HBXIP and LSD1 Scaffolded by IncRNA Hotair Mediate Transcriptional Activation by c-Myc
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Abstract

C-Myc is regarded as a transcription factor, but the basis for its function remains unclear. Here, we define a long noncoding RNA (IncRNA)/protein complex that mediates the transcriptional activation by c-Myc in breast cancer cells. Among 388 c-Myc target genes in human MCF-7 breast cancer cells, we found that their promoters could be occupied by the oncoprotein HBXIP. We confirmed that the HBXIP expression correlated with expression of the c-Myc target genes cyclin A, eIF4E, and LDHA. RNAi-mediated silencing of HBXIP abolished c-Myc-mediated upregulation of these target genes. Mechanistically, HBXIP interacted directly with c-Myc through the leucine zippers and recruited the IncRNA Hotair along with the histone demethylase LSD1, for which Hotair serves as a scaffold. Silencing of HBXIP, Hotair, or LSD1 was sufficient to block c-Myc–enhanced cancer cell growth in vitro and in vivo. Taken together, our results support a model in which the HBXIP/Hotair/LSD1 complex serves as a critical effector of c-Myc in activating transcription of its target genes, illuminating long-standing questions on how c-Myc drives carcinogenesis. Cancer Res; 76(2); 293–304. ©2015 AACR.

Introduction

Cancer cells are characterized by acquisition of several characteristics that enable them to become tumorigenic, in which the uncontrolled proliferation is one of the most fundamental characteristics of cancer cells (1, 2). The activation of oncogenes and their cooperative effects play crucial roles in the process of tumorigenesis and cell proliferation. The oncogene-mediated reprogramming of gene expression, which enables the cell to acquire disorder of metabolism, cell cycle, and other aspects, is a hallmark of cancer cells (1, 3, 4). Importantly, the c-Myc proto-oncogene, which has been implicated in the pathogenesis of most types of human tumors (1, 5), affects multiple cell biologic processes, including cell cycle, protein synthesis, cell adhesion, cytoskeleton, metabolism, and miRNA regulation (6–8). c-Myc protein binds to a DNA sequence called E-box (CACGTG) and dimerizes with Max to mediate approaching a helix-loop-helix (HLH) zipper dimerization motif, by which the transcriptional factor c-Myc in breast cancer remains poorly understood.

Given that transcriptional activation is linked to the chromatin environment, histone H3 modification is the major pattern of chromatin remodeling (21). Lysine-specific demethylase 1 (LSD1)–mediated demethylation of H3K4 leads to local DNA oxidation as a driving force in the assembly of the c-Myc–induced transcription initiation complex (22, 23). The epigenetics is involved in the c-Myc–mediated transcription (24, 25). Long noncoding RNAs (IncRNA) are emerging as new players in the cancer paradigm demonstrating potential roles in both oncogenic and tumor-suppressive pathways. These novel genes accomplish remarkable biologic functions in a variety of human cancers (26). IncRNA HOX transcript antisense intergenic RNA (Hotair), identified as a 2.2 kilobase ncRNA residing in the HOXC locus, can bind to LSD1. Hotair serves as a scaffold of histone modification complexes comprising LSD1 and polycomb-repressive complex 2 (PRC2), resulting in the malignance of multiple tumors (27, 28). It has been reported that IncRNA PRNCR1 and PCEGM1 are involved in the androgen–receptor transcriptional complex (29). However, whether Hotair is involved in the c-Myc–induced transcription activation is not well documented.

In this study, we try to gain insights into the mechanism of c-Myc–mediated transcription activation. Interestingly, our...
data show that HBXIP acting as a coactivator directly interacts with transcription factor c-Myc. HBXIP and LSD1 form a complex that is scaffolded by Hotair to activate c-Myc in the promoter of c-Myc target genes, leading to transcription activation of c-Myc target genes in breast cancer cells. Thus, our finding provides new insights into the mechanism by which c-Myc functions in carcinogenesis.

Materials and Methods

Cell lines, transfection, and siRNA

Immortalized breast cell line HBL-100 and breast cancer cell lines MCF-7, T47D, LM-MCF-7, and SK-BR3 were cultured in RPMI Medium 1640 (Invitrogen) with 10% FCS. MDA-MB-463, MDA-MB-231, and HEK293T cells were cultured in DMEM (Invitrogen) supplemented with 10% FCS. The siRNAs targeting HBXIP mRNA were reported previously (16, 19). The siRNAs targeting c-Myc mRNA, Hotair, and LSD1 mRNA were listed in Supplementary Table S1 (28). Cells were transfected with corresponding plasmids or siRNAs using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s instructions.

