RESEARCH ARTICLE

# Let-7c Inhibits NSCLC Cell Proliferation by Targeting HOXA1

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## Abstract

**Objective: The aim of the present study was to explore mechanisms by which let-7c suppresses NSCLC cell proliferation. Methods: The expression level of let-7c was quantified by qRT-PCR. A549 and H1299 cells were transfected with let-7c mimics to restore the expression of let-7c. The effects of let-7c were then assessed by cell proliferation, colony formation and cell cycle assay. Mouse experiments were used to confirm the effect of let-7c on tumorigenicity *in vivo*. Luciferase reporter assays and Western blotting were performed to identify target genes for let-7c. Results: HOXA1 was identified as a novel target of let-7c. MTS, colony formation and flow cytometry assays demonstrated that forced expression of let-7c inhibited NSCLC cell proliferation by inducing G1 arrest *in vitro*, consistent with inhibitory effects induced by knockdown of HOXA1. Mouse experiments demonstrated that let-7c expression suppressed tumorigenesis. Furthermore, we found that let-7c could regulate the expression of HOXA1 downstream effectors CCND1, CDC25A and CDK2. Conclusions: Collectively, these results demonstrate let-7c inhibits NSCLC cell proliferation and tumorigenesis by partial direct targeting of the HOXA1 pathway, which suggests that restoration of let-7c expression may thus offer a potential therapeutic intervention strategy for NSCLC.**

**Keywords:** Let-7c - NSCLC - HOXA1 - G1 arrest

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## Introduction

MicroRNAs (miRNAs) represent a class of endogenous, single-strand and highly conserved non-coding small RNAs. They post-transcriptionally regulate protein expression by directly binding the 3'-UTR of target genes and thus play critical roles in numerous biological processes, including development, apoptosis, life span and metabolism (Bartel, 2004).

Let-7 was the second miRNA to be identified after lin-4 in Caenorhabditis elegans (Pasquinelli et al., 2000). To date, thirteen members of the let-7 family have been identified in the human genome (Pasquinelli et al., 2000; Roush et al., 2008). Previous studies have demonstrated that let-7 is underexpressed in various cancers and is related to the initiation, progression and prognosis of cancer (Takamizawa et al., 2004; Barh et al., 2010). Furthermore, restoration of its expression prevented tumor growth by targeting various oncogenes, including Ras, C-myc and HMGA2 (Johnson et al., 2007; Mayr et al., 2007; Kumar et al., 2008; Kim et al., 2009).

Let-7c is one member of the let-7 family and maps to 21q11-21, a region frequently deleted in lung cancer (Yamada et al., 2008). Let-7c is poorly expressed in various cancers, including lung, liver and prostate (Navarro et al., 2009; Shimizu et al., 2010; Nadiminty et al., 2012). Direct evidence for the potential of let-7c as a tumor suppressor was demonstrated by functional studies in several cancer cell lines. For example, the ectopic expression of let-7c promoted the granulocytic differentiation of AML cell lines and primary blasts by targeting PBX2. Restoration of let-7c also inhibited cell proliferation, clonogenicity and the anchorage-independent growth of PCa cells in vitro and led to decreased metastasis in colorectal cancer by targeting MMP11 and PBX3 (Han et al., 2012; Nadiminty et al., 2012; Pelosi et al., 2012). In lung cancer, let-7c inhibited growth and cell cycle progression in vitro (Johnson et al., 2007; Esquela-Kerscher et al., 2008). However, mechanisms in which let-7c inhibits proliferation and tumorigenesis of NSCLC remain large unknown.

In the current study, HOXA1 was identified as a novel target of let-7c. Furthermore, we demonstrated that let-7c inhibited NSCLC cell proliferation and tumorigenesis by directly targeting the 3'UTR of HOXA1 mRNA, which subsequently reduced the expression of CCND1, CDC25A and CDK2.

## Materials and Methods

*Cell culture*

A549, H1299, 16HBE and HEK293 cells were purchased from American Type Culture Collection (ATCC) and maintained in RPMI-1640 supplemented with

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10% fetal bovine serum, 100 U/ml penicillin and 100 μg/mL streptomycin at 37°C with 5% $CO_2$.

