



Original Articles

DMBX1 promotes tumor proliferation and regulates cell cycle progression via repressing OTX2-mediated transcription of p21 in lung adenocarcinoma cell

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ABSTRACT

Lung adenocarcinoma (LUAD) was the predominant histological subtype of lung cancer, with poor prognosis. By analyzing the TCGA dataset, we found that DMBX1 (diencephalon/mesencephalon homeobox 1), a member of the bicoid sub-family of homeodomain-containing transcription factors, was overexpressed in LUAD and correlated with poorer prognosis and more advanced clinicopathological features of LUAD patients. Silencing of DMBX1 inhibited proliferation of LUAD and induced G1/S cell cycle arrest, whereas ectopic expression of DMBX1 enhanced tumor growth of LUAD and promoted G1/S cell cycle exit. Further we found that the function of DMBX1 was dependent on p21 (CDKN1A), a key regulator of G1/S cell cycle progression. Co-IP assay revealed that DMBX1 directly bound to another homeobox transcription factor, OTX2. ChIP and luciferase reporter assay confirmed that OTX2 directly interacted with the promoter region of p21 to enhance its transcription, and DMBX1 repressed OTX2-mediated transcription of p21. Our study reveals that DMBX1 plays an oncogenic role in LUAD by repressing OTX2-mediated transcription of p21 and the results may provide new therapeutic targets for LUAD patients.

1. Introduction

Lung cancer is one of the most common malignancies and also is the leading cause of cancer-related death globally [1,2]. As the predominant histological subtype of lung cancer, lung adenocarcinoma (LUAD) accounts for approximately 40% of lung cancer cases [3]. Despite tremendous advances have been made in the diagnosis and treatment of LUAD [4], the prognosis of LUAD patients remains poor, with a 5-year overall survival rate less than 20% [5]. The molecular etiology of LUAD is complicated and multiple genomic alterations function in cancer growth [6,7]; however, the functional impact of most genomic alterations on tumor growth of LUAD remains unknown [8].

Better understanding of these alterations is critical for the advance of diagnostic markers and therapeutic targets for LUAD patients.

The progression of cancer is closely linked to activation of oncogenes and inactivation of tumor suppressor genes [9–12]. To screen for novel functional oncogenes in LUAD, we analyzed The Cancer Genome Atlas (TCGA) and found that DMBX1 (diencephalon/mesencephalon homeobox 1) might be a potential oncogene in LUAD and correlated with the prognosis of LUAD patients. DMBX1 is located on Chromosome 1p33 and encodes a member of the bicoid sub-family of homeodomain-containing transcription factors [13]. It has been well known that transcription factors control the rate of transcription of genetic information from DNA to messenger RNA and many of them function as

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proto-oncogenes or tumor suppressors in cancers [14–16]. DMBX1 was firstly identified as a novel member of the homeobox family in 2002 [17]. The follow-up studies showed that DMBX1 played an important role in postnatal survival, growth [18], the development of the limb [19], the central nervous system [20–22] and was essential in agouti-related protein action [23]. Many homeobox genes play a role in early embryonic development and meanwhile participate in the tumorigenesis of cancers [24–26], just like the role of carcino-embryonic antigen (CEA) in gastroenteric tumors [27]. It makes great sense to further study the function and mechanism of DMBX1 in LUAD.

In recent years DMBX1 was found to regulate cell cycle exit and differentiation of progenitor cells during midbrain and retinal development [28–30]. It is well-known that a well-balanced cell cycle progression is necessary for cell proliferation, while dysregulation of the cell cycle components may lead to tumor formation [31]. Consistently with the research in progenitor cells, we found that DMBX1 promoted tumor proliferation and regulated cell cycle progression in LUAD. Further study indicated that DMBX1 exerted its oncogenic role via repressing OTX2-mediated transcription of p21, a well-known cyclin-dependent kinase inhibitor and a tumor suppressor. Together, the current study revealed DMBX1 as an important regulator of tumor growth in LUAD and might provide diagnostic markers and therapeutic targets for LUAD patients.

2. Materials and methods

2.1. Bioinformatics analysis

The two TCGA dataset, named TCGA_LUNG_exp_HiseqV2-2015-02-24 and TCGA_LUAD_exp_Hi-SeqV2-2015-02-24, were downloaded from the UCSC Cancer Browser (<https://genomecancer.ucsc.edu/>) [32]. The normalized gene expression was obtained from “genomicMatrix” file and the clinicopathological information was acquired from “clinical_data” file. For Reactome pathway analysis, a total of 112 genes (Table S1) highly co-expressed (Spearman's correlation score > 0.35) with DMBX1 in LUAD (<http://www.cbioportal.org/>) [33] were submitted to Reactome Pathway Database (<https://reactome.org/>) [34]. The String Database (<https://string-db.org/>) [35] was used to predict functional associations between DMBX1 and other proteins. And we analyzed the binding site between transcription factor and DNA promoters through the Jaspas Database (<http://jaspardev.genereg.net/>) [36].

2.2. Cell culture

Lung adenocarcinoma cell lines (NCI–H1299, SPC-A1, PC9, A549) and human bronchial epithelial cell (HBE) were purchased from Shanghai Institutes for Biological Science, China. NCI–H1299, PC9 and A549 cells were cultured in RPMI 1640 medium (KeyGene, Nanjing, China), and SPC-A1 and HBE cells were cultured in DMEM medium (KeyGene, Nanjing, China), supplemented with 10% fetal bovine serum. All cells were cultured at 37 °C in a humidified incubator containing 5% CO₂.

2.3. Patient tissue samples

A total of 60 paired LUAD tissues and adjacent non-tumor tissues were collected from patients who had undergone curative surgical resection in the department of thoracic surgery, Jiangsu Cancer Hospital, from 2014 to 2017. All tissues were confirmed by experienced pathologists and the clinical information of these patients were obtained from their medical records. This study was approved by Ethics Committee of Jiangsu Cancer Hospital and informed consent was obtained from all patients.

2.4. RNA extraction and qRT-PCR

Total RNA was extracted from tissue samples or cultured cells with TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Reverse transcription was performed to generate cDNA using a Reverse Transcription Kit (Takara, Cat: RR036A, KeyGEN). SYBR Select Master Mix (Applied Biosystems, Cat: 4472908, KeyGEN, Nanjing, China) was used for qRT-PCR and primers are shown in Table S2.

2.5. Western blotting

Cultured cells were lysed with lysis buffer (RIPA, KeyGEN) containing protease inhibitors (PMSF, KeyGEN) on ice, and protein concentration was determined using a BCA Kit (KeyGEN). Western blotting were obtained utilizing 20–40 µg of lysate protein. The following primary antibodies were used: anti-β-actin (Cell Signaling Technology; Cat: 3700; 1:1000), anti-DMBX1 (Santa Cruz Biotechnology; Cat: sc-515294; 1:1000), anti-p21 (Cell Signaling Technology; Cat: 2947; 1:1000), anti-CDK4 (Cell Signaling Technology; Cat: 12790; 1:1000), anti-CDK6 (Cell Signaling Technology; Cat: 3136; 1:1000), anti-CyclinD1 (Santa Cruz Biotechnology; Cat: sc-246; 1:1000), anti-CyclinE1 (Abcam; Cat: ab3927; 1:1000), anti-p27 (Abcam; Cat: ab32034; 1:1000), anti-phospho-Rb (Cat: Ser780) (Cell Signaling Technology; Cat: 9307; 1:1000), anti-phospho-Rb (Ser795) (Cell Signaling Technology; Cat: 9301; 1:1000), anti-OTX2 (Santa Cruz Biotechnology; Cat: sc-514195; 1:1000).