Chromatin immunoprecipitation assays and the overlap of HBXIP and c-Myc binding

Chromatin immunoprecipitation (ChIP) assays were performed using the EpiQuik Chromatin Immunoprecipitation Kit from Epigentek Group Inc. MCF-7 cells were lysed 24 hours after transfection. Protein/DNA complexes were immunoprecipitated by HBXIP or LSD1 antibodies, using normal rabbit IgG as a negative control. The primers used for PCR amplification were flanking the E-box in the promoters of cyclin A, elf4E, and LDHA (30–32). The ChIP-DNA Selection and Ligation (ChIP-DLS) and data analysis of HBXIP-bound genes were performed by CapitalBio Corporation according to a protocol from Aviva Systems Biology. Experiments were repeated three times, and the results were analyzed using MAS (http://bioinfo.capitalbio.com/mas/login.do) with a P value cutoff of 1.0 × 10−5 for promoter identification. According to previous report (33), the datasets of ChIP-seq for c-Myc were used. According to the accession number and the gene name, 1,348 top bound genes in c-Myc datasets were used to merge with the HBXIP-bound genes for overlapping. The gene pathways were analyzed by using the KEGG database of DAVID Bioinformatics Resources 6.7 (http://david.abcc.ncifcrf.gov/).

Immunohistochemistry staining

Breast cancer tissue microarrays were purchased from Xian Aomei Biotechnology. IHC staining was performed as described previously (16). The negative control was performed as above protocol without using primary antibody. The staining level of HBXIP, cyclin A, elf4E, and LDHA was classified into three groups (0, 0% stained; 1, 1%–49% stained; 2, 50%–100% stained). A multiplied score (intensity score × percentage score) lower than 1 was considered to be negative staining (−). 1 and 2 were considered to be moderate staining (+), and 4 was considered to be intense staining (++)

Real-time PCR and Western blot analysis

Total 40 cases of breast cancer tissues were collected from patients undergoing resection of breast cancer in Tianjin First Center Hospital (Tianjin, China). Total RNAs from breast cancer tissues or cells were extracted using TRIzol reagent (Invitrogen) according to the instructions (16). First-strand cDNA was synthesized by PrimeScript reverse transcriptase (TaKaRa Bio) according to the manufacturer’s instructions. Primers used to test HBXIP expression were obtained from reports (16). Real-time PCR was performed as described previously (16). Primers used in the study were listed in Supplementary Table S2. For Western blot analysis (16), total protein lysate was extracted from tissues or cells with RIPA buffer (Solarbio). The protein samples were subjected to SDS-PAGE and then transferred to a nitrocellulose membrane, blocked with 5% non-fat milk, and incubated with primary antibodies for 2 hours at room temperature. After incubation with secondary antibody for 1 hour, the membrane was visualized by ECL (Millipore). The antibodies used in this study were listed in Supplementary Table S3. All experiments were repeated 3 times.

Constructs

The plasmids, such as pCMV-Tag2B, pCMV-HBXIP, pCDNA3.1, pDNA-HBXIP, pGEX-4T1, pGEX-HBXIP, and pCMV-HBXIP-LZM, were used in our previous studies (16, 34, 35). The E-box reporter was constructed by inserting 6 × E-box into the pGL3-Basic vector (36). pCDNA-Hotair vector with full-length Hotair was constructed by Genomics. Human HBXIP, c-Myc, and the fragments of HBXIP were PCR-amplified from cDNA of total RNAs in MCF-7 cells. The resulting products were cloned into the multiple cloning sites of the vectors, such as pEGFP-C2, pCMV-Tag2B, pET28, pDNA3.1, pDNA-HA, and pGEX-4T1, respectively.

Luciferase reporter gene assays

The plasmids of Renilla luciferase reporter vector pRL-TK were used as described previously (16). Luciferase activities were normalized to Renilla luciferase activity. All the assays were performed in triplicate. Cells were seeded into 24-well plates and cultured for 24 hours before transfection. The luciferase activities of E-box reporter and Gal4-c-Myc reporter were determined 36 hours after transfection using a dual-luciferase reporter gene assay kit (Promega).

