

Downregulation of the lncRNA RP11-432I5.4 inhibits tumorigenesis in the JAK2V617F-positive classic myeloproliferative neoplasms

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Research

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Abstract

Background: Although aberrant expression of long non-coding RNA (lncRNA) is associated with many human cancers, little is known about its role in the JAK2V617F-positive classic myeloproliferative neoplasms (cMPNs).

Methods: In this study, we performed a comprehensive analysis of lncRNAs in human cMPNs cells using a lncRNA cDNA microarray and identified the lncRNA RP11-432I5.4 acted as an effective factor in JAK2V617F-positive cMPN cells.

Results: The lncRNA RP11-432I5.4 showed higher expression in cMPN patients, especially higher in JAK2V617F-positive cells compared with JAK2V617F-negative cells. Overexpression of lncRNA RP11-432I5.4 increased the proliferation of JAK2V617F-positive cells while downregulation of it decreased proliferation, promoted apoptosis and triggered S phase arrest of JAK2V617F-positive cells. Furthermore, in a mouse xenograft model, the silencing of lncRNARP11-432I5.4 repressed tumor formation *in vivo*.

Conclusions: Taken together, these results revealed that lncRNA RP11-432I5.4 plays an important role in cMPN tumorigenesis and may be a potential novel target for treatment of JAK2V617F-positive cMPN patients.

Background

Classic myeloproliferative neoplasms (cMPNs), including polycythemia vera (PV), essential thrombocythemia (ET), and primary myelofibrosis (PMF), are clonal disorders characterized by the overproduction of one kind or more of the blood cells. Somatic mutations are responsible for the clonal expansion of cells in cMPNs and more than 85 percent of the patients appear to have a mutation in one of the following three genes: JAK2, MPL, or CALR(1–3). Other somatic mutations, such as LNK, CBL, TET2, ASXL1, IDH, IKZF1, EZH2, or DNMT3A are also identified in some cMPN patients(4–7). Janus kinase 2 (commonly called JAK2) is a non-receptor tyrosine kinase(8). Mutation in exon 14 of JAK2 substitutes a bulky phenylalanine for a conserved valine at position 617 in the JH2 or pseudokinase domain (Val617Phe, V617F), which negatively regulates the kinase domain. The JAK2V617F mutation causes cytokine-independent activation of JAK-STAT pathway and accounts for hypersensitivity of hematopoietic progenitor cells in cMPNs to growth factors and other cytokines(9–11).

Mammalian genomes are pervasively transcribed to produce thousands of long non-coding RNAs (lncRNAs)(12, 13). LncRNAs are transcripts longer than 200 nucleotides that have little or no protein-coding capacity(14, 15). Nuclear lncRNAs can silence or activate gene expression in cis or in trans manner(16), guide DNA methylation and histone modification(17), interplay with nuclear domains(18), perform gene-control through sequestration(19) and regulate higher-order chromosomal interactions(20). Aberrant expression of many lncRNAs is involved in the progression of CML, a BCR-ABL-positive MPN. For example, in CML, lncRNA-BGL3 functions as a competitive endogenous RNA for binding microRNAs to cross-regulate phosphatase and tensin homolog (PTEN) expression, and Bcr-Abl-mediated cellular

transformation critically requires silence of tumor-suppressor lncRNA-BGL3(21). Overexpression of lncRNA-H19 contributes to the progression of CML by enhancing cell survival, colony formation, and inhibiting cell apoptosis(22). LncRNA X-inactive specific transcript (Xist) is required for hematopoietic stem cell survival and function. Deleting Xist causes marrow fibrosis, leukemia, and histiocytic sarcoma(23). However, whether lncRNAs contribute to the pathogenesis and progression of BCR-ABL-negative cMPNs, especially JAK2V617F-positive cMPNs, remains to be explored. Hence, we undertook a comprehensive study examining lncRNA expression in JAK2V617F-positive cMPN patients and querying the functional consequences of lncRNA expression.

In this study, using lncRNA microarray analysis, we found that numerous lncRNAs are differentially expressed in JAK2V617F-positive cMPN patients compared to their JAK2V617F-negative counterparts. One of them, lncRNA RP11-432I5.4 (NCBI accession number HG508247), was identified to be greatly upregulated in JAK2V617F-positive MPN samples. Loss- or gain-of-function analysis indicated that lncRNA RP11-432I5.4 promoted tumorigenesis and progression, playing a vital role in JAK2V617F-positive cMPNs. This study demonstrated the mechanism of how lncRNA RP11-432I5.4 influences JAK2V617F-positive cMPNs and provides a potential strategy for the treatment of JAK2V617F-positive cMPNs.

Methods

Patients and Cell lines

A total of 12 samples from cMPN patients (six JAK2V617F-positive and six JAK2V617F-negative) were included in the lncRNA microarray experiment. All patients were recruited from Shanghai Tongji Hospital. The cMPN diagnosis was defined according to World Health Organization (WHO) criteria(24). All subjects had given written informed consent in accordance with the Declaration of Helsinki.