*miRNA and siRNA transfection*

Let-7c mimics (let-7c), small interfering RNA targeting HOXA1 (si-HOXA1) and negative control (NC) were purchased from GenePharma (Shanghai, China). Cells were transfected with 100 nM let-7c, si-HOXA1 or NC using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instruction. The target sequence of si-HOXA1 is 5'-GTTGTGGTCCAAGCTATGG-3' according to a previous report (Zhu et al., 2005).

*Total RNA extraction and quantitative RT-PCR (qRT-PCR)*

Total RNA, including miRNA and mRNA, was extracted from cells or tissue using TRIzol® reagent (Invitrogen), according to the manufacturer's instructions.

Let-7c expression was quantified using a miRNA-specific Taqman MiRNA Assay kit (Applied Biosystems, Foster City, CA, USA). U6 small nuclear RNA was used as an internal control.

To detect mRNA expression levels, total RNA was reverse-transcribed using the First-Strand cDNA Synthesis kit (Fermentas). mRNA levels were determined by qRT-PCR using SYBR green I (Invitrogen). β-actin was used as an internal control. All primer sequences are obtained from previous reports (Suzuki et al., 2001; Jemal et al., 2007; Wang et al., 2010; Feng et al., 2011).

All qRT-PCR tests were performed using an ABI 7500 Sequence Detection System. The relative expression fold change of mRNA and miRNAs were calculated using the $2^{-\triangle\triangle Ct}$ Method.

*Lentiviral vector and generation of stable Cell Lines*

A let-7c lentivirus was purchased from Genepharma. This vector consisted of the pre-hsa-let-7c sequence cloned into the GLVH1/GFP (LV-let-7c) plasmid. A GLVH1/GFP plasmid expressing a scrambled RNA was used as a control (LV-NC). Stable H1299 cell lines expressing let-7c or NC were generated by transfecting with LV-let-7c or LV-NC, respectively.

*MTS assay*

Cells were seeded into 96-well plates ($3\times10^3$ viable cells/well) and incubated for 24 h, 48 h, 72 h and 96 h. At the indicated time points, cell viability was measured using the CellTiter 96® Aqueous Non-Radioactive Cell Proliferation Assay (MTS) kit (Promega, Madison, WI, USA), according to the manufacturer's instruction.

*Colony formation assay*

Cells were collected and seeded into 10 $cm^2$ culture plates (500 cells per for each plates) for colony formation. Media were replaced every 3-4 days, and after 12-14 days of incubation, colonies were stained in with 0.1% crystal violet solution (prepared in 20% ethanol).

*Flow cytometry for cell cycle analysis*

Cells were seeded into 6-well plates at a concentration of $2\times10^5$ cells/well and allowed to adhere overnight. Cells were serum-starved for 24 h in RPMI supplemented with 0.5% FBS and then restored to RPMI with 10% FBS for an additional 24 h. The cells were then collected for cell cycle profile analysis by flow cytometry, according to the previous report (Yin et al., 2012).

*Western Blot*

Cells or tissue were lysed in RIPA buffer with 1 mM PMSF (Sigma, St. Louis, MO, USA). The BCA Protein Assay Kit (Pierce, Rockford, Italy) was used to detect the total protein concentration. Western blot was performed as previously described (Simoncini et al., 2000). The following primary antibodies were used: rabbit polyclonal antibodies against HOXA1, CCND1, CDC25A or CDK2 and a mouse monoclonal antibody against β-actin (all from Cell Signaling Technology, Beverly, USA).

*Construction of 3'UTR reporter plasmids and luciferase reporter assays*

The 3'UTR of HOXA1 was amplified by PCR using the following primer sequences: Forward, 5'-TTCCAGAACCGCCGAATGA-3'; Reverse, 5'-TCCCAGCCCAAGGAGATGC-3'. The 3'UTR segment was inserted into the pGL3.0 control vector (Promega) using the XBAI site. A mutated 3'-UTR fragment of HOXA1 was synthesized after point mutations were made in several bases within the binding sites. Both constructs were confirmed by sequencing.