2.6. siRNA and plasmid construction and cell transfection

The siRNAs targeting DMBX1, p21 and OTX2 were conducted and purchased from RiboBio, Guangzhou, China. **The full-length cDNA of human DMBX1, p21 and OTX2 were PCR-amplified and cloned into the expression vector pENTER (Vigene Biosciences, Shandong, China).** The siRNAs were transfected into LUAD cells using Imax (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. The plasmid vectors were extracted using DNA Midiprep kits (E.Z.N.A Endo-Free Plasmid Mini Kit II, OMEGA, Shanghai, China) and transfected with Lipofectamine 3000 (Invitrogen, Carlsbad, CA, USA). All siRNA sequences are shown in Table S3.

2.7. Cell proliferation assays

All the cell proliferation assays were performed 24 h after transfection. For the Cell Counting Kit-8 (CCK-8) assay, cells were plated in 96-well plates at a density of 2000 cells/100 µL and the absorbance at 450 nm was measured every 24 h. For Real Time xCELLigence analysis system (RTCA), 8000 cells/100 µL were seeded in E-plates, and the plates were locked into the RTCA DP device in the incubator to calculate the “cell index” value. In colony formation assay, a total of 200 cells were placed in a fresh 6-well plate and the cells were stained with 0.1% crystal violet solution after 10–14 days. For 5-ethynyl-2'-deoxyuridine (EdU) assay (EdU Apollo[®]488 In Vitro Imaging Kit, RiboBio, Guangzhou, China), 10,000 cells/100 µL were plated in 96-well plates. Then the cells were incubated with 100 µL 50 µM EdU solution for 2 h and fixed with 50 µL 4% paraformaldehyde. And 50 µL 2 mg/mL glycine was added to neutralize paraformaldehyde and the cells were washed with 100 µL 0.5% TritonX-100. After that the cells were stained with 100 µL 1 × Apollo solution and 100 µL 1 × Hoechst33342 solution. After washed with 100 µL PBS for 3 times, images were obtained from fluorescence microscope for further calculation of proliferation rates.

2.8. Cell cycle analysis

Cells were digested with 0.25% trypsin-EDTA and fixed with 70%

ethanol for 12 h at 4 °C. The ethanol-suspended cells were centrifuged and stained with PI staining solution for 10 min in the dark at 37 °C. A FACSCalibur flow cytometer was used to detect cell cycle distribution. The percentage of cells in G1, S, and G2-M phases were counted and compared.

2.9. Co-immunoprecipitation (Co-IP)

Pierce™ Co-Immunoprecipitation Kit (Pierce™ Co-Immunoprecipitation Kit, Thermo Scientific Pierce, 26149) was used for Co-IP analysis. Firstly, anti-DMBX1 antibody (Santa Cruz Biotechnology; sc-515294), anti-OTX2 antibody (Santa Cruz Biotechnology; sc-514195) and normal mouse IgG (Santa Cruz Biotechnology; sc-2025) were separately cross-linked with AminoLink Plus Coupling Resin by incubating in a rotator for 120 min at room temperature. The compound were centrifuged in spin column to fix the antibody-resin mix. After washing with Quenching Buffer, Sodium Cyanoborohydride Solution, 1 × Coupling Buffer and Wash Solution, the resin-immobilized antibodies were ready for Co-immunoprecipitation. A549 cells were harvested, washed with Modified Dulbecco's PBS and lysed with IP Lysis Buffer. Cell lysates were pretreated with Pierce Control Agarose Resin to remove non-specific combination. For Co-immunoprecipitation, the antibody-cross-linked resin were washed with IP Wash Buffer for 2 times and added with cell lysates. The immunoprecipitation reaction was performed in a rotator at 4 °C overnight. After that, immunoprecipitation products were eluted with Elution Buffer and analyzed using western blotting.

2.10. Chromatin immunoprecipitation (ChIP)

A549 cells were cross-linked in 4% paraformaldehyde by incubating for 10 min at room temperature, followed by 10 × glycine for additional 5 min. After washing with PBS twice, cells were added with pre-cooling PBS (containing cocktail) and scraped into a centrifuge tube. The cells were centrifuged for 10 min at 800 g at 4 °C, then added with 500 μL cell lysis buffer (containing 2.5 μL cocktail) and incubated on ice for 15 min. Next, cell precipitates were resuspended in 500 μL nucleus lysis buffer (containing 2.5 μL cocktail). DNA was sheared by sonication to yield soluble chromatin. Then 5 μg anti-DMBX1 antibody (Santa Cruz Biotechnology; Cat: sc-515294), 5 μg anti-OTX2 antibody (Proteintech, Cat: 13497-1-AP) or 5 μg mouse IgG (Santa Cruz Biotechnology; Cat: sc-2025) were added with 100 μL Dilution buffer and 20 μL protein A/G Plus-agarose beads, followed by rotating for 30 min at room temperature. For immunoprecipitation, the above complex were added with chromatin and incubated at 4 °C overnight. The immunoprecipitation products were precipitated on magnetic frame and washed with 500 μL low salt buffer, high salt buffer, LiCl buffer, and TE buffer successively, all for 5 min at 4 °C. For DNA purification, immunoprecipitation products were added with ChIP elution buffer (containing proteinase K) and then was heated at 62 °C for 2 h and 95 °C for 10 min. The cooled beads were wiped out on magnetic frame and the supernatant were transferred into a new tube. In the last step, the supernatant were added with 100 μL Bind reagent A and put in an adsorption column to centrifuge at 12000g for 30 s. The adsorption column was then washed with 500 μL Wash reagent B and DNA were eluted with 50 μL Elution buffer C. ChIP DNA samples were subjected to PCR amplification with primers specific to p21 promoter region. PCR products were then used for agarose gel electrophoresis. The sequence of primers used are shown in Table S4 and GAPDH was used as a control.

2.11. Luciferase reporter assay

The p21 promoter region (−2000bp) was amplified and cloned into luciferase reporter plasmid (pGL3-basic). The p21 promoter-LUC plasmid or mutant-type plasmid were co-transfected with CMV expression plasmids or siRNAs in HEK293T cells, and CMV-empty vector

or nonsense siRNA (si-NC) were used as negative control. In addition, CMV-Renilla was used as an internal control and Firefly/Renilla value was used to measure luciferase activity. After transfection for 48 h, cells were harvested and assessed for luciferase activity using the Dual Luciferase Reporter Assay System (Promega, Madison, WI, USA).

2.12. Animal studies

All animal studies were conducted in accordance with NIH animal use guidelines and protocols were approved by Nanjing Medical University Animal Care Committee. Before tumor transplantation, LUAD tumor cells were transfected with shRNAs or expression plasmids. The transfection was performed by transient transfection according to the specification of Lipofectamine 3000 (Invitrogen, Carlsbad, CA, USA). The sh-NC and empty vector pENTER were used as controls and totally 10 μg plasmid vectors were transfected into cells for each group. The sequences of shRNAs are shown in Table S5. We used 6 mice per group and 2 × 10⁶ LUAD tumor cells in 100 μL sterile PBS were injected subcutaneously to each mouse at age of 6–8 weeks old. Tumors were harvested at 6 weeks after injection. The weight of tumor was measured on the scale and tumor volume was estimated using calipers ($[\text{length} \times \text{width}^2]/2$).

2.13. Immunohistochemistry

Tissue sections were deparaffinized and rehydrated through graded alcohol. Endogenous peroxidase activity was blocked by incubation in 3% H₂O₂ and antigen retrieval was carried out with 0.01 M citrate buffer (pH 6.0). Immunostaining was performed using following antibodies: anti-DMBX1 (Invitrogen, Cat: PA5-55425, 1:100), anti-OTX2 (Proteintech, Cat: 13497-1-AP, 1:100), anti-p21 (Proteintech, Cat: 10355-1-AP, 1:400).