Three cell lines were used in the study. HEL, a human JAK2V617F-expressing cell line, and K562 (a human wild type JAK2-expressing cell line) were cultured in RPMI-1640 and IMDM medium (Gibco, Grand Island, NY, USA) respectively with 10% fetal bovine serum at 37°C in a humidified atmosphere of 5% CO₂. 293T cells were cultured in high-glucose Dulbecco's modified Eagle's medium (Gibco, Grand Island, NY, USA) with 10% fetal bovine serum at 37 °C in a humidified atmosphere of 5% CO₂.

LncRNA microarray analysis

The lncRNA cDNA microarray was from Arraystar (Arraystar, Rockville, MD, USA). Bone marrow mononuclear cells aspirated from the 12 cMPN patients were isolated by Ficoll-Hypaque density gradient centrifugation. Total RNA of every patient was extracted from bone marrow mononuclear cells using Trizol reagent (Invitrogen, Carlsbad, CA, USA) following the manufacturer's instruction. Integrity and concentration of RNA were assessed after RNA extraction and prior to sample labeling. Agilent Quick Amp Labeling Kit was used for sample labeling. Hybridization was performed in Agilent's SureHyb Hybridization Chambers. After washing, slides were scanned with the Agilent DNA Microarray Scanner.

Data was extracted using Agilent Feature Extraction software. Further data analysis was performed using Agilent GeneSpring GX v11.5.1 software. Subgroup analysis was performed using home-made scripts. The cDNA synthesis, labelling, hybridization, and data analysis were carried out as previously described(25).

Quantitative real-time polymerase chain reaction (qRT-PCR)

Specific quantitative real-time PCR (qRT-PCR) assays for lncRNA RP11-432I5.4 were performed and used GAPDH as the internal control. Reverse transcription was performed to synthesize cDNA. qRT-PCR reactions were carried out using the ABI7500 system (Applied Biosystems, CA). Primers for qRT-PCR are listed as following (Table 1).

Table 1
Primers for qRT-PCR of lncRNA RP11-432I5.4

Name	Primer	Sequence (5'-3')	Size(bp)
lncRNA RP11-432I5.4	Forward	ATAACTGCCAGGGCCTACAC	86
	Reverse	TGATCCCTGACAGTCCCTTGA	
GAPDH	Forward	TGACTTCAACAGCGACACCCA	121
	Reverse	CACCCTGTTGCTGTAGCCAAA	

Cells transfection

Lentiviral BR-V108 RNAi vectors expressing shRNA against lncRNA RP11-432I5.4 (5'-CCGGTACCAATAGGTTAACGAGATTCTCGAGAATCTGCTAACCTATTGGTATTTTG-3') and scramble sequence (5'-CCGGGCGCGCTTGTAGGATTGCTCGAGAATCTGCTAACCTATTGGTATTTTG-3') used as a negative control were constructed. High-titer lentiviral vector stock was produced in 293T cells with the RNAi vectors, pHelper1.0, and pHelper2.0 co-transfected. HEL and K562 cells were infected by freshly prepared viruses and maintained with 0.8ug/ml hygromycin. LncRNA RP11-432I5.4 expression vector or the corresponding empty vector (Shanghai Biosciences, co. LTD, China) were used to produce lentivirus by the same way and HEL and K562 cells were infected and maintained with 0.8ug/ml hygromycin.

Cell proliferation assay

Infected HEL and K562 cells at logarithmic growth phase were cultured in 96-well plates at density of 2000 cells per well with 3 repeated wells arranged and culture in the aforementioned conditions. After the cells were cultured for 24 h, cell counting kit-8(CCK-8, 10 µl, 5 mg/ml, Sigma, USA) reagent was added into each well at dark and incubated at 37°C for 4 h. The optical density (OD) of each well at a 450 nm wavelength was detected by a microplate reader.

Apoptosis assay

Cells were stained with Annexin V (eBioscience, Thermo Fisher Scientific, USA). Fluorescence-activated cell sorter (FACS) analyses were performed on a fluorescence-activated cell sorter (Millipore, Guava easy

Cyte HT, USA) and data was analyzed using FlowJo 7.6.1.

Xenograft mouse model

Animal studies were approved by the ethics committee of Tongji Hospital of Tongji University and were carried out in accordance with guidelines for animal care and protection and protocols. K562 or HEL cells with lncRNA RP11-432I5.4 stably knockdowned were obtained by infection with lncRNA RP11-432I5.4 shRNA lentiviral. Then cells (5×10^6 per mouse, 9 mice per group) infected by lncRNA shRNA lentiviral or the control lentiviral were subcutaneously injected into both back flanks of male nude mice (6 weeks old of age and around 20 g in weight). Tumor growth was monitored and measured in weight at the indicated days post-inoculation by a caliper. Thirty-four days after injection, mice were sacrificed and xenograft tumor weight was measured.

Statistical analysis

All data represented are the mean \pm standard deviation from at least 3 independent experiments. The data was analyzed with two-tailed Student's t tests, and differences with $p < 0.05$ were considered statistically significant.