HEK293 cells were plated into 24-well plates at a concentration of $5\times10^4$ cells/well and allowed to grow overnight. 200 ng luciferase reporter plasmid and 20 ng of pRL-TK (Promega) were transfected with let-7c or NC. Twenty-four hours after transfection, luciferase activity was measured using the dual luciferase reporter assay system (Promega) on a SIRIUS luminometer (Berthold Detection System, Pforzheim, Germany). Firefly luciferase activity was normalized to Renilla luciferase activity for each transfected well.

*In vivo assays*

Male BALB/c nude mice aged 4–6 weeks were purchased from the Laboratory Animal Centre of Hunan experiment center. All procedures were performed according to internationally accepted ethical guidelines. LV-let-7c-H1299 or LV-NC-H1299 cells ($2\times10^6$ cells/mice) were injected subcutaneously into the right flanks. After the mice were sacrificed, expression levels of let-7c, HOXA1, CCND1, CDC25A and CDK2 were measured by qRT-PCR and/or Western blot.

*Statistical analysis*

Data were expressed as mean ± SD of at least three independent experiments. Differences were analyzed by Student's t-test and a two-tailed P value $<0.05$ was considered significant. Statistical analysis was performed using SPSS18.

## Results

*NSCLC cells lose let-7c and express high level of HOXA1*

To detect whether let-7c levels were underexpressed

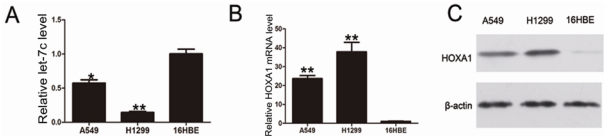


**Figure 1. NSCLC Cells Lose Let-7c and Express High Level of HOXA1.** (A) QRT-PCR analysis of let-7c in A549, H1299 and 16HBE cells. (B, C) The mRNA (B) and protein (C) expression levels of HOXA1 in A549, H1299 and 16HBE cells detected by qRT-PCR and Western blot. *p<0.05, **p<0.01, compared with 16HBE cells

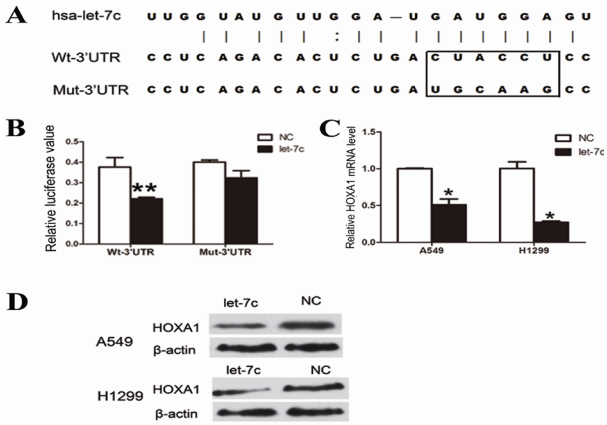


**Figure 2. HOXA1 is a Direct Target of Let-7c in NSCLC Cells.** (A) The HOXA1 3'UTR with wild-type or mutated let-7c binding site based on the Targetscan database. (B) Analysis of luciferase activity. (C, D) HOXA1 mRNA and protein levels were analyzed by qRT-PCR (C) and Western blot (D) 48 h after transfection with let-7c or NC. *p<0.05, **p<0.01, compared with the NC group

in NSCLC cells, we measured let-7c expression levels using qRT-PCR. As shown in Figure 1A, let-7c was downregulated in two NSCLC cell lines (A549 and H1299), compared with human bronchial epithelial cell line, 16HBE, demonstrating that let-7c is likely involved in the tumorigenesis of NSCLC. This result is consistent with previous studies (Johnson et al., 2005; Johnson et al., 2007).

Furthermore, we compared the HOXA1 mRNA and protein levels of A549 and H1299 cells with 16HBE cells by qRT-PCR and Western blot. We found that HOXA1 was upregulated in A549 and H1299 cells compared to 16HBE cells (Figure 1B, 1C).

