

FIGURE 3. **Knockdown of the *MALAT1* by RNA interference decreased *LTBP3* transcription and TGF-β1 secretion.** MM-MSCs1, MM-MSCs2, MM-MSCs4, or MM-MSCs5 was transfected with two independent siRNA against *MALAT1* or with control siRNA (*siControl*) for 48 h. *A*, quantitative real-time PCR analysis was performed to determine *MALAT1* and *LTBP3* levels, with GAPDH used as an internal normalization control. *Error bars* represent mean ± S.D. *B*, LTBP3 levels were determined by immunoblotting. *C*, TGF-β1 was quantified by ELISA.

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. *MALAT1* was overexpressed more than 11- and 14-fold, respectively (Fig. 4*A*). As shown in Fig. 4, *B* and *C*, overexpres-

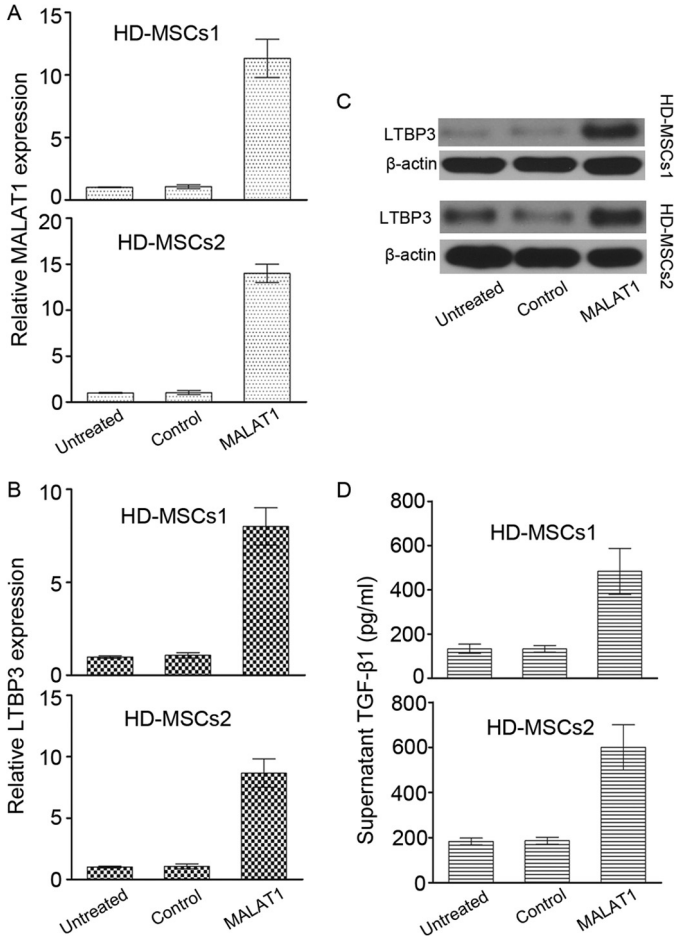

FIGURE 4. **Overexpression of *MALAT1* increased *LTBP3* transcription and TGF-β1 secretion.** HD-MSCs1 or HD-MSCs2 was infected with control or *MALAT1*-expressing lentivirus for 48 h. *A* and *B*, quantitative real-time PCR analysis was performed to determine *MALAT1* (*A*) and *LTBP3* (*B*) levels, with GAPDH used as an internal normalization control. *Error bars* represent mean ± S.D. *C*, LTBP3 levels were determined by immunoblotting. *D*, TGF-β1 was quantified by ELISA.

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. 4*D*). 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. 5*A*). 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. 5*B*). 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. 5*C*) ($p < 0.01$). To provide further confirmation, we examined 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. 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. 5*D*) ($p < 0.01$). 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. 5*C*). As shown in Fig. 5*E*, the endogenous Sp1 binding site 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