2.14. Statistical analysis

Data were presented as the mean ± SD. Student's T-test was used to determine statistical significance between two groups. Correlation between DMBX1 expression and other genes were assessed using Spearman's correlation score. The association between the expression level of DMBX1 and patient survival was plotted using the Kaplan–Meier method. Chi-square test was performed to analyze the relationship between DMBX1 and clinical pathological characteristics. $P < 0.05$ was considered statistically significant. And data were graphically displayed using GraphPad Prism v.5.0 for Windows (GraphPad Software, Inc., La Jolla, CA, USA).

3. Results

3.1. DMBX1 was identified as a potential oncogene in LUAD

To screen for aberrant-expressed genes in LUAD, we analyzed two TCGA dataset, TCGA_LUNG_exp_HiseqV2-2015-02-24 and TCGA_LUAD_exp_Hi-SeqV2-2015-02-24. Three candidate genes (PITX2, DMBX1 and HOXC13) were identified using the following screening criteria: fold change > 10 (Tumor/Normal) and tumor expression > 3 (Fig. 1A). PITX2 and HOXC13 have been studied in lung cancer [37–40] but DMBX1 remains functionally unknown. Further analysis of TCGA dataset revealed that DMBX1 was up-regulated in LUAD (Fig. 1B). By ranking the expression of DMBX1 in totally 511 LUAD tissues from high to low, we defined the upper quartile as 'high DMBX1 expression' (n = 128) and the lower quartile as 'low DMBX1 expression' (n = 128). Survival analysis showed that patients with high DMBX1 expression had a more dismal overall survival (Fig. 1C). The chi-squared test suggested that the DMBX1 level was related to T stage (p = 0.017356) and KRAS mutation status (p = 0.02632) (Table 1). For the 60 paired LUAD and normal tissues collected from Jiangsu Cancer Hospital,

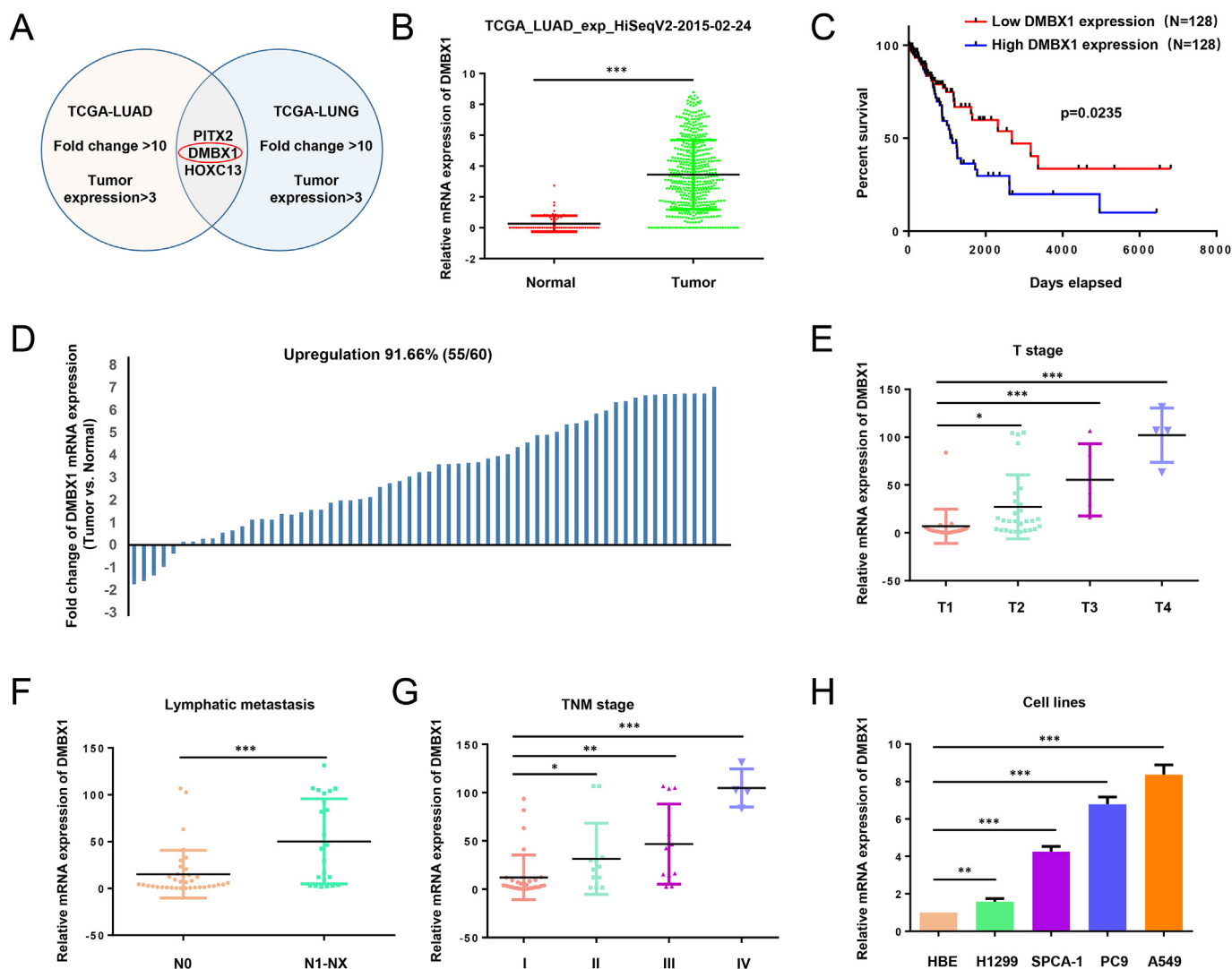


Fig. 1. DMBX1 is overexpressed in lung adenocarcinoma and correlates with clinical pathological characteristics. A. Venn Diagram for gene screening. By analyzing the two TCGA datasets, three potential oncogenes were obtained using the following screening criteria: genes with fold change > 10 (Tumor/Normal) and tumor expression > 3. B. The mRNA expression of DMBX1 in lung adenocarcinoma tissues is higher than normal lung tissues in TCGA dataset. C. Lung adenocarcinoma patients with low expression of DMBX1 have a higher percentage of overall survival compared with high DMBX1 expression patients. D. DMBX1 is upregulated in 91.66% of 60 lung adenocarcinoma tissues collected from our own hospital (normalized to normal tissues). E. Compared with T1 tumors, DMBX1 is significantly upregulated in T2, T3, and T4 tumors. F. Tumors with lymphatic metastasis shows higher expression of DMBX1 than those with no metastasis. G. By comparing the expression of DMBX1 in tumors with different TNM stage, DMBX1 shows higher expression in tumors with more advanced TNM stage. H. The mRNA expression of DMBX1 in lung adenocarcinoma cell lines (H1299, SPCA-1, PC9, A549) is higher than normal lung epithelial cells (HBE). (*P < 0.05; **P < 0.01; ***P < 0.001).

DMBX1 mRNA expression was increased in 91.66% (55/60) of LUAD tissues (Fig. 1D) and correlated with more advanced clinical stages (Fig. 1E, F, G). In addition, DMBX1 mRNA level was higher in LUAD cell lines (H1299, SPCA-1, PC9 and A549) compared with Human Bronchial Epithelial Cells (HBE) (Fig. 1H).