*HOXA1 is a novel target of let-7c*

Next, we sought to identify mRNA targets of let-7c that might play a role in the tumorigenesis of NSCLC. One target predicted by the web-based TargetScan and miRanda algorithms is HOXA1. Furthermore, HOXA1 was upregulated in A549 and H1299 cells compared to 16HBE cells, as shown in Figure 1B and 1C. We proposed that HOXA1 is a direct target of let-7c. To determine whether let-7c directly regulates HOXA1, we constructed luciferase reporter vectors including the wild-type 3'UTR of HOXA1 (Wt-3'UTR) and a corresponding 3'UTR with mutations in the predicted binding site of let-7c (Mut-3'UTR) (Figure 2A). As shown in Figure 2B, let-7c significantly decreased luciferase activity in HEK293 cells transfected with Wt-3'UTR, compared with NC. However, let-7c-mediated repression of luciferase

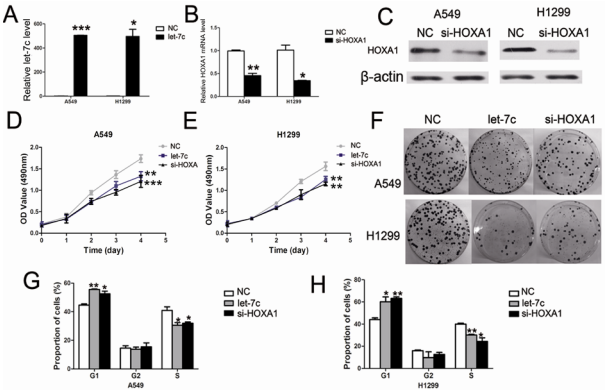


**Figure 3. Figure 3. Overexpression of Let-7c Inhibits NSCLC Cell Proliferation.** (A) Let-7c expression levels were measured in A549 and H1299 cells transfected with let-7c or NC for 48 h by qRT-PCR. (B, C) Analysis of qRT-PCR and Western blot to show HOXA1 mRNA and protein expression levels in si-HOXA1-transfected A549 and H1299 cells 48 h post-transfection, compared with NC-treated cells. (D, E) A549 (D) or H1299 (E) cells were transfected with let-7c, si-HOXA1 or NC for 24 h. Cell viability was detected at the indicated time points (post transfection) by MTS assays. (F) Colonies formed by cells transfected with let-7c, si-HOXA1 or NC for 24 h were shown 12-14 days after plating. (G, H) Effect of let-7c on the cell cycle of A549 (G) and H1299 (H) cells. *p<0.05, **p<0.01, ***p<0.001, compared with the NC-treated group

activity was abolished by the mutant putative binding site. Furthermore, we investigated the effect of let-7c on endogenous HOXA1 mRNA and protein expression levels. As shown in Figure 2C and 2D, the mRNA and protein expression levels of HOXA1 were downregulated by transfection with let-7c in A549 and H1299 cells compared with NC-treated cells. Collectively, these results suggest that let-7c has a general regulatory effect on HOXA1 in NSCLC cells by directly targeting the 3'UTR of its mRNA.

*Enforced expression of let-7c inhibits NSCLC cell proliferation and induces G1 arrest*

To further explore the biological significance of let-7c in NSCLC cells, MTS assay, colony formation assay and flow cytometry were performed in A549 and H1299 cells. Compared with the NC group, let-7c-transfected cells exhibited a more significant reduction in cell viability (Figure 3D, 3E). To assess the long-term effect of let-7c on cell growth, we performed a colony formation assay. As shown in Figure 3B, let-7c-treated A549 and H1299 cells displayed fewer and smaller colonies than NC-treated cells.

We further examined the cell cycle to determine possible mechanisms of let-7c-induced growth inhibition. Flow cytometry analysis of the cell cycle revealed an increase in the proportion of G1 phase cells and a decrease of S phase cells in the let-7c-transfected group (Figure 3G, 3H). These results demonstrated that let-7c might inhibit growth by inducing G1 arrest in A549 and H1299 cells.
In the present study, we have demonstrated that HOXA1 is a target of let-7c. To investigate whether let-7c exerts its anti-cancer function by targeting HOXA1, we examined whether the knockdown of HOXA1 by treatment with si-HOXA1 could recapitulate the suppressive effects of