Results

The lncRNA RP11-432I5.4 is upregulated in JAK2V617F-positive cells

To investigate the role of lncRNAs in JAK2V617F-positive cMPNs, we first examined lncRNA expression profiles in six JAK2V617F-positive cMPN cases and six JAK2V617F-negative cMPN cases using the lncRNA-cDNA microarray. Transcriptome profiling identified 675 upregulated lncRNAs and 788 downregulated lncRNAs by standard of more than 1.5-fold change in JAK2V617F-positive MPN patients compared to their JAK2V617F-negative counterparts. With most significantly changed expression, lncRNA RP11-432I5.4 was selected for further research (Fig. 1A and 1B). The lncRNA RP11-432I5.4 was determined by open reading frame finder from NCBI and displayed no coding potential. To confirm the association between the JAK2V617F mutation and dysregulated expression of lncRNA RP11-432I5.4, we examined the expression level of lncRNA RP11-432I5.4 in JAK2V617F-positive human erythroleukemia (HEL) cells and wild-type JAK2-expressing K562 cells. It's shown that the expression level of lncRNA RP11-432I5.4 in HEL cells is higher than that of K562 cells (Fig. 1C). These results provided strong evidence that lncRNA RP11-432I5.4 may contribute in JAK2V617F-positive cMPNs, while the exact mechanism still needs to be illustrated.

Upregulation of lncRNA RP11-432I5.4 promoted cell proliferation of JAK2V617F-positive cells in vitro

To uncover the physiological role of lncRNA RP11-432I5.4 in cMPNs, we stably overexpressed lncRNA RP11-432I5.4 in JAK2V617F-positive HEL cells and wild-type JAK2-expressing K562 cells. Then the efficiency of lncRNA RP11-432I5.4 overexpression was detected by qRT-PCR. Compared with the corresponding empty-vector control groups, overexpression efficiency of lncRNA RP11-432I5.4 in HEL cells and K562 cells are 6.43 folds and 2.05 folds respectively (Fig. 2A and 2B). Cell Counting Kit-8 (CCK-8) assay showed that overexpression of lncRNA RP11-432I5.4 in HEL cells significantly promoted cell proliferation, while the proliferation of K562 cells was not much changed (Fig. 2C and 2D). Promoted cell proliferation in lentivirus-infected HEL cells rather than K562 cells showed that upregulation of lncRNA RP11-432I5.4 promoted cell proliferation especially in JAK2V617F-positive cells.

Downregulation of lncRNA RP11-432I5.4 inhibited cell proliferation and triggered S phase arrest in JAK2V617F-positive cells in vitro

To further investigate the potentially functional significance of lncRNA RP11-432I5.4 in JAK2V617F-positive cMPNs, we examined whether knockdown of lncRNA RP11-432I5.4 affects cell proliferation in both HEL cells and K562 cells. As expected, siRNAs targeting lncRNA RP11-432I5.4, whose interference specificities were evaluated by qRT-PCR (Fig. 3A and 3B), dramatically reduced cell proliferation of JAK2V617F-positive HEL cells, while K562 cells showed no statistically significant differences (Fig. 3C and 3D). Next, we investigated the effect of knockdown of lncRNA RP11-432I5.4 on cell cycle distribution of HEL and K562 cells using flow cytometry analysis. As shown in Fig. 3E and 3F, lncRNA RP11-432I5.4 knockdown induced significant S phase arrest in HEL cells, in which the G1 phase was reduced from 51.6–13.9% and S phase was raised from 35.41–66.48%, while cell cycle distribution of K562 cells was little affected. These results showed that Downregulation of lncRNA RP11-432I5.4 inhibited cell proliferation and triggered S phase arrest in JAK2V617F-positive cells.

Downregulation of lncRNA RP11-432I5.4 promoted cell apoptosis in JAK2V617F-positive cells in vitro

To investigate the role of lncRNA RP11-432I5.4 in cell apoptosis of JAK2V617F-positive cells, flow cytometry analysis and western blotting were performed to evaluate the cell apoptosis and apoptosis-related proteins in lncRNA RP11-432I5.4-downregulated HEL and K562 cells. The HEL cells with lncRNA RP11-432I5.4-knockdowned showed significant apoptosis in comparison to K562 cells with downregulated lncRNA RP11-432I5.4, and the differences were statistically significant ($P < 0.001$) (Fig. 4A and 4B). Western blotting was performed to detect the apoptosis-related proteins in HEL cells and showed that knockdown of lncRNA RP11-432I5.4 significantly induced dephosphorylation of Akt and mTOR, and increased the levels of cleaved-caspase-3 in comparison to the control group (Fig. 4C). These results indicated that knockdown of lncRNA RP11-432I5.4 can promote the apoptosis of JAK2V617F-positive cells.