3.2. DMBX1 promotes tumor proliferation and regulates cell cycle progression in LUAD in vitro

To further explore the function of DMBX1 in LUAD, two siRNAs (si1-DMBX1 and si2-DMBX1) and pENTER-plasmid (oe-DMBX1) were used to respectively knockdown or overexpress DMBX1. In A549 cells, both siRNAs effectively decreased the expression of DMBX1 (Fig. 2A) and knockdown of DMBX1 inhibited LUAD cell proliferation, validated by Cell Counting Kit-8 (CCK-8) assay (Fig. 2B), Real Time xCELLigence analysis system (RTCA) (Figure 2C), 5-ethynyl-2'-deoxyuridine (EdU) assay (Fig. 2D) and colony formation assay (Fig. 2E). As previous studies have reported that DMBX1 regulates cell cycle exit during body

development [28–30], we performed cell cycle analysis to further investigate the biological function of DMBX1 in LUAD. The results showed that knockdown of DMBX1 induced G1/S cell cycle arrest in A549 cells (Fig. 2F). And in H1299 cells, ectopic expression of DMBX1 (Fig. 2G) promoted LUAD cell proliferation (Fig. 2H–K and Fig. S1) and accelerated G1/S cell cycle progression (Fig. 2L). These results suggest that DMBX1 plays a critical role in proliferation and cell cycle progression of LUAD cells.

3.3. DMBX1 exerts its oncogenic role in LUAD via modulating p21 expression

As DMBX1 is a functionally unknown gene, we first performed Reactome pathway analysis to predict its role in LUAD. The results showed that genes highly co-expressed with DMBX1 were involved in “Cell Cycle” and “Genetic Transcription Pathway” (Fig. 3A). Combined with previous data, DMBX1 might participate in the regulation of G1/S cell cycle progression. We therefore examined whether DMBX1 could

Table 1
Correlation between DMBX1 expression and clinical/pathological characteristics in TCGA.

Characteristics	low level of DMBX1 expression number (n = 128)	high level of DMBX1 expression number (n = 128)	χ^2	p-Value
Age (years)			0.57	0.451259
≤65	67	73		
>65	61	55		
Sex			1.9	0.168076
Male	65	54		
Female	63	74		
Primary Tumor			10.15	0.017356*
T1	52	36		
T2	65	65		
T3	8	16		
T4	3	11		
Metastasis			0.17	0.682646
M0	91	88		
M1-MX	37	40		
Lymph node			1.38	0.501912
N0	85	76		
N1	23	27		
N2-NX	20	25		
TNM stage			3.54	0.315316
I	72	63		
II	26	38		
III	22	22		
IV	8	5		
EGFR mutation			4.34	0.114305
Yes	6	6		
No	58	42		
Undetermined	64	80		
KRAS mutation			7.27	0.02632*
Yes	11	25		
No	37	39		
Undetermined	80	64		

*Significant correlation.

modulate expression of G1-related cyclins and CDKs genes, such as p21, CDK4, CDK6, CCND1, CCNE1 and p27. The qPCR and western blotting assay indicated that p21 was significantly upregulated in DMBX1-knockdown A549 cells (Fig. 3B and D) and it was remarkably down-regulated in DMBX1-overexpressed H1299 cells (Fig. 3C and D). Beyond that, we analyzed the phosphorylation status of Rb1, a marker for G1-S transition. Phosphorylated Rb levels were decreased after DMBX1 knockdown and increased following DMBX1 overexpression (Fig. 3D). Furthermore, modulating of p21 expression by specific siRNA or ectopic expression plasmid (Fig. 3E and F) reversed the effect of DMBX1 on proliferation (Fig. 3G and H) and cell cycle exit (Fig. 3I and J). The data reveals that the oncogenic role of DMBX1 in LUAD is p21-dependent.

3.4. The function of DMBX1 in LUAD can be restored by OTX2

Transcription factors control the rate of transcription of genes by binding to a specific DNA sequence [41]. Using the Jaspar database, we found that DMBX1 had no potential binding sites within the promoter region of p21 (Fig. 5A). And ChIP assay revealed that chromatin immunoprecipitated by anti-DMBX1 antibody have no p21 promoter DNA fragment (Fig. 5B). Besides the above-mentioned mechanism, transcription factors may also recruit coactivator or corepressor proteins to form the transcription factor-DNA complex and regulate downstream gene expression [42]. It has been reported that DMBX1 functions as a

transcription repressor by binding the consensus motif of Otx1, Otx2, and Crx [20]. Using the String Database, 6 proteins were predicted to directly bind to DMBX1, including OTX2 (Fig. 4A). Interestingly, researchers have demonstrated that OTX2 directly activates cell cycle genes and OTX2 silencing blocks G1-S cell cycle transition in medulloblastoma cells [43]. Thus, we sought to determine whether DMBX1 binds to OTX2 and suppress its transcription of p21. Confirmed by Co-immunoprecipitation, DMBX1 indeed bond to OTX2 in LUAD cells (Fig. 4B). OTX2 silencing downregulated p21 expression (Fig. 4C and E) while ectopic expression of OTX2 markedly increased p21 levels (Fig. 4D and E). Furthermore, CCK-8 and cell cycle analysis revealed that OTX2 was able to rescue the effect of DMBX1 on proliferation (Fig. 4F and G) and cell cycle progression (Fig. 4H and I) in LUAD. Taken together, these findings suggest that the oncogenic role of DMBX1 is dependent on OTX2.

3.5. DMBX1 represses OTX2-mediated transcription of p21

Predicted by Jaspar Database, OTX2 but not DMBX1 has potential binding sites within p21 promoter region (Fig. 5A). In ChIP assay, chromatin was sonicated into DNA fragments with size of 500–1500bp (Fig. S2). Chromatin immunoprecipitation was then performed with anti-OTX2 antibodies, anti-DMBX1 antibody and control IgG, followed by PCR amplification of the p21 promoter region. The results showed that OTX2 but not DMBX1 specifically bound to the p21 promoter DNA. Intriguingly, the binding of OTX2 to the p21 promoter was greatly reduced by ectopic expression of DMBX1, indicating that DMBX1 represses OTX2-mediated transcription of p21 (Fig. 5B and C). To further confirm this, the p21 promoter region (–2000bp) was amplified and cloned into luciferase reporter vector (pGL3-basic) (Fig. 5D). A mutant-type p21-Luc reporters was also generated (Fig. 5E). Consistent with our results in ChIP assay, overexpression of OTX2 promoted transcriptional activity of the p21 promoter and DMBX1 overexpression rescued the effect (Fig. 5F). In addition, silencing OTX2 inhibited transcriptional activity of the p21 promoter and knockdown of DMBX1 restored the effect (Fig. 5G). These results indicate that DMBX1 inhibits OTX2-mediated transcription of p21 via the interaction with OTX2.

3.6. DMBX1 promotes LUAD tumor growth in vivo

Xenograft tumor models were used to explore the oncogenic role of DMBX1 *in vivo*. A549 or H1299 cells transfected with the indicated expression plasmids or shRNAs were injected intramuscular in the left hind limb muscle of mice and tumor nodules were harvested at 6 weeks after injection (Fig. 6A and B). Silencing DMBX1 inhibited tumor growth (volume and weight) *in vivo* and knockdown of either OTX2 or p21 could restore this effect (Fig. 6C). Moreover, overexpression of DMBX1 evidently promoted tumor proliferation *in vivo*, which was greatly reversed by ectopic expression of either OTX2 or p21 (Fig. 6D). Immunohistochemistry was then performed to detect the protein levels of DMBX1, OTX2 and p21 in the corresponding tumor xenografts (Fig. 6E and F). Taken together, these results indicate that DMBX1 promotes LUAD tumor growth *in vivo* via the OTX2-p21 axis.