Immunofluorescence staining and confocal microscopy

Immunofluorescence staining was performed as described previously (35). After transfection, the cells were fixed with paraformaldehyde, and permeabilized with 0.1% Triton X-100 in PBS. After blocking in PBS containing 3% BSA, the cells were incubated with primary antibodies at room temperature. After washing with PBS, the cells were incubated with fluorophore-conjugated secondary antibody (DAKO) and DAPI. After washing with PBS, slides were mounted with glycerol and observed under a confocal microscopy (Leica TCS SP5).

Coimmunoprecipitation assay and GST pull-down

The cells were harvested and lysed in a lysis buffer (50 mmol/L Tris- HCl, pH 8.0, 100 mmol/L NaCl, 50 mmol/L sodium fluoride, 1% Nonidet P-40, 1 mmol/L dithiothreitol, 1 mmol/L Na3VO4, 1 mmol/L Microcystin-LR, 1 mmol/L phenylmethysulfonyl fluoride, 10 mg/mL leupeptin, and 10 mg/mL aprotinin). The lysates were incubated with antibodies/protein G–conjugated agarose beads (Millipore) or ANTI-FLAG M2 affinity gel beads (Sigma). The precipitates were washed six times with ice-cold lysis buffer,
resuspended in PBS, followed by Western blot analysis. The GST pull-down was performed according to published protocols (16). Glutathione beads were recovered by a brief centrifugation and washed six times with lysis buffer, followed by Western blot analysis.

**Electrophoresis mobility shift assays**

The electrophoresis mobility shift assay (EMSA) protocol was described in detail (16, 17). Nuclear extracts were prepared with MCF-7 cells using the EpiQuik Nuclear Extraction Kit (Epigentek). Probes were generated by annealing single-strand oligonucleotides containing the E-box (37). The primary antibody (1 μg) was incubated with nuclear extracts on ice before probes were added into the binding buffer. Samples were incubated on ice and then separated by electrophoresis on nondenaturing polyacrylamide gel, and then the gel was dried and subjected to autoradiography.

**RNA immunoprecipitation assays**

RNA immunoprecipitation (RIP) assays were performed in native conditions as described (29). Briefly, MCF-7 cell nuclei were pelleted and lysed. The lysates were passed through a 27.5 gauge needle 4 times to promote nuclear lysis. The supernatant was incubated with 4 μg primary antibody of rabbit anti-HBXIP or mouse anti–c-Myc (Supplementary Table S3) with 40 μL protein G–conjugated agarose beads (Millipore) or ANTI-FLAG M2 affinity gel beads (Sigma). The RNA/antibody complex was washed by NT2 buffer (50 mmol/L Tris-HCl, pH 7.4, 150 mmol/L NaCl, 1 mmol/L MgCl2, 0.05% NP-40). The RNA was extracted by TRIzol (Invitrogen) according to the manufacturer’s protocol and subjected to real-time PCR analysis.

**MTT assays**

Cell growth assays were carried out using MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) reagent (Sigma) as described previously (38). In brief, transfected cells were trypsinized, counted, and plated into 96-well plates. After incubating different time periods, MTT was added directly to each well, followed by incubation for 4 hours, and then the supernatant was removed and 100 μL of dimethyl sulfoxide was added to stop the reaction. Absorbance at 490 nm was measured using an ELISA reader system (Labsystem, Multiskan Ascent). All experiments were performed in triplicate.

**Animal transplantation**

All experimental procedures involving animals were in accordance with the Guide for the Care and Use of Laboratory Animals (NIH publications nos. 80–23, revised 1996) and were performed according to the institutional ethical guidelines for animal experiment. MCF-7 cells (1 × 106) transiently transfected with corresponding plasmids and siRNAs were subcutaneously injected into the flanks of 4-week-old male BALB/c athymic nude mice, respectively. Tumor growth was monitored every 4 days. After 26 days, the mice were sacrificed, necropsies were performed, and tumors were weighed. Tumor volume (V) was monitored by measuring the length (L) and width (W) using calipers and calculated according to the formula (L × W²) × 0.5 (16).