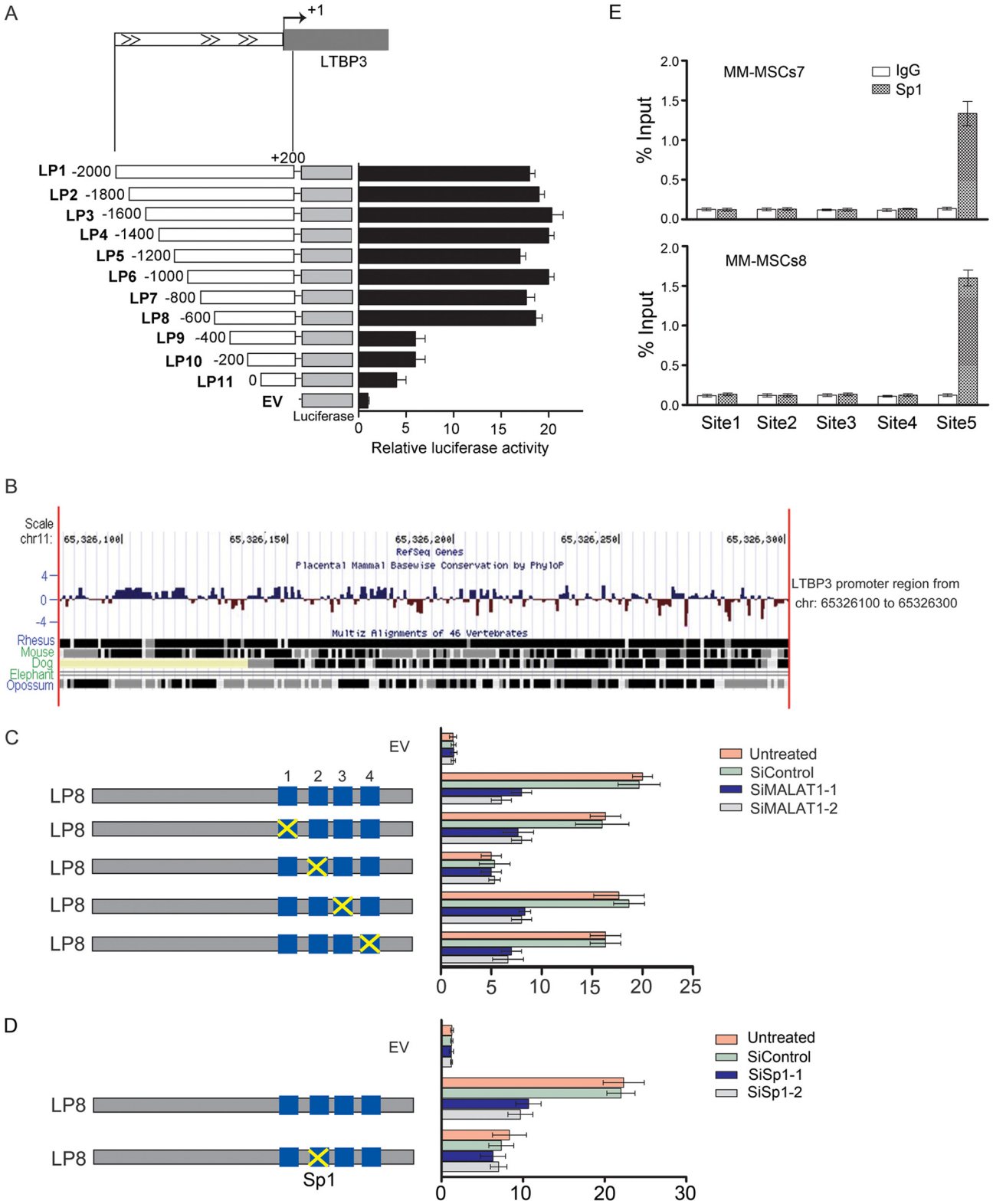

FIGURE 5. **Identification of *MALAT1* core element in the *LTBP3* promoter.** *A*, luciferase reporter activity of promoter constructs of *LTBP3* on chromosome 11q13.1 in MSCs2 cells. Schematic presentation of *LTBP3* promoter deletion constructs is shown on the *left*. The *arrow* above construct LP1 indicates the position of the transcription start site +1. 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 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.