4. Discussion

The poor prognosis of lung adenocarcinoma is largely due to late diagnosis and indistinct mechanism of cancer development [44,45]. And cancer growth is the consequence of multiple, cooperative genomic alterations, including epigenetic alteration, genomic instability and transcriptional alterations [8,46]. Numerous alterations have been catalogued by whole genome sequencing, but the effect of most alterations still remains unknown [47]. In the past decades, significant efforts have been made in treatment and management of lung adenocarcinoma on the basis of genomic alterations (EGFR, KRAS, ALK) [48–50]; however, drug resistance turned into the main obstacle for

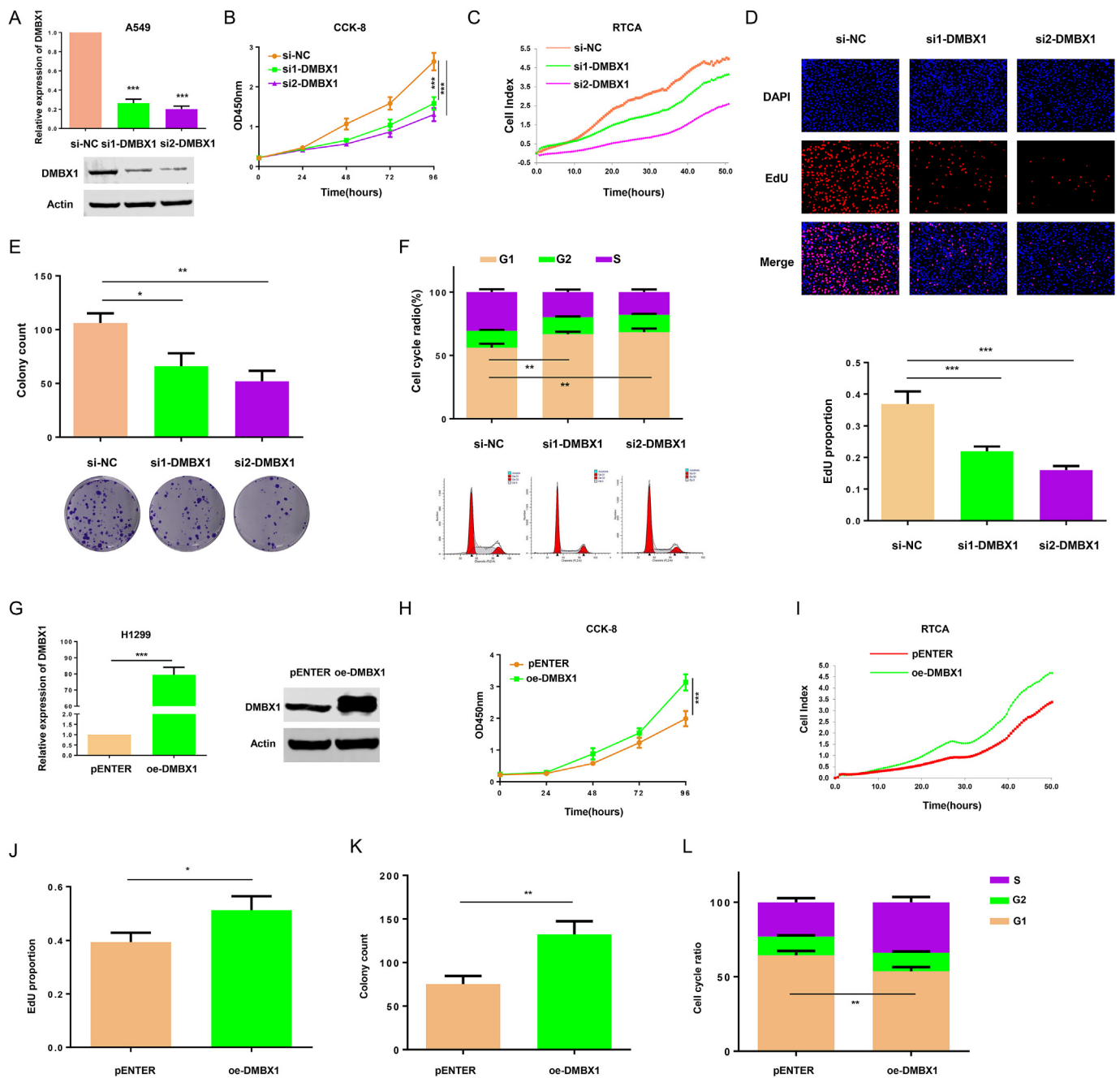


Fig. 2. Knockdown of DMBX1 suppresses proliferation of lung adenocarcinoma *in vitro*, while ectopic expression of DMBX1 shows reverse effect. A. Two specific siRNA (si1-DMBX1, si2-DMBX2) were transfected into lung adenocarcinoma cell lines A549, and both effectively downregulated the expression of DMBX1 in mRNA and protein levels compared with negative control (si-NC). B. In CCK-8 assay, knockdown of DMBX1 inhibited growth of A549 cell lines. C. Real Time Cellular Analysis (RTCA) revealed that knockdown of DMBX1 inhibited proliferation of A549 cells. D. A549 cells transfected with siRNA showed a lower proportion of EdU relative to si-NC. E. In colony formation assay, colony numbers of si-DMBX1 group were less than control group. F. Knockdown of DMBX1 induced G1/S cell cycle arrest. G. The pENTER vector containing total coding sequence of DMBX1 were transfected into H1299 cell lines, and the mRNA and protein level of DMBX1 were increased compared with control (pENTER). H–K. Overexpression of DMBX1 promoted proliferation of lung adenocarcinoma in H1299 cells. L. Ectopic expression of DMBX1 caused an increase of cells in S phase. (*P < 0.05; **P < 0.01; ***P < 0.001; n. s., no significant).

targeted therapy [51,52]. Therefore, there is a compelling need to identify novel key molecular drivers of LUAD and thus to provide the new clinical targets for LUAD therapy.

In this study, we identified that DMBX1 promoted LUAD tumor proliferation and abnormally-expressed DMBX1 correlated with clinical pathological characteristics and overall survival of LUAD patients. However, DMBX1 did not play a similar role in lung squamous carcinoma (LUSC) (Fig. S3), suggesting that DMBX1 might specifically function in LUAD. As a member of homeobox gene family, DMBX1 is

defined as a homeodomain-containing transcription factor that plays a role in brain and sensory organ development [20]. Homeobox genes are a cluster of genes containing homeobox (a homologous DNA sequence, around 180 base pairs long) and are involved in the regulation of patterns of anatomical development (morphogenesis) in animals, fungi and plants [53,54]. Many homeobox genes are specifically expressed in particular tissues during embryogenesis and regulate the formation of many body structures by control the transcription of targeted genes [55]. Expression of homeobox genes is spatial and temporal, while