**Statistical analysis**

Each experiment was repeated at least three times. Statistical significance was assessed by comparing mean values (±SD) using the Student t test for independent groups, assumed for P < 0.01 ("*"). Correlation between expression levels of HBXIP and cyclin A (or eIF4E and LDHA) in tumors was explored using the Pearson correlation coefficient. Statistical analysis of tissue array was performed with the χ² test or Kruskal–Wallis test using the SPSS software program (SPSS).

**Results**

The expression levels of HBXIP are positively associated with those of cyclin A, eIF4E, and LDHA of c-Myc–induced genes in breast cancer

It has been reported that c-Myc is a crucial proto-oncogenic transcription factor in cancer (1, 5). HBXIP functions as an oncogenic transcriptional co-activator in breast cancer (19, 35). Either HBXIP or c-Myc contains a leucine-zipper domain (5, 15). Accordingly, we supposed that HBXIP as a transcriptional co-activator might activate c-Myc–induced genes in breast cancer cells. Compared with the data of c-Myc ChIP-seq (33, 39), we found that 28.8% (388/1,348)/16.8% (388/2,311) for c-Myc/HBXIP-target gene promoters could be occupied by both HBXIP and c-Myc in the cells (Fig. 1A and Supplementary Table S4), suggesting that HBXIP may be involved in the c-Myc–induced gene expression. However, bioinformatics analysis showed that these common target–involved pathways were different from those of the c-Myc target genes, which could not be occupied by HBXIP. Interestingly, we observed that the common genes were associated with cell cycle, whereas the c-Myc target genes, which could not be occupied by HBXIP, were related to ribosome biosynthesis (Supplementary Tables S5 and S6; Supplementary Fig. S1A and S1B). Moreover, we selected three common target genes of HBXIP and c-Myc (cyclin A, eIF4E, and LDHA) to investigate the effect of HBXIP on c-Myc–induced gene transcription. As expected, ChIP assays validated that HBXIP could occupy the promoters of above three genes in MCF-7 cells (Fig. 1B). We also observed that the expression levels of HBXIP exhibited parallel changes with the three genes in six breast cell lines (Fig. 1C). Moreover, IHC staining showed that the positive rate of HBXIP was positively associated with that of cyclin A (or eIF4E and LDHA) in breast cancer tissues (pairing χ² analysis, Fig. 1D and Supplementary Tables S7–S9). Furthermore, real-time PCR revealed that the expression levels of HBXIP mRNA were positively related to those of cyclin A, eIF4E, and LDHA mRNAs in breast cancer tissues (P < 0.01; Pearson correlation, Fig. 1E–G). Thus, we conclude that the expression levels of HBXIP are positively associated with those of cyclin A, eIF4E, and LDHA of c-Myc–induced genes in breast cancer.

HBXIP is required for the upregulation of cyclin A, eIF4E, and LDHA

Next, we are interested in whether HBXIP is able to upregulate c-Myc target genes in breast cancer cells. As expected, real-time PCR assays showed that the overexpression of HBXIP could upregulate the expression of cyclin A, eIF4E, and LDHA at the levels of mRNA in a dose-dependent manner in MCF-7, T47D, and ER-negative MDA-MB-231 cells (Fig. 2A; Supplementary Fig. S2A and S2B). Moreover, Western blot analysis validated the data at the protein levels in the system (Fig. 2B; Supplementary Fig. S2C and S2D). Then, we constructed an E-box luciferase reporter to test the effect of HBXIP on c-Myc–induced transcription (36). Our data revealed that HBXIP triggered the E-box reporter activities in MCF-7 and T47D cells (Fig. 2C and Supplementary Fig. S2E), suggesting that
HBXIP is able to activate the E-box element in the promoters of c-Myc target genes in breast cancer cells. Moreover, the silence of c-Myc by siRNA was able to abolish the HBXIP-mediated expression of above three c-Myc target genes at the levels of mRNA and protein in MCF-7 cells (Fig. 2D and E). Conversely, the depletion of HBXIP by siRNA could result in the disruption of c-Myc–enhanced cyclin A, eIF4E, and LDHA at the levels of mRNA and protein in MCF-7, T47D, and MDA-MB-231 cells (Fig. 2F and G and Supplementary Fig. S2F–S2I), suggesting that HBXIP contributes to the transcriptional activation mediated by c-Myc. Thus, we conclude that HBXIP is required for the upregulation of cyclin A, eIF4E, and LDHA in breast cancer.