LncRNA RP11-432I5.4 is required for tumorigenesis of JAK2V617F-positive cells *in vivo*

Based on the above studies, we found that downregulation of lncRNA RP11-432I5.4 inhibited cell proliferation and promoted apoptosis of JAK2V617F-positive cells *in vitro*. Therefore, we examined whether knockdown of lncRNA RP11-432I5.4 could make a dent in tumor growth *in vivo* by subcutaneously injecting lncRNA RP11-432I5.4-knockdowned HEL or K562 cells into nude mice and monitoring tumor growth on day 25, 27, 30, 32 and 34 after inoculation. After 34 days, the mice were euthanized and tumors were weighed. The results showed that tumors grew much faster in lncRNA RP11-432I5.4-knockdowned K562 cells engrafted mice (Fig. 5A and 5B). The tumor diameters of lncRNA RP11-432I5.4-knockdowned K562 cells engrafted mice were significantly larger than those of the HEL cells group on day 34, and almost no tumor formation was observed in lncRNA RP11-432I5.4-knockdowned HEL cells engrafted mice (Fig. 5B). The average tumor weight of mice inoculated with lncRNA RP11-432I5.4-knockdowned K562 cells was 110.6 ± 62.9 mg, which was significantly higher than that of the HEL cells group (Fig. 5C). Injection of lncRNA RP11-432I5.4-knockdowned HEL cells into nude mice showed that there was almost no subcutaneous tumor formation. Taken together, these results suggested that downregulation of lncRNA RP11-432I5.4 significantly attenuated tumorigenicity in JAK2V617F-positive cells rather than K562 cells (wild-type JAK2-expressing), which was consistent with the data obtained in the proliferation and apoptosis assays *in vitro*.

Discussion

Recently, increasing numbers of lncRNAs have been identified as vital components of human genome controlling cell differentiation and critical functions(26–29). Numerous studies have found that dysregulated expression of lncRNA was associated with leukemia cell proliferation, differentiation, and apoptosis(30, 31). However, the functional relevance of lncRNAs and their regulation during JAK2V617F mutation-mediated tumorigenesis in cMPNs remain enigmatic. Here, we investigated the role of lncRNAs in JAK2V617F-positive cMPN patients. We found that the novel lncRNA RP11-432I5.4 was significantly increased in JAK2V617F-positive cMPN patients. Upregulation of lncRNA RP11-432I5.4 promoted cell proliferation and downregulation of it inhibited cell proliferation and triggered S phase arrest as well as promoted cell apoptosis in JAK2V617F-positive HEL cells *in vitro*. Moreover, HEL cells with suppressed lncRNA RP11-432I5.4 showed significantly attenuated tumorigenesis in nude mice. Collectively, the results of this study present that lncRNA RP11-432I5.4 plays a vital role in tumorigenesis and progress of JAK2V617F-positive cMPNs.

JAK2V617F mutation is considered as an important driven mutation of the cMPNs(32). It directly results in the constitutive activation of the JAK/STAT pathway and cause the disease. But many studies have indicated that other factors including the epigenetic regulation are also involved in the pathogenesis of cMPNs. The results of our examination showed that the expression of lncRNA RP11-432I5.4 was markedly higher in JAK2V617F-positive cells collected from cMPN patients than in wild-type JAK2-

expressing cells. As it's well documented that dysregulation of lncRNA is a hallmark of various cancers and aberrant expression of lncRNA is correlated with hematopoietic malignancies, we hypothesized that lncRNA RP11-432I5.4 might be related to the pathogenesis of JAK2V617F-positive cMPN. Thus, we conducted *in vitro* study using JAK2V617F-positive cell line, HEL, and the results showed that upregulation of lncRNA RP11-432I5.4 promoted cell proliferation, while downregulation of lncRNA RP11-432I5.4 inhibited cell proliferation, triggered S phase arrest, and promoted cell apoptosis significantly. Moreover, the same effects were not observed in JAK2V617F-negative K562 cells when the lncRNA RP11-432I5.4 was upregulated or downregulated. It has been reported that apoptosis and cell cycle alteration are responsible not only for tumor development and progression but also for tumor resistance to therapies(33, 34). Here we found that aberrant expression of lncRNA RP11-432I5.4 may affect cell cycle and apoptosis of HEL cells, and this influence on cell phenotype is specifically correlated with JAK2V617F mutation, which means lncRNA RP11-432I5.4 may act as a new specific therapeutic target of JAK2V617F-positive cMPNs.

Therefore, to further investigate whether lncRNA RP11-432I5.4 plays a role in tumorigenesis *in vivo*, we subcutaneously inject lncRNA RP11-432I5.4-knockdowned HEL or K562 cells into nude mice. The results showed that downregulation of lncRNA RP11-432I5.4 significantly attenuated tumorigenicity of JAK2V617F-positive cells rather than wild-type JAK2-expressing cells. As lncRNAs play a vital role in hematopoietic tumorigenesis in combination with recurring mutations or gene rearrangements(35), and some lncRNAs are emerging as key modulators of gene activity(36), these data hinted that lncRNA RP11-432I5.4 could be important regulator of JAK2V617F-induced gene dysregulation, and lncRNA RP11-432I5.4 may synergize with JAK2V617F to play a pivotal role in pathogenesis of cMPNs.

Taken together, our study identified a JAK2V617F-related lncRNA, RP11-432I5.4, which is dysregulated in JAK2V617F-positive cells and act as a tumor-promote gene in classic MPNs. LncRNA RP11-432I5.4 may serve as a novel therapeutic target especially in JAK2V617F-positive cMPNs. However, we understand that our cMPN patients sample size is limited. Another large-scale study may improve the persuasiveness of the results we obtained from the cMPN sample. In addition, whether the dysregulation of lncRNA RP11-432I5.4 influences JAK2V617F-induced cMPNs tumorigenesis *in vivo* still remains elusive. Further studies are needed to address these issues and to determine the therapeutic significance of lncRNA RP11-432I5.4 in cMPNs.