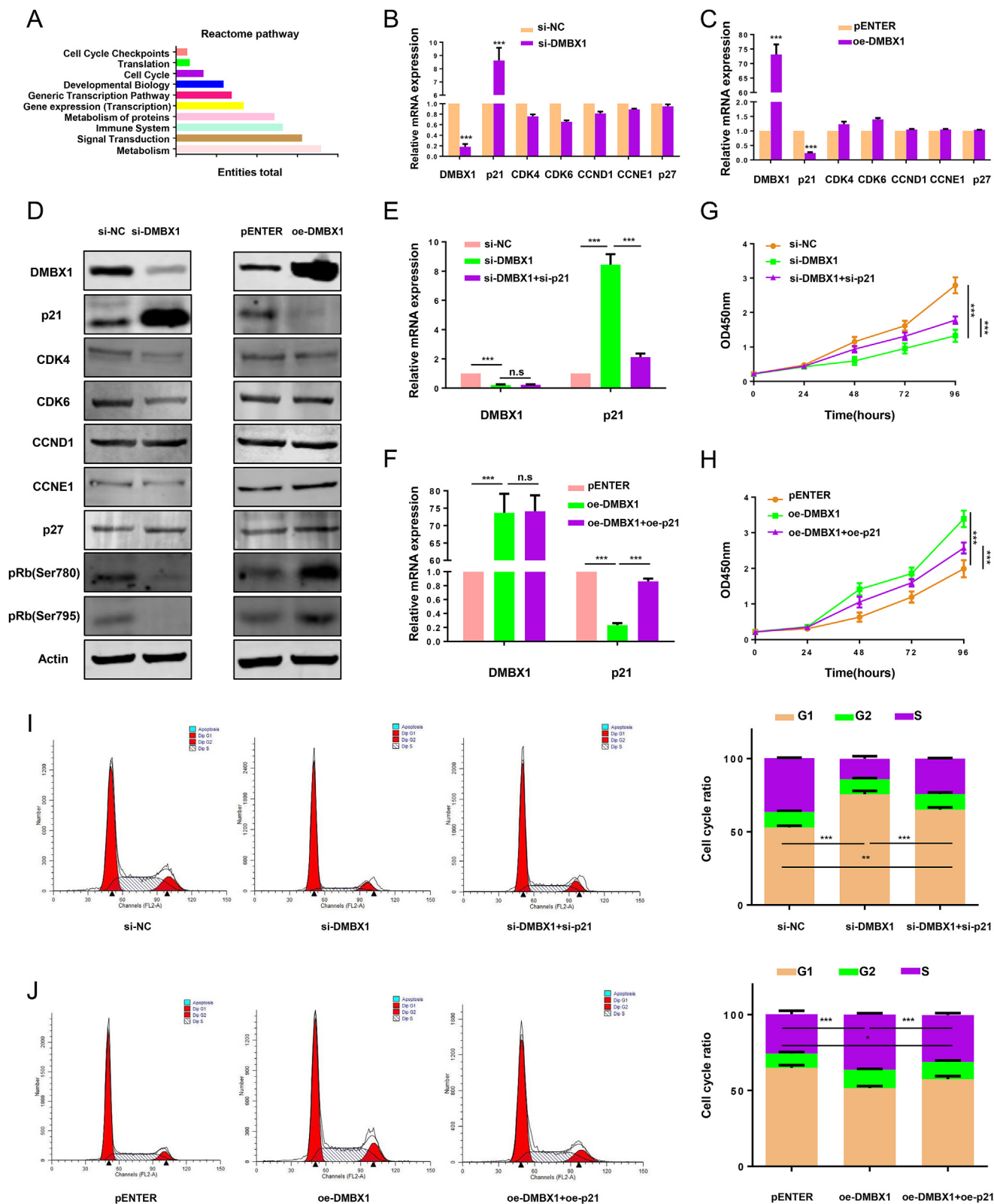


Fig. 3. DMBX1 exerts its oncogenic role via repressing the expression of p21. **A.** Reactome pathway analysis revealed that genes co-expressed with DMBX1 were involved in “Cell Cycle” and “Genetic Transcription Pathway”, which were consistent with our previous results. **B–C.** The expression of G1/S-related cell cycle genes was measured by qRT-PCR after knockdown and overexpression of DMBX1, and p21 mRNA varied most significantly. **D.** Western blot showed that knockdown of DMBX1 increased the expression of p21 and decreased the phosphorylation of Rb, while overexpression of DMBX1 had an opposite result. **E–F.** siRNA and overexpression plasmids of p21 were used to rescue the expression of p21 in lung adenocarcinoma cell lines. **G–H.** CCK-8 assay suggested that rescue of p21 reversed the effect of DMBX1 on proliferation of lung adenocarcinoma. **I–J.** Cell cycle was detected by flow cytometry and results showed that rescue of p21 reversed the effect of DMBX1 on G1/S cell cycle transition of lung adenocarcinoma. (* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; n. s., no significant).

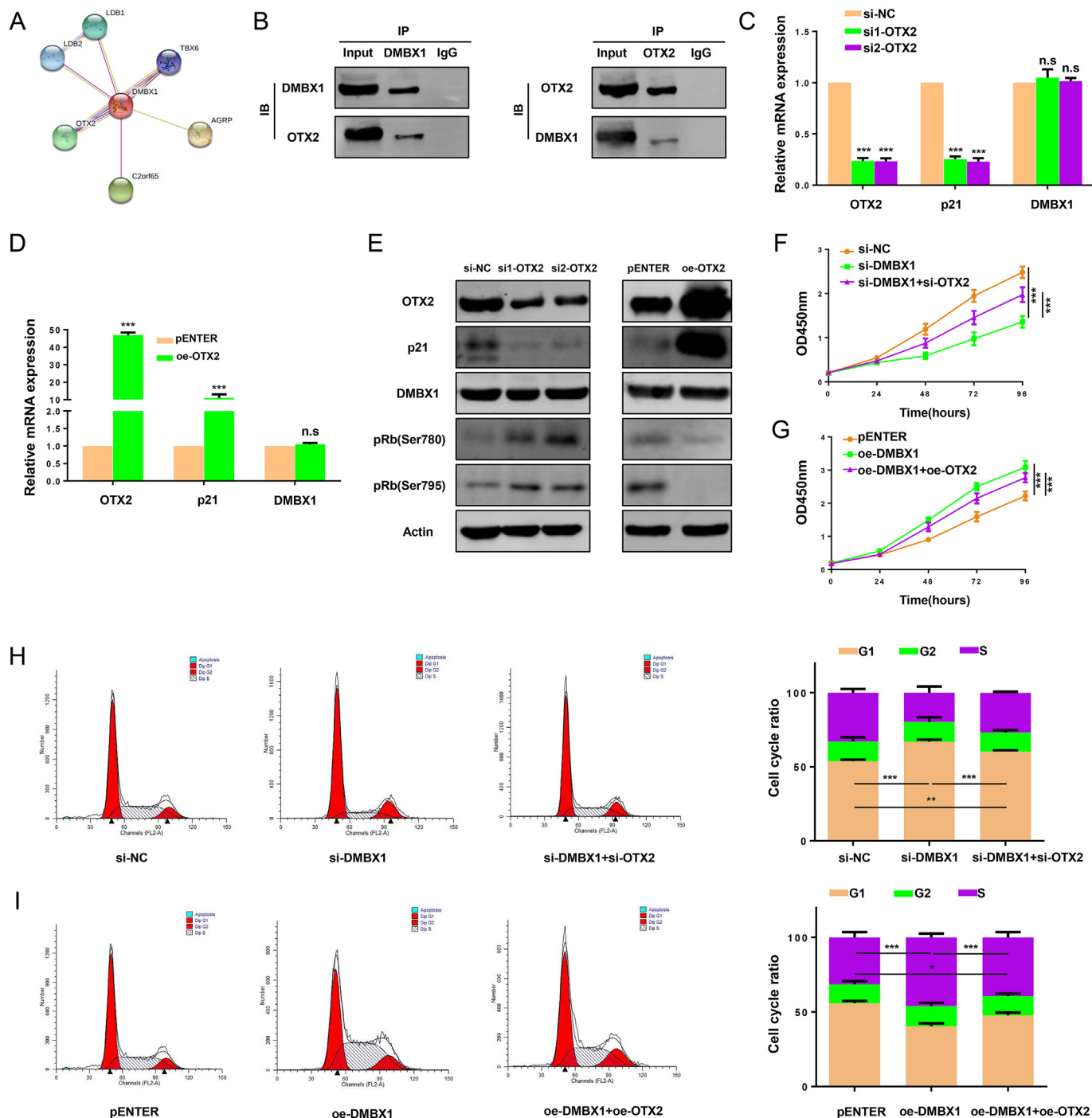


Fig. 4. OTX2 directly binds to DMBX1 and antagonizes its role in lung adenocarcinoma. A. OTX2 is predicted to directly bind DMBX1 in STRING database. B. Co-immunoprecipitation assay suggested that DMBX1 bound OTX2 in lung adenocarcinoma cell lines. C-D. Knockdown of OTX2 decreased the mRNA expression of p21 while ectopic of OTX2 increased its expression. E. Knockdown of OTX2 decreased the protein level of p21 and increased the phosphorylation level of Rb, while overexpression of OTX2 had an opposite result. F-G. OTX2 antagonized the influence of DMBX1 on proliferation of lung adenocarcinoma. H-I. OTX2 reversed the effect of DMBX1 on G1/S cell cycle transition of lung adenocarcinoma. (* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; n. s, no significant).