HBXIP directly interacts with c-Myc to coactivate c-Myc

Given that either HBXIP or c-Myc contained a leucine-zipper domain (5, 15), we tried to investigate whether HBXIP was able to interact with c-Myc through leucine zipper. Indeed, confocal images validated that most of HBXIP and c-Myc colocalized in the nucleus in MCF-7 and T47D cells (Fig. 3A and Supplementary Fig. S3A). Coimmunoprecipitation (Co-IP) assays validated that the endogenous or exogenous c-Myc could interact with HBXIP in MCF-7 cells (Fig. 3B and Supplementary Fig. S3B). To further verify whether HBXIP directly interacted with c-Myc, GST pull-down assays were performed. We found that the c-Myc protein with His-tag could be pulled down by GST-HBXIP, but not by the control GST (Fig. 3C), suggesting that HBXIP directly binds to c-Myc. Then, we tried to map the binding domain of HBXIP interacting with c-Myc. According to the secondary structure of HBXIP (Fig. 3D; ref. 40), we constructed three fragments of HBXIP (aa 1–55, aa 82–173, and aa 56–173) and leucine-zipper mutant of HBXIP (HBXIP LZM). Co-IP assays showed that either full-length or the fragment aa 82–173
Figure 2.
HBXIP is required for the upregulation of cyclin A, eIF4E, and LDHA. A, the mRNA levels of HBXIP, cyclin A, eIF4E, and LDHA were tested by real-time PCR in MCF-7 cells. B, the protein levels of above genes were tested by Western blot analysis in MCF-7 cells. C, the relative luciferase activities of E-box reporter were tested by dual-luciferase system in MCF-7 cells. D, the mRNA levels of HBXIP, c-Myc, cyclin A, eIF4E, and LDHA were tested by real-time PCR in MCF-7 cells. E, the expression levels of above genes were tested by Western blot analysis in MCF-7 cells. F, the mRNA levels of c-Myc, HBXIP, cyclin A, eIF4E, and LDHA were tested by real-time PCR in MCF-7 cells. G, the expression levels of above genes were tested by Western blot analysis in MCF-7 cells. All experiments were repeated at least three times. Statistically significant differences are indicated. **, P < 0.01, Student t test.
56–173 of HBXIP containing leucine zipper could interact with the HA-tagged c-Myc in HEK293T cells (Fig. 3E), but others failed to work in the cells. GST pull-down assays further validated the same result (Fig. 3F), suggesting that the leucine zipper is required for HBXIP binding to c-Myc. Furthermore, EMSA revealed that a supershift band was observed when the HBXIP antibody was added (Fig. 3G, lane 7), suggesting that HBXIP is able to interact with the E-box element in the promoters of c-Myc target genes. Then, we constructed a plasmid encoding the c-Myc protein fused to the Gal4 DNA-binding domain to test the interaction of HBXIP with c-Myc. Functionally, HBXIP significantly increased the luciferase activities of Gal4-c-Myc in a dose-dependent manner in HEK293T cells (Fig. 3H), suggesting that HBXIP may serve as a coactivator of c-Myc. Thus, we conclude that HBXIP directly interacts with c-Myc to coactivate c-Myc in breast cancer cells.