Conclusions

LncRNA RP11-432I5.4 played an important role in pathogenesis of JAK2V617F-positive classic MPNs but not in that with wild type JAK2. LncRNA RP11-432I5.4 is dysregulated in JAK2V617F-positive cells and act as a tumor-promote gene in classic MPNs. This lncRNA might cooperate with JAK2V617F mutation to promote pathogenesis of MPNs, and may be a potential novel target for treatment of JAK2V617F-positive MPN patients.

Abbreviations

lncRNA
long non-coding RNA
cMPNs
classic myeloproliferative neoplasms
PV
polycythemia vera
ET
essential thrombocythemia
PMF
primary myelofibrosis
JAK2
Janus kinase 2
V617F
Val617Phe
PTEN
phosphatase and tensin homolog
Xist
lncRNA X-inactive specific transcript
WHO
World Health Organization
qRT-PCR
Quantitative real-time polymerase chain reaction
CCK-8
cell counting kit-8
FACS
Fluorescence-activated cell sorter

Declarations

Ethics approval and consent to participate

All subjects had given written informed consent in accordance with the Declaration of Helsinki. Animal studies were approved by the ethics committee of Tongji Hospital of Tongji University and were carried out in accordance with guidelines for animal care and protection and protocols.

Consent for publication

Not applicable.

Availability of data and materials

The data used and/or analysed during the current study are available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

Jie Zhou performed the cell experiments and was a major contributor in writing the manuscript. Hao Wu collected the samples from MPN patients. Wen-Jun Zhang helped with the analyses of data. Jian-Fei Fu and Ai-Bin Liang provided the idea of this project. All authors read and approved the final manuscript.