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

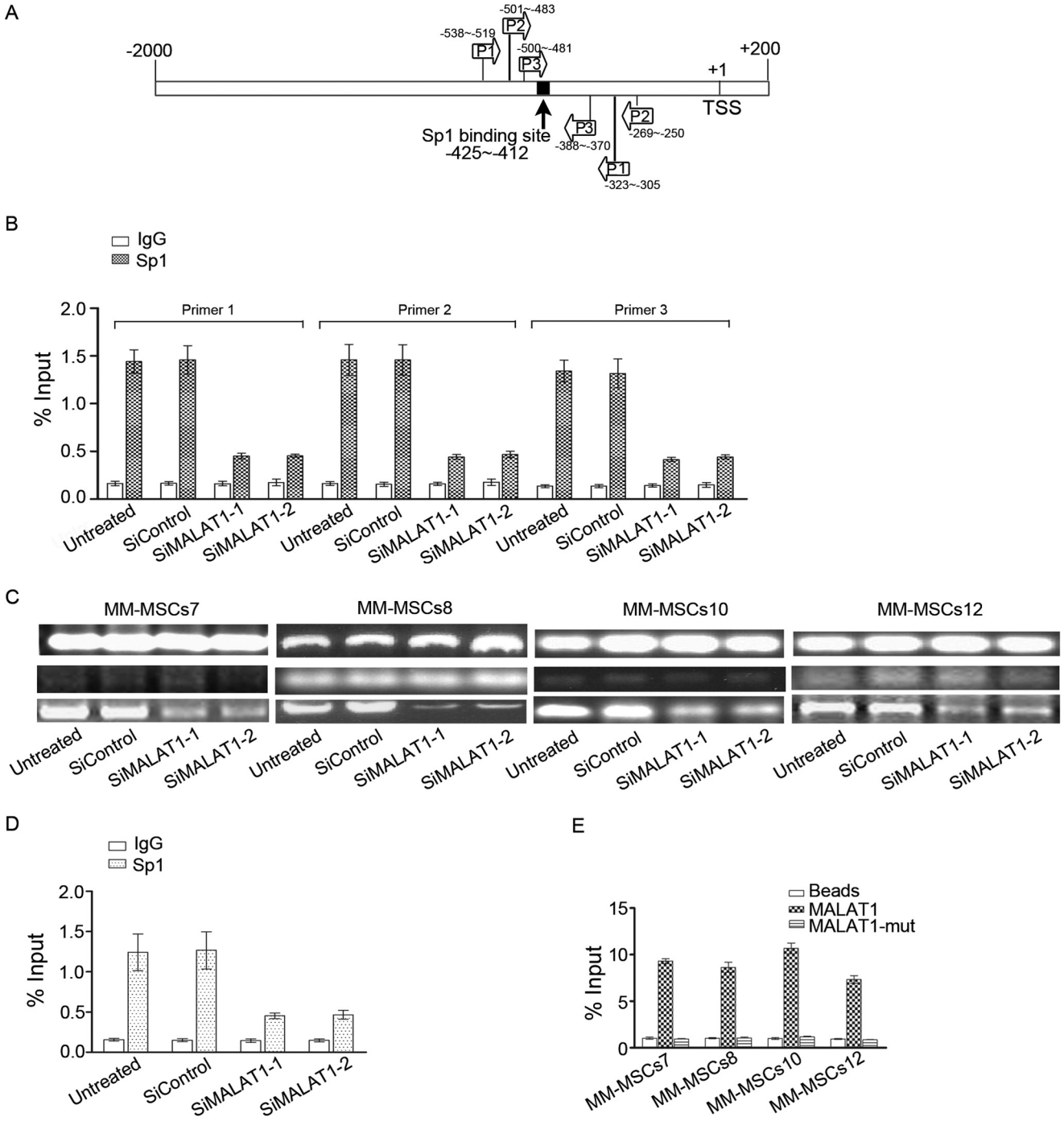

FIGURE 6. **lncRNA *MALAT1* in recruitment of the Sp1 on *LTBP3* promoter *in vivo*.** *A*, schematic representation of the primer sets specific for *LTBP3* promoter. *B*, MSCs were untreated or treated with control siRNA or with siRNA targeting *MALAT1*, and ChIP assays were performed with the Sp1 antibody 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. 6*D*). 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. 6*E*) ($p < 0.01$). The data strengthen the evidence for the interaction of the Sp1 with *MALAT1*. These results clearly implicated that lncRNA *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 lncRNA *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 lncRNAs 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, lncRNA *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

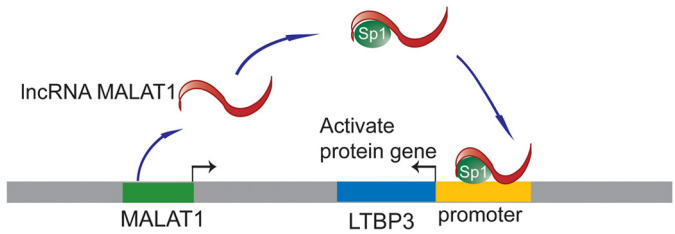

FIGURE 7. **A model proposing that *MALAT1* and Sp1 form a complex, ultimately affecting transcription of the *LTBP3* gene.**

to Sp1 consensus sequences. We propose that the lncRNA *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 lncRNA *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 lncRNAs.

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 lncRNA. An ultimate goal should be the use of cis- and trans-acting lncRNAs 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 lncRNAs as key regulators of genome expression in physiological conditions and diseases will be required.

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