HBXIP serves as a linker of c-Myc and LSD1 in the complex of c-Myc/HBXIP/LSD1

Usually, transcription factors function in a complex manner in the activation of target genes. It has been reported that LSD1-mediated demethylation of H3K4 acts as a driving force in the assembly of the c-Myc–induced transcription initiation complex (22, 23); however, the mechanism remains unclear. Real-time PCR assays showed that the mRNA levels of LSD1 in the breast cancer cell lines were higher than those in the immortalized breast cell HBL-100 (Supplementary Fig. S4A). Then, we evaluated the relationships between LSD1 and HBXIP (or c-Myc) in breast cancer. Our data showed that the expression levels of LSD1 mRNA were positively associated with those of HBXIP and c-Myc mRNAs in breast cancer tissues (P < 0.01, Pearson correlation, Supplementary Fig. S4B and S4C). Therefore, we supposed that c-Myc/HBXIP/LSD1 might...
form a complex. Confocal images showed that HBXIP, c-Myc, and LSD1 could colocalize in the nucleus of the MCF-7 cells (Fig. 4A). It has been reported that LSD1 is an RNA-interacting protein (27). Interestingly, we found that both HBXIP and c-Myc could be immunoprecipitated by LSD1 antibody when RNase inhibitor was added into the lysis buffer (Fig. 4B). But, it did not work when RNase A was added in the system (Fig. 4C). In addition, LSD1 could also be immunoprecipitated by either HBXIP or c-Myc antibody when the lysis buffer was treated with RNase inhibitor (Fig. 4D), but it failed to work when RNase A was added (Fig. 4E), suggesting that LSD1 indirectly interacts with c-Myc/HBXIP, in which RNA is required for the complex. Moreover, the depletion of HBXIP by siRNA could disrupt the interaction of c-Myc with LSD1 in MCF-7 cells when the RNAs were protected by RNase inhibitors (Fig. 4F), suggesting that HBXIP links c-Myc and LSD1 in the complex of c-Myc/HBXIP/LSD1. ChIP assays further showed that the silence of HBXIP by siRNA resulted in the decrease of LSD1 occupation in the promoters of above three c-Myc target genes in MCF-7 cells (Fig. 4G), suggesting that HBXIP recruits LSD1 to the promoters of c-Myc target genes. Thus, we conclude that HBXIP serves as a linker in the complex of c-Myc/HBXIP/LSD1 in breast cancer cells, in which RNA is required for the complex.
lncRNA Hotair scaffolds HBXIP and LSD1 to form a complex of c-Myc/HBXIP/Hotair/LSD1 in activation of c-Myc–induced transcription

Given that RNAs might be involved in the c-Myc/HBXIP/LSD1 complex as above and lncRNA Hotair interacted with LSD1 in cancer cells (27), we supposed that Hotair might be involved in the c-Myc/HBXIP/LSD1 complex. Then, we provided evidence that the levels of Hotair in the breast cancer cell lines were higher than those in the immortalized breast HBL-100 cell (Supplementary Fig. S5A), and the levels of Hotair were positively associated with those of HBXIP (or c-Myc) in breast cancer tissues (P < 0.01, Pearson correlation, Supplementary Fig. S5B and S5C) by using real-time PCR assays. Strikingly, co-IP assays showed that the depletion of Hotair by siRNA could block the interaction of LSD1 with HBXIP/c-Myc in MCF-7 cells (Fig. 5A), suggesting that Hotair is required for the interaction of HBXIP/c-Myc with LSD1. Next, we concerned whether LSD1-linked Hotair could interact with HBXIP/c-Myc. RIP assays showed that Hotair could be immunoprecipitated by HBXIP or c-Myc in MCF-7 and T47D cells (Fig. 5B). Moreover, silence of HBXIP by siRNA could disrupt the interaction of Hotair with c-Myc in MCF-7 cells, whereas silence of c-Myc by siRNA failed to influence the interaction of Hotair with HBXIP in the cells (Fig. 5C), suggesting that Hotair scaffolds HBXIP, but not c-Myc, and LSD1 in the complex of c-Myc/HBXIP/Hotair/LSD1. Furthermore, depletion of Hotair by siRNA could block the occupation of LSD1 in the promoters of c-Myc target genes (cyclin A, eIF4E, and LDHA) in MCF-7 cells (Fig. 5D and Supplementary Fig. S5D), suggesting that Hotair recruits LSD1 to the promoters of c-Myc target genes. In addition, depletion of HBXIP by siRNA could disrupt the occupation of LSD1 in the promoters of above genes when both c-Myc and Hotair were overexpressed in MCF-7 cells (Fig. 5D and Supplementary Fig. S5D), suggesting that the complex of c-Myc/HBXIP/Hotair/LSD1 might be required for the activation of c-Myc target genes. Functionally, we validated that the overexpression of c-Myc failed to upregulate above c-Myc target genes at the levels of mRNA or protein when Hotair was silenced by siRNA in MCF-7, T47D, and MDA-MB-231 cells (Fig. 5E and F; Supplementary Fig. S5E–S5H). The overexpression of both c-Myc and Hotair failed to upregulate above genes at the levels of mRNA or protein when HBXIP was silenced by siRNA in MCF-7 and MDA-MB-231 cells (Fig. 5E and F; Supplementary Fig. S5E–S5H), suggesting that both HBXIP and Hotair are required for the activation of c-Myc target genes. Furthermore, we randomly selected 10 of the 388 common targets of HBXIP and c-Myc to test whether Hotair was involved in the activation of c-Myc target genes. We found that overexpression of Hotair was able to upregulate these 10 genes in MCF-7 cells (Supplementary Fig. S5I), supporting that Hotair plays a role in the activation of c-Myc target genes. Thus, we conclude that lncRNA Hotair scaffolds HBXIP and LSD1 to form a complex of c-Myc/HBXIP/Hotair/LSD1 in activation of c-Myc–induced transcription in breast cancer cells.