inappropriate expression of these genes may involve in carcinogenesis and cancer development, just as the role of carcino-embryonic antigen (CEA) in gastroenteric tumors [27]. For instance, CDX2, a homeobox gene playing a role in early embryonic development of the intestinal tract, participates in the tumorigenesis of colorectal cancer and can serve as a prognostic biomarker [56,57]. NKX2-1, a thyroid-specific transcription factor involved in morphogenesis, sustains EGFR survival signaling and affects drug resistance in lung adenocarcinoma [58,59]. ZEB1, associated with posterior polymorphous corneal development, is

also linked to the progression the ovarian cancer [60], pancreatic cancer [61], glioblastoma [62] and breast cancer [63]. In accordance with these research, we found that DMBX1, a brain development-associated gene, was tremendously expressed in LUAD and influenced tumor proliferation. Our findings may implicate DMBX1 as a “cancer-encephalic protein”, a novel definition for a number of genes involving in brain development and also regulating cancer progression. OTX2, which plays a role in brain, craniofacial, sensory organ development and is also suspected of having an oncogenic role in medulloblastoma,

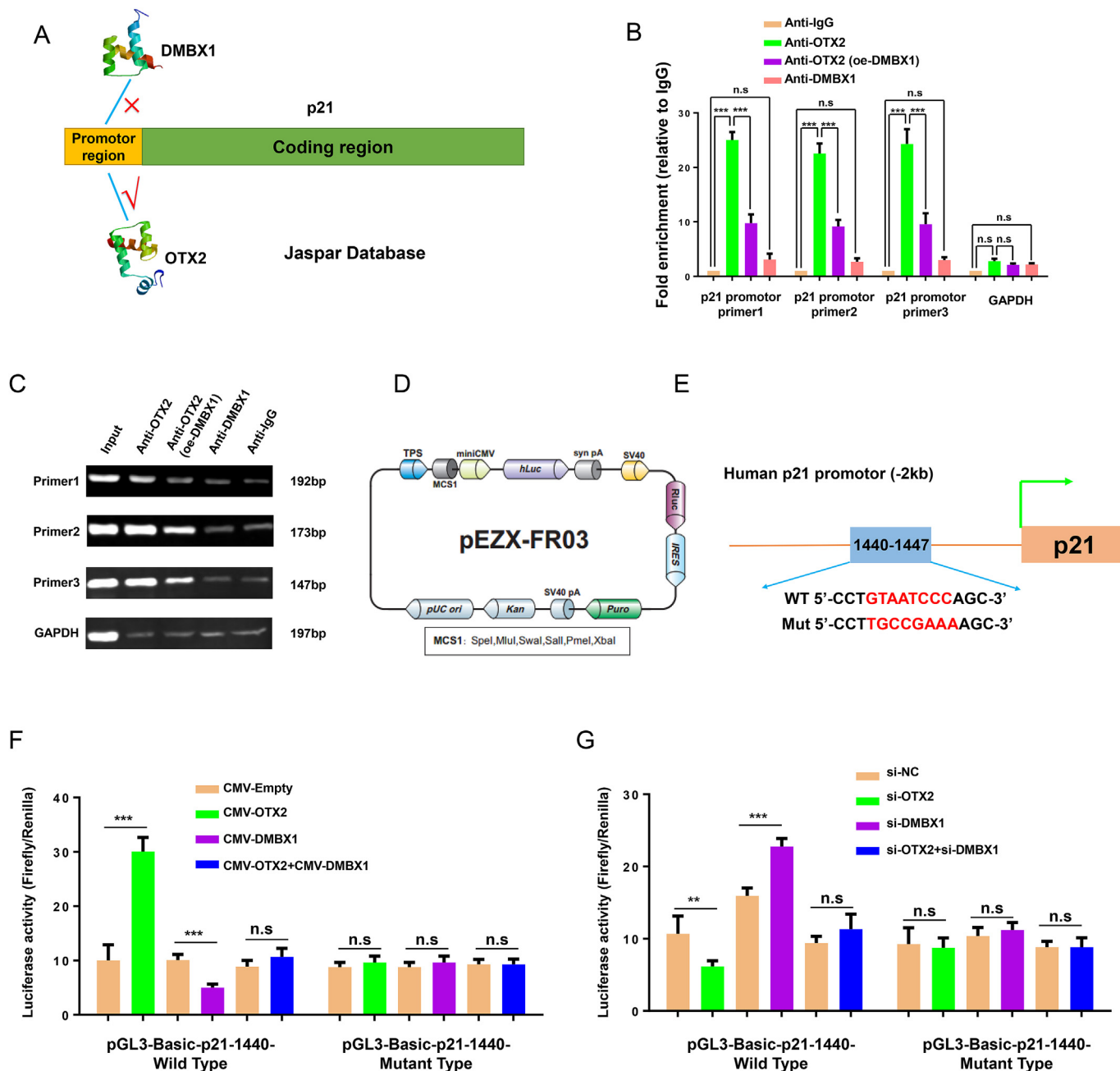


Fig. 5. DMBX1 represses OTX2-mediated transcription of p21. **A.** By analyzing the Jaspar database, OTX2 but not DMBX1 is predicted to have physical connection with p21 promoter region (the upstream 2000bp of coding region is regarded as promoter region). **B–C.** 3 primers were designed for the promoter region of p21 (GAPDH was used as a control primer) and direct binding of OTX2 but not DMBX1 to the p21 promoter region were shown by ChIP assay. And overexpression of DMBX1 inhibited the binding of OTX2 with p21 promoter region. **D.** Map of reporter gene plasmid for luciferase reporter gene assay. **E.** Schematic illustration of OTX2 binding site on p21 promoter region and the mutant type were presented. **F.** Overexpression of OTX2 significantly increased wild type but not mutant p21 promoter activity in 293T cells, and the effect can be inhibited by DMBX1. **G.** Knockdown of OTX2 by siRNA significantly reduced wild type but not mutant p21 promoter activity in 293T cells, and the effect can be reversed by silencing DMBX1. (*P < 0.05; **P < 0.01; ***P < 0.001; n.s, no significant).

can be also a carcino-encephalic protein [64,65]. Likewise OTX1, DBX2, and ADNP are members of carcino-encephalic protein family [24–26]. Clarifying the role and pathway of carcino-encephalic proteins in cancers may provide new biomarkers for diagnosis and prognosis.