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Figures

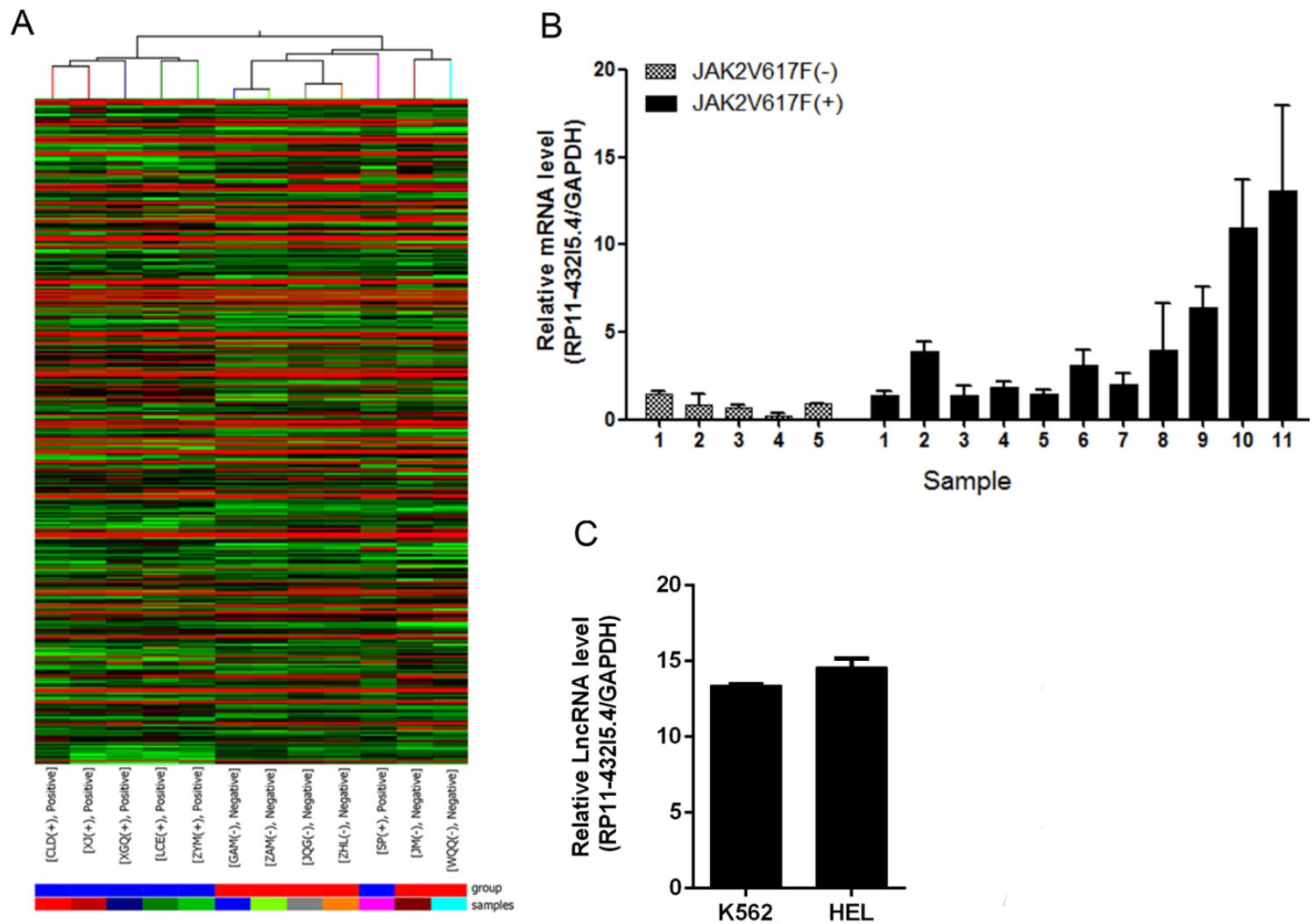


Figure 1

LncRNA RP11-432I5.4 is identified as a functional lncRNA in classic MPNs. (A) Hierarchical clustering analysis of lncRNAs differentially expressed in JAK2V617F-positive or -negative MPN patients (difference greater than 1.5-fold change; $P<0.05$). (B) The expression of lncRNA RP11-432I5.4 in JAK2V617F-positive or -negative MPN patients was detected by qRT-PCR. (C) Expression of lncRNA RP11-432I5.4 was detected by qRT-PCR in K562 and HEL cells.

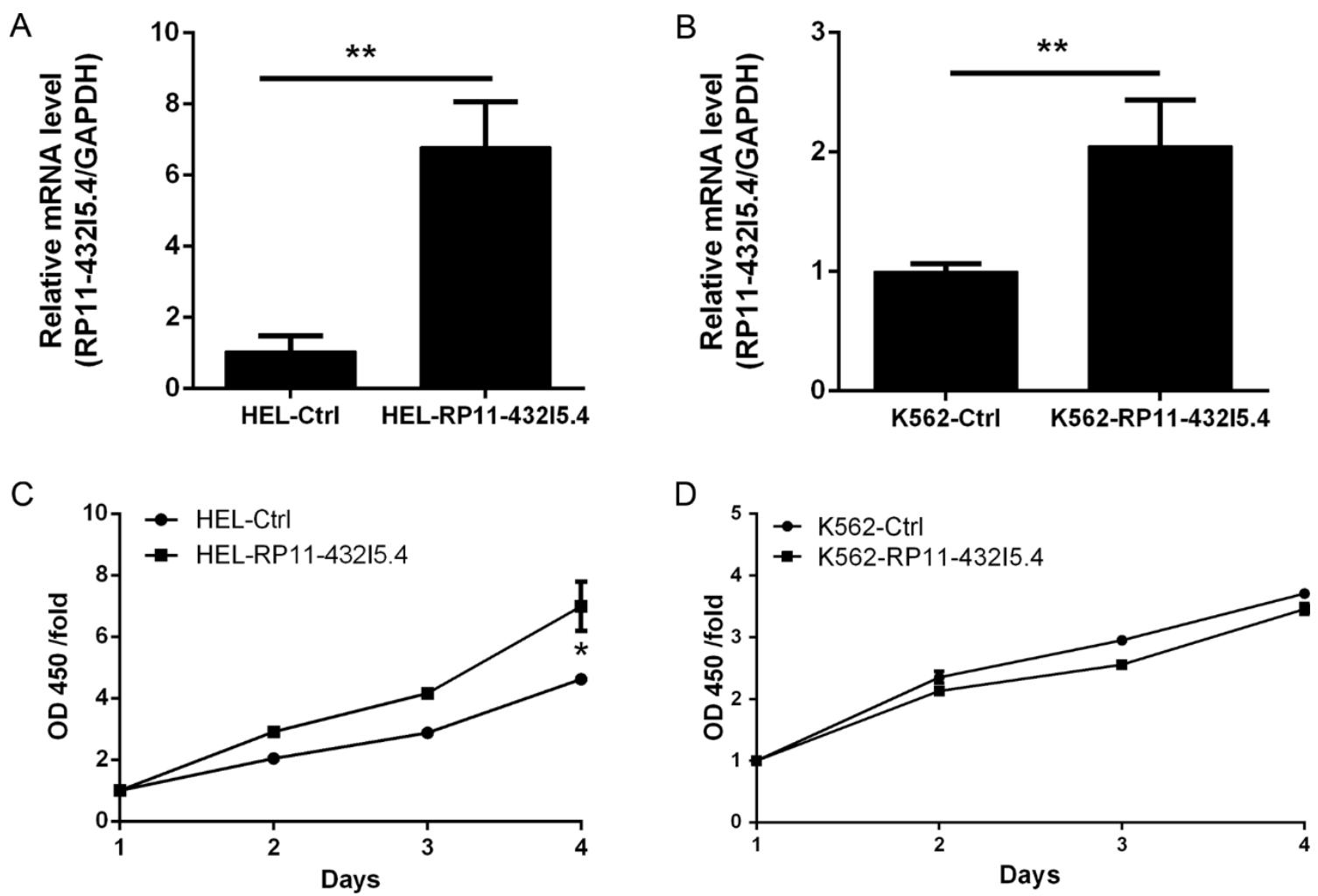


Figure 2

LncRNA RP11-432I5.4 promoted cell proliferation in HEL cells. (A), (B) HEL and K562 cells were infected with LncRNA RP11-432I5.4 overexpression lentivirus or control vector and the expression levels were detected by qRT-PCR. (C), (D) CCK-8 assay was used to analyze the cell proliferation at different time points. (*P<0.05, **P<0.01 compared to control group).