The complex of c-Myc/HBXIP/Hotair/LSD1 contributes to the c-Myc–enhanced growth of breast cancer cells in vitro and in vivo

Next, we evaluated the role of the complex of c-Myc/HBXIP/Hotair/LSD1 in c-Myc–enhanced proliferation of breast cancer cells. RNA interference efficiency of LSD1-1 and LSD1-2 siRNAs was examined, and we found that siRNA LSD1-2 was able to powerfully knockdown the expression of LSD1 (Supplementary Fig. S6A). MTT assays showed that the knockdown of HBXIP (or Hotair and LSD1) by siRNA remarkably attenuated the proliferation of MCF-7 and T47D cells in vitro when c-Myc was overexpressed in the cells (Fig. 6A and Supplementary Fig. S6B). Moreover, the knockdown of HBXIP (or Hotair and LSD1) markedly reduced the growth of tumors in nude mice in vivo when c-Myc was overexpressed in MCF-7 cells (Fig. 6B–D). Interestingly, the expression levels of c-Myc target genes (cyclin A, eIF4E, and LDHA) were significantly reduced in the system when HBXIP (or Hotair) was silenced in the c-Myc–overexpressed tumor tissues (Supplementary Fig. S6C). It is well known that Ki67 is a marker of cell proliferation (41). IHC staining showed that the levels of Ki67 were consistent with the tumor volumes as well (Fig. 6E). Therefore, we conclude that the complex of c-Myc/HBXIP/Hotair/LSD1 contributes to the c-Myc–enhanced growth of breast cancer cells in vitro and in vivo.

Discussion

The transcription initiation complex, including transcription factors, coactivators, and others, is necessary for the transcription of genes in eukaryotes. The proto-oncogene transcription factor c-Myc is a central regulator in the tumorigenesis (1, 3, 5). Our group has reported that the oncoprotein HBXIP is an oncogenic transcriptional coactivator in breast cancer (16, 42). Recently, it has been reported that lncRNA PRNCR1 and PCGEM1 are involved in the androgen–receptor transcriptional complex (29). In the present study, we are interested in whether HBXIP and lncRNAs contribute to the transcriptional regulation of c-Myc–induced genes.

The c-Myc target genes are estimated to comprise about 15% of all human genes, which are involved in multiple cell biologic processes (6). In this study, we are interested in whether HBXIP is involved in the transcriptional regulation of c-Myc–induced genes. As expected, we observed that there were 388 common target genes of c-Myc and HBXIP in MCF-7 cells, in which the c-Myc–induced genes were 28.8% (388/1,348) and HBXIP-induced genes were 16.8% (388/2,311). It suggests that HBXIP is associated with the transcription of c-Myc–target genes. Then, we validated that HBXIP was able to modulate c-Myc–induced genes in breast cancer cells, such as cyclin A, eIF4E, and LDHA, which were out of 388 genes. Moreover, we found that the expression levels of HBXIP were significantly positively correlated with those of above three c-Myc target genes in clinical breast cancer tissues. Functionally, we showed that HBXIP was required for the c-Myc–induced target genes in the cells, and HBXIP was able to increase the activities of E-box luciferase reporter. Our previous reports showed that several c-Myc target genes, such as cyclin D1, cyclin E, and telomerase reverse transcriptase, could be regulated by HBXIP (18, 43), which is consistent with this finding. Next, we try to identify the mechanism by which HBXIP enhances the transcription of c-Myc–induced genes in breast cancer cells. Interestingly, we showed that HBXIP could directly interact with c-Myc, in which the fragment aa 56–173 with the leucine zipper of HBXIP was responsible for the interaction. It has been reported that c-Myc protein binds to a DNA sequence called E-box (CACGTC, ref. 44). And other HLH-Zip transcription factors, such as ChREBP, SREBP, NRF1, Clock, and HIF, are able to recognize the E-box as well (5, 45, 46). Moreover, we revealed that HBXIP was able to interact with the E-box in the promoters of c-Myc–induced genes. It further supports that HBXIP is a coactivator of transcriptional factor c-Myc, which is consistent with our reports that HBXIP is able
Figure 5. 
Intronic RNA Hotair scaffolds HBXIP and LSD1 in the complex to activate the c-Myc-induced transcription. A, the interaction of LSD1 with c-Myc (or HBXIP) was measured by co-IP assays in MCF-7 cells treated with both RNase inhibitors and siHotair. B, the interaction of HBXIP (or c-Myc) with Hotair was detected by RIP-qPCR assays in MCF-7 cells or T47D cells. C, the interaction of HBXIP (or c-Myc) with Hotair was detected by RIP-qPCR assay in MCF-7 cells treated with siRNA of c-Myc (or HBXIP). D, the occupancy of LSD1 in the promoter of LDHA was tested by ChIP-qPCR in MCF-7 cells. E, the RNA levels of c-Myc, HBXIP, Hotair, cyclin A, eIF4E, and LDHA were tested by real-time PCR in MCF-7 cells. F, the protein levels of those genes were tested by Western blot in MCF-7 cells. Statistically significant differences are indicated. **, P < 0.01, Student t test.
to coactivate transcription factors, such as TF-IID, STAT3, SP1, CREB, and E2F1, in breast cancer cells (15–20).