Moreover, our results showed that DMBX1 exerts its oncogenic role via regulating cell cycle, especially by repressing OTX2-mediated transcription of p21. As known, a well-balanced cell cycle progression is necessary for cell proliferation and the dysregulation of the cell cycle components may lead to tumor formation [66]. P21, also called cyclin-dependent kinase inhibitor 1 (CDKN1A), is associated with linking DNA damage to cell cycle arrest via regulating the activity of CDK and

phosphorylation of Rb [67–69]. We found that knockdown of DMBX1 induced G1/S cell cycle arrest while ectopic expression of DMBX1 promoted G1/S cell cycle progression. In addition, DMBX1 inhibited the expression of p21 and alteration of OTX2 or p21 partially reversed the oncogenic role of DMBX1. As an important tumor suppressor gene, p21 was regulated by a variety of molecules, including p53 [70], MLK3 [71], TRIB2 [72], and other regulatory network [73,74]. Moreover, it has been reported that DMBX1 exerted its biological effects in other ways, such as regulating CCND1 [29] or Agouti-Related Peptide [75]. These might be the reason why knockdown or overexpress p21/OTX2 could not totally reverse the effect caused by DMBX1 knockdown or

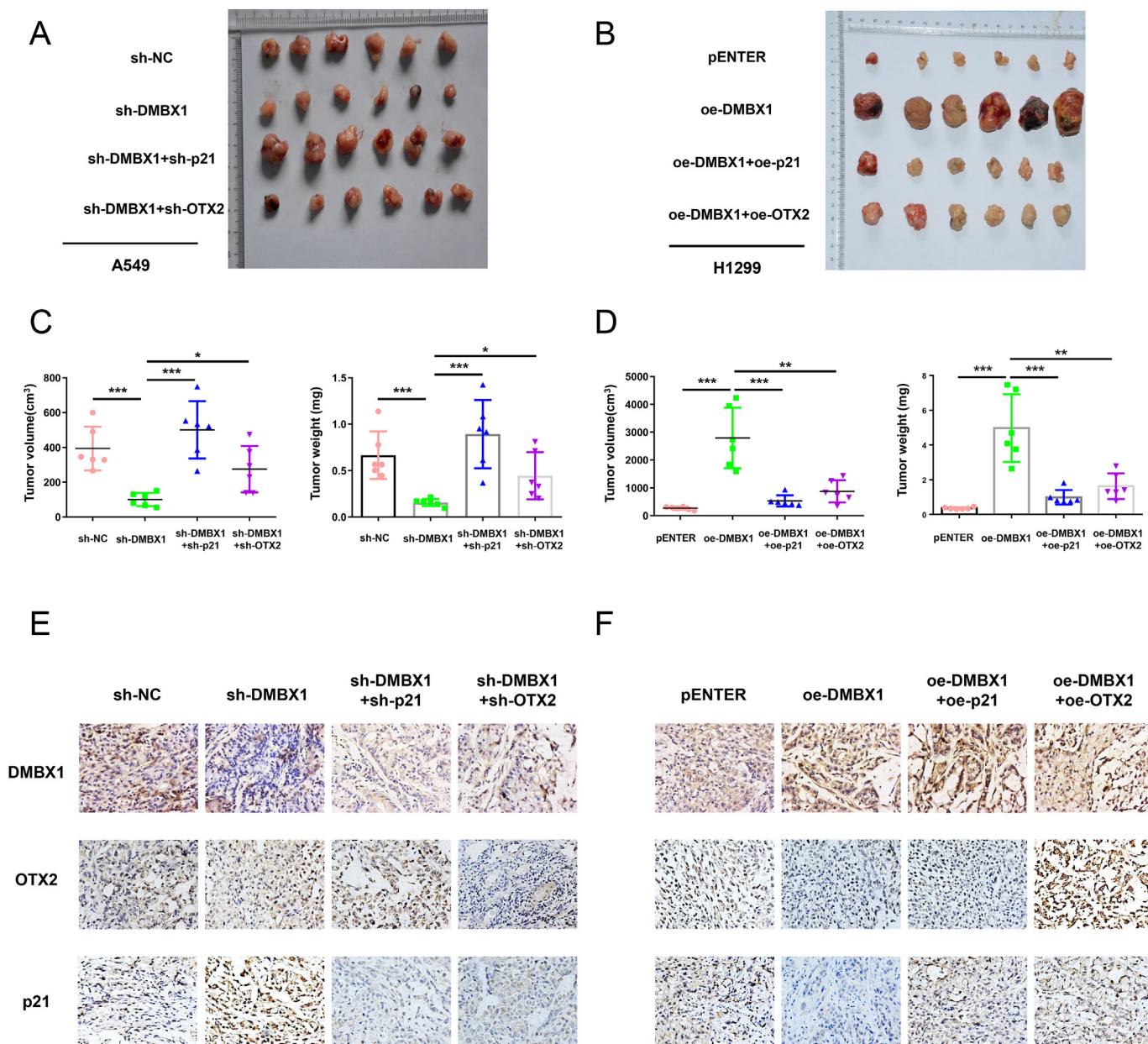


Fig. 6. DMBX1 promotes proliferation of lung adenocarcinoma *in vivo* and the effect can be antagonized by OTX2 and p21. A-B. Harvested tumor nodules are shown. C-D. The volume and weight of tumor nodules of each group are measured and the results revealed that DMBX1 promoted proliferation of lung adenocarcinoma *in vivo*, of which reversed by altering the expression of OTX2 and DMBX1. E-F. Immunohistochemistry of tumor nodules by specific antibody were shown. DMBX1 inhibited the p21 protein expression while OTX2 upregulated it. (*P < 0.05; **P < 0.01; ***P < 0.001; n. s, no significant).

overexpression. However, in our study, the role of DMBX1 in lung adenocarcinoma was relied on, or at least, partially relied on p21 and OTX2. DMBX1 is a homeodomain-containing transcription factor but bioinformatics analysis and ChIP assay revealed that DMBX1 did not directly bind to the promoter region of p21. Transcription factors also regulated downstream genes by influencing the transcriptional activity of other transcription factors, of which we called coactivator or corepressor [42]. For example, Foxp2 could inhibit Nkx2.1-mediated transcription of SP-C via interactions with the Nkx2.1 homeodomain [76]; and nuclear factor-κB2 represses Sp1-mediated transcription at the CD99 promoter [77]. In previous study, DMBX1 has been shown by electrophoretic mobility shift assay to bind to the TAATCC motif, the consensus binding sequence for Otx1, Otx2, and Crx [20]. Furthermore, OTX2 has been proved to activate cell cycle genes and OTX2 silencing blocks G1-S cell cycle transition in medulloblastoma cells [43]. Consistently, we found that DMBX1 bound to OTX2 and repressed its

transcription of p21.

In summary, our study proved that overexpressed DMBX1 repressed OTX2-mediated transcription of p21, thus activated Cyclin-CDK complex and accelerated G1/S cell cycle transition, leading to the increased proliferation of lung adenocarcinoma (Fig. S4). Our findings indicate that DMBX1 may represent a potential biomarker and a promising therapeutic target for LUAD. And further exploration of “carcino-encephalic protein” in cancers is of great prospects.

List of abbreviations

LUAD: lung adenocarcinoma; Co-IP: co-immunoprecipitation; ChIP: chromatin immunoprecipitation; TCGA: The Cancer Genome Atlas.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of data and material

All data generated or analyzed during this study are included in this published article and its supplementary information files.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

Lin Xu, Yi Shen and Binhui Ren conceived, designed, and supervised the study. Jing Luo performed the experiments and wrote the manuscript. Kaichao Liu and Yu Yao provided technical support and analyzed the data. Qi Sun and Xiufen Zheng performed the statistical analyses. Bqing Zhu performed experiments and edited the manuscript.

All authors read and approved the final version of the manuscript.

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Not applicable.

Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.canlet.2019.03.045>.

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