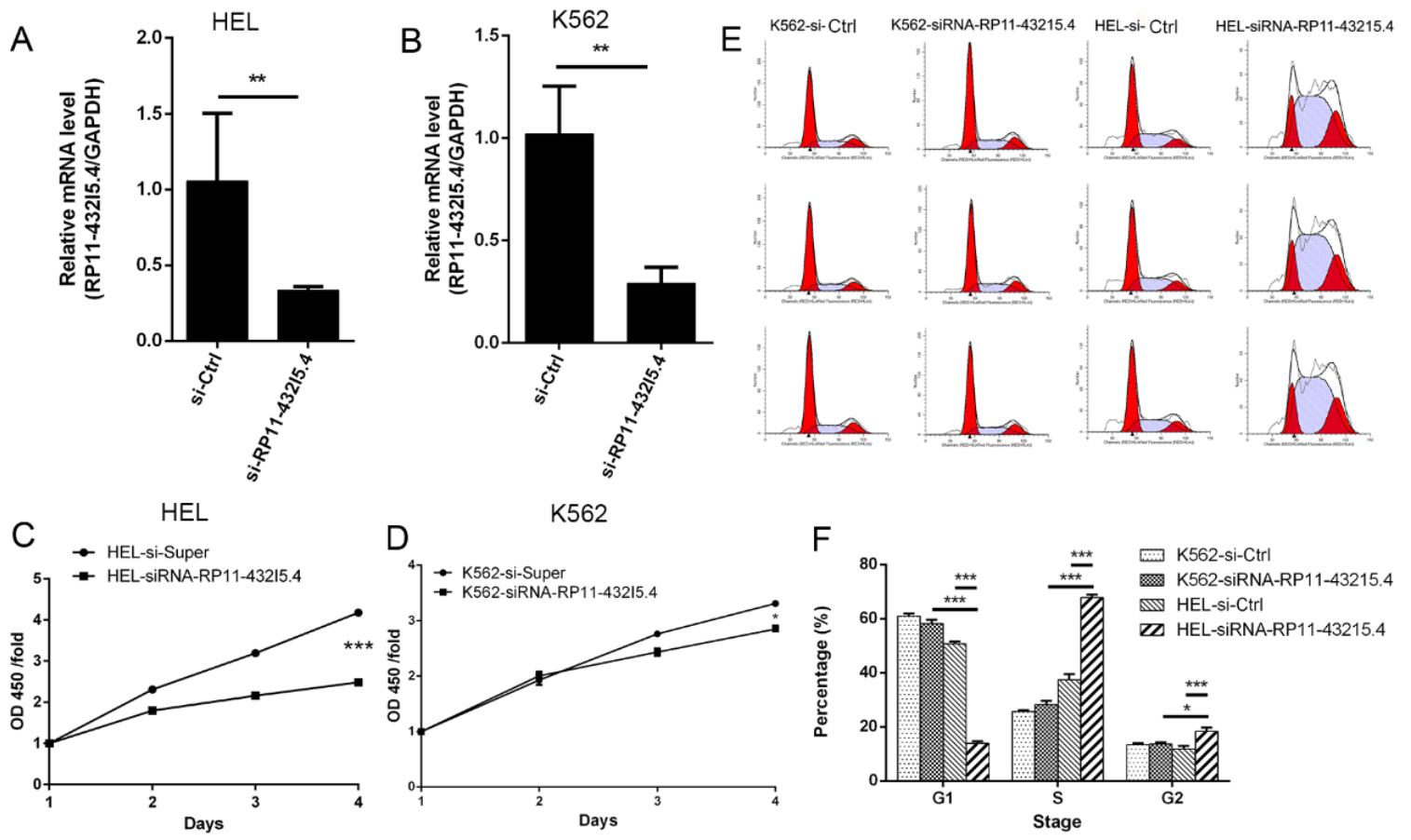
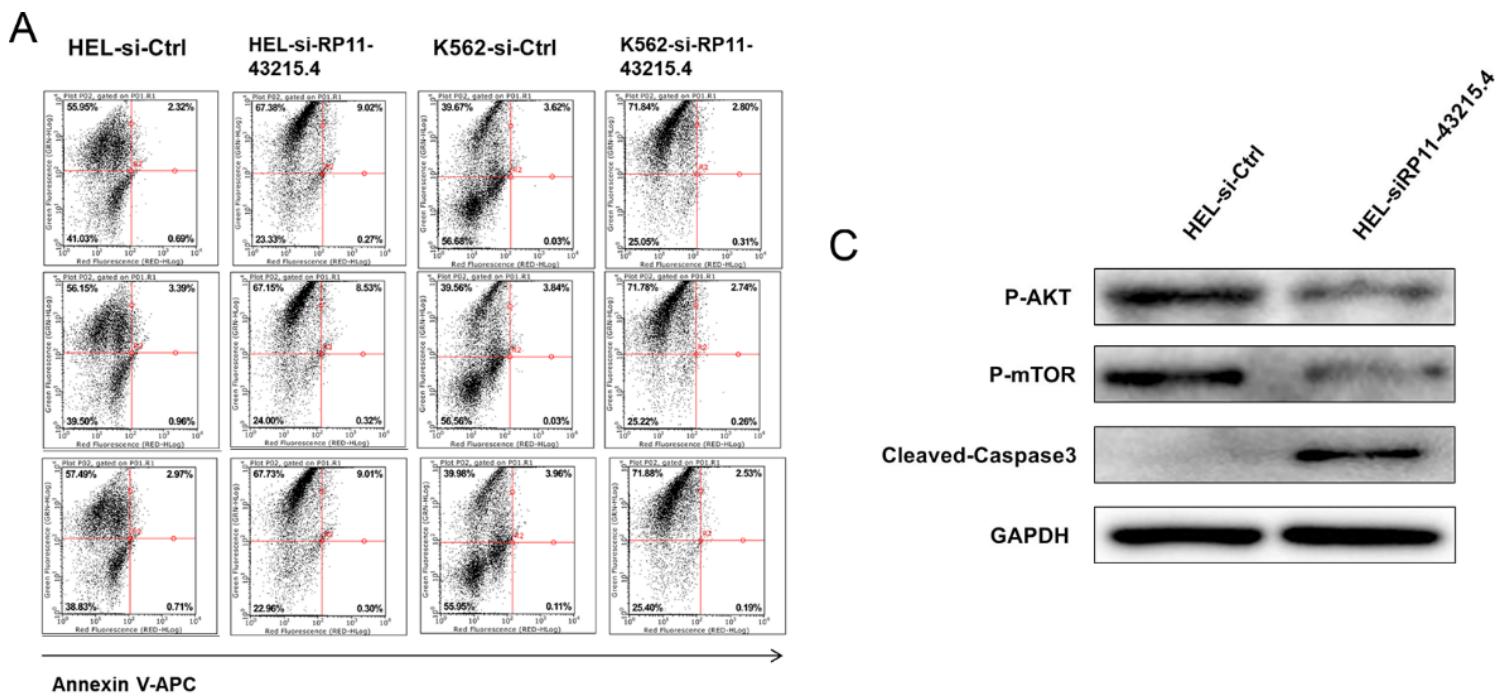
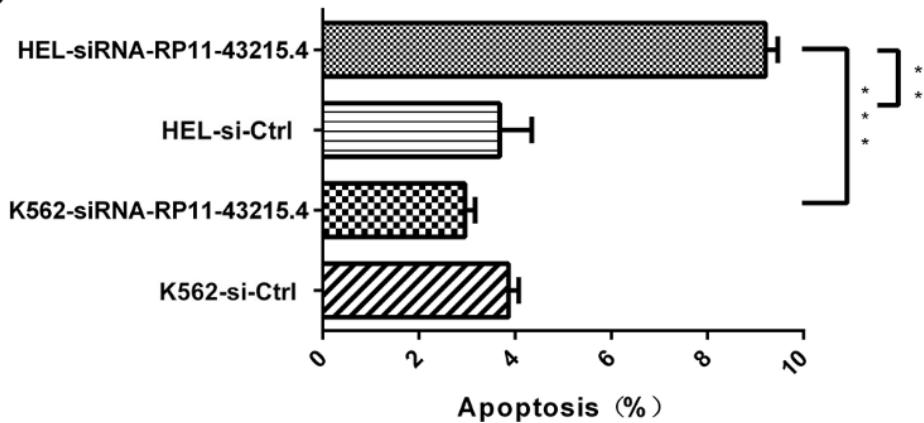