Methylated H3 marks the promoter and the E-box chromatin in Myc-induced genes (47, 48). It has been reported that LSD1-mediated demethylation of H3K4 leads to the assembly of the c-Myc–induced transcription initiation complex (22, 23). Therefore, we supposed that a complex containing c-Myc, HBXIP, LSD1, and others might be involved in the event. As expected, our data showed that HBXIP linked c-Myc and LSD1 in the complex of c-Myc/HBXIP/LSD1. It has been reported that LSD1 is an RNA-interacting protein (27). Accordingly, we concerned that RNAs might be involved in the complex as well. Interestingly, RNase A could block the interaction of c-Myc (or HBXIP) with LSD1. It suggests that RNAs are required for the interaction of LSD1 with...
c-Myc (or HBXIP) in the complex. It has been reported that IncRNA Hotair serves as a scaffold recruiting PRC2 and LSD1 in the promotion of cancer metastasis (27, 28, 49). Strikingly, we found that Hotair acted as a scaffold for HBXIP and LSD1 in our system. More importantly, we identified that HBXIP was responsible for the recruitment of Hotair to c-Myc to form the transcriptional complex. This finding is consistent with above reports that Hotair serves as a scaffold modulating the interaction of proteins (27). Thus, in this study, we report that Hotair serves as a scaffold in the transcriptional complex of c-Myc–induced genes. Our data are consistent with the report that IncRNAs contribute to transcription complex, for instance PRNCR1 and PCGEM1 are involved in the androgen–receptor transcriptional complex (29). It has been reported that PRC2 is able to interact with Hotair (27, 50). We concerned whether EZH2, a component of PRC2, was one of members of the complex of c-Myc/HBXIP/Hotair/LSD1. However, our data showed that HBXIP failed to interact with EZH2 (Supplementary Fig. S5).

Taken together, we summarize a model that a complex of c-Myc/HBXIP/Hotair/LSD1 contributes to the c-Myc–mediated transcriptional activation in breast cancer, in which HBXIP acts as a coactivator through direct interaction with c-Myc, and IncRNA Hotair scaffolds HBXIP and LSD1 to form the complex, leading to the activation of transcription of the c-Myc–induced genes (Fig. 7). Thus, our finding provides new insights into the mechanism by which the oncogenic transcriptional factor c-Myc functions in carcinogenesis, illuminating long-standing questions on how c-Myc drives carcinogenesis.

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.

**Authors’ Contributions**

Conception and design: Y. Li, X. Zhang, L. Ye

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Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): Y. Li, Z. Wang, H. Shi, H. Li, L. Li, R. Fang, X. Cai, B. Liu

Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): Y. Li, Z. Wang, X. Zhang, L. Ye

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Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): Y. Li, Z. Wang, X. Zhang, L. Ye

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HBXIP and LSD1 Scaffolded by IncRNA Hotair Mediate Transcriptional Activation by c-Myc

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