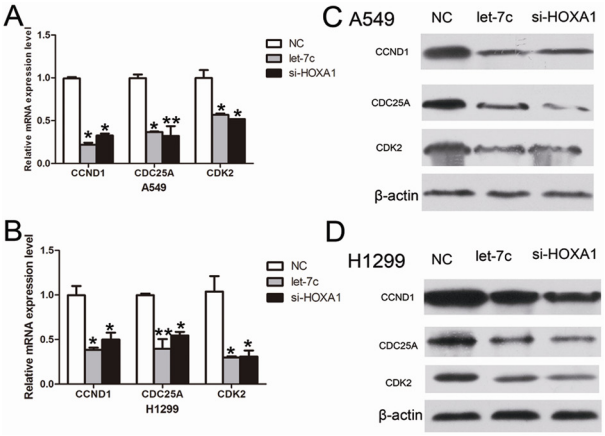


**Figure 4. Figure 4. Let-7c Reduces the Expression of CCND1, CDC25A and CDK2.** (A, B) QRT-PCR analysis of CCND1, CDC25A and CDK2 mRNA expression in A549 (A) and H1299 (B) cells transfected with let-7c, si-HOXA1 or NC for 48 h. (C, D) Western blot demonstrating protein levels of CCND1, CDC25A and CDK2 in A549 (C) and H1299 (D) cells transfected with let-7c, si-HOXA1 or NC for 48 h. *p<0.05, **p<0.01, compared with the NC-treated group

let-7c in A549 and H1299 cells. We found that siRNA knockdown of HOXA1 in A549 and H1299 cells led to significant cell growth inhibition and G1 arrest, similar to that induced by let-7c (Figure 3D-3H). These results indicate that the reduction of HOXA1 expression could mimic the inhibitory effects of let-7c on NSCLC cells, suggesting that targeting HOXA1 may be a novel mechanism mediating the tumor suppressor function of let-7c in A549 and H1299 cells.

*Let-7c reduces the expression of the HOXA1 downstream effectors CCND1, CDC25A and CDK2.*

Previous studies have confirmed that CCND1, CDC25A and CDK2 are involved in regulation of G1/S transition (Jinno et al., 1994; Aleem et al., 2005; Liu et al., 2008). CCND1 has been reported to be a downstream target of HOXA1 in A549 cells (Cho et al., 2012). Microarray profiles have demonstrated that CDC25A and CDK2 were modulated by HOXA1 in MCF-10 cells (Mohankumar et al., 2007). We therefore sought to determine whether the HOXA1-mediated downstream effectors were also impacted by let-7c. We found that the knockdown of HOXA1 reduced the expression of CCND1, CDC25A and CDK2 in A549 (Figure 4A, 4C) and H1299 cells (Figure 4B, 4D), which is similar to previous studies (Mohankumar et al., 2007; Cho et al., 2012). Furthermore, the ectopic expression of let-7c downregulated the mRNA and protein levels of CCND1, CDC25A and CDK2 in A549 (Figure 4A, 4C) and H1299 cells (Figure 4B, 4D). Collectively, these findings demonstrate that let-7c could regulate the expression of HOXA1 downstream effectors CCND1, CDC25A and CDK2.

*Let-7c suppresses tumorigenesis in vivo*

To further study the role of let-7c in tumorigenesis, we evaluated the effect of let-7c overexpression on tumor growth in vivo. LV-let-7c-H1299 or LV-NC-H1299 cells were injected subcutaneously into the right dorsal flanks of nude mice.

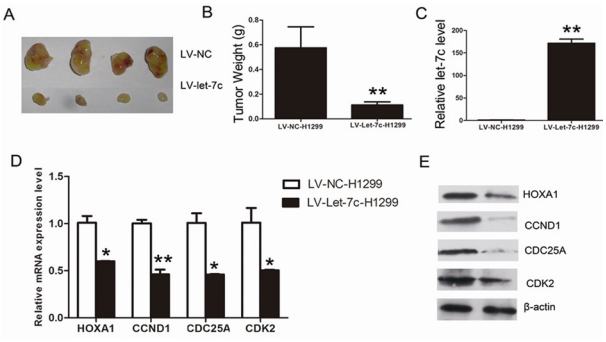


**Figure 5. Figure 5. Let-7c Suppresses Tumor Growth in vivo.** (A) Photograph of xenograft tumor masses from nude mice. (B) The average weight of xenograft tumors. (C) Let-7c levels as assessed by qRT-PCR. (D, E) The HOXA1, CCND1, CDC25A and CDK2 expression levels as assessed by qRT-PCR (D) and Western blot (E) *p<0.05, **p<0.01, compared with the LV-NC-H1299 group

Thirty days after injection, the average tumor weight was also significantly reduced in the LV-let-7c-H1299 group, compared with the LV-NC-H1299 group (Figure 5B). Moreover, we measured the expression of let-7c, HOXA1, CCND1, CDC25A and CDK2 in harvested tumor tissues. Similar to the in vitro, the let-7c levels were significantly upregulated (Figure 5C), and HOXA1, CCND1, CDC25A and CDK2 levels were decreased (Figure 5D, 5E) in tumors derived from LV-let-7c-H1299 cells, as compared to LV-NC-H1299 cells.

## Discussion

In the present study, HOXA1 was identified as a novel target of let-7c. Ectopic expression of let-7c significantly suppressed NSCLC cell proliferation and induced G1 arrest in vitro, which is consistent with the previous study (Johnson et al., 2007). We also demonstrated that the overexpression of let-7c could inhibit tumorigenesis in vivo. Furthermore, we demonstrated that HOXA1 and its downstream effectors, CCND1, CDC25A and CDK2, contributed to the inhibitory effect of let-7c on NSCLC cells. Admittedly, we could not exclude other additional factors within the complicated network regulating cell proliferation and tumorigenesis that we observed because a single miRNA could target multiple messenger RNAs (Bartel, 2004).

HOXA1 is a member of the homeodomain-containing transcription factor family, which plays a pivotal role during development (Chen et al., 2003). Previous studies have suggested that HOXA1 is overexpressed in various cancers, including breast, liver, oral and lung cancer (Chariot et al., 1996; Abe et al., 2006; Bitu et al., 2012; Zha et al., 2012). The forced expression of HOXA1 promoted oncogenic transformation by modulating the p44/42 MAP kinase pathway and bcl-2 in MCF-10 cells (Mohankumar et al., 2007). The knockdown of HOXA1 reduced cell growth in bladder and lung cancer cells (Cho et al., 2012). Furthermore, high HOXA1 expression was correlated with poor prognosis in oral squamous cell and hepatocellular carcinomas. On the other side, HOXA1 was verified as a target of miR-10a, and the reduction of HOXA1 expression promoted invasiveness of pancreatic cancer

(Ohuchida et al., 2012). Therefore, the function of HOXA1 is likely complicated, functioning as a proto-oncogene or oncogene, depending on specific conditions and in different types of cancer. In the present study, HOXA1 is found to be unregulated in A549 and H1299 cells compared to 16HBE cells. Additionally, the knockdown of HOXA1 inhibited growth of cells and induced G1 arrest. These results demonstrate that, similar to previous studies, HOXA1 plays a role as a proto-oncogene in NSCLC cells (Abe et al., 2006; Cho et al., 2012).

Let-7c has been shown to modulate the expression of CCND1 at both the mRNA and protein levels, although the study did not confirm whether let-7c could direct modulate CCND1 through the 3'UTR of its mRNA (Gong et al., 2011). CCND1 has been verified as a direct target of let-7b (Schultz et al., 2008). CDC25A was reported to be a direct target of let-7 (Johnson et al., 2007). Bioinformatic analysis predicts that let-7c could target CCND1 and CDC25A. We speculated that let-7c could also modulate CCND1 and CDC25A directly through the 3'UTRs of their mRNA transcripts. In the present study, we demonstrated that both the ectopic expression of let-7c and knockdown of HOXA1 by si-HOXA1 downregulated CCND1, CDC25A and CDK2. Taken together, we proposed that let-7c could modulate CCND1 and CDC25A, not only through knockdown of HOXA1 indirectly but also by directly interacting with their 3'UTRs. Further experiments are needed to confirm whether CCND1 and CDC25A are direct targets of let-7c.

In summary, the current study provides, for the first time, an important link between let-7c-mediated tumor growth inhibition, G1-phase cell cycle arrest of NSCLC cells and the downregulation of HOXA1/CCND1, CDC25A and CDK2. Restoration of let-7c expression may thus provide a potential therapeutic intervention strategy for NSCLC.

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