Figure 3

Silencing of lncRNA RP11-43215.4 inhibits HEL cell proliferation and triggered S phase arrest. (A), (B) K562 and HEL cells were infected with lncRNA RP11-43215.4 siRNA or control vector and the knockdown efficiency were detected by qRT-PCR. (C), (D) CCK-8 assay was used to analyze the cell proliferation of HEL and K562 cells at different time points. (E), (F) Flow cytometry assay was applied to measure the cell cycle of HEL and K562 cells (*P<0.05, **P<0.01, ***P < 0 .001 compared to control group).



B



C

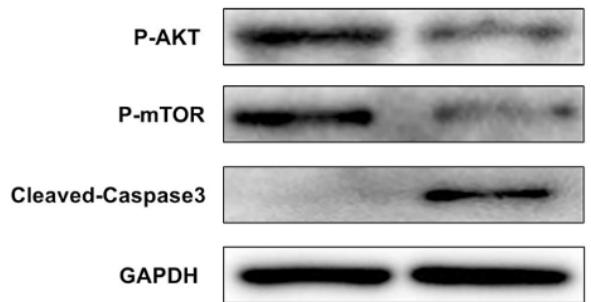


Figure 4

Suppression of lncRNA RP11-43215.4 promoted apoptosis of HEL cells. (A), (B) Cells apoptosis of lncRNA RP11-43215.4-downregulated cells was measured by flow cytometry assay. (C) Cells apoptosis-related proteins were examined by western blotting assay (*P< 0.05, **P< 0.01 compared to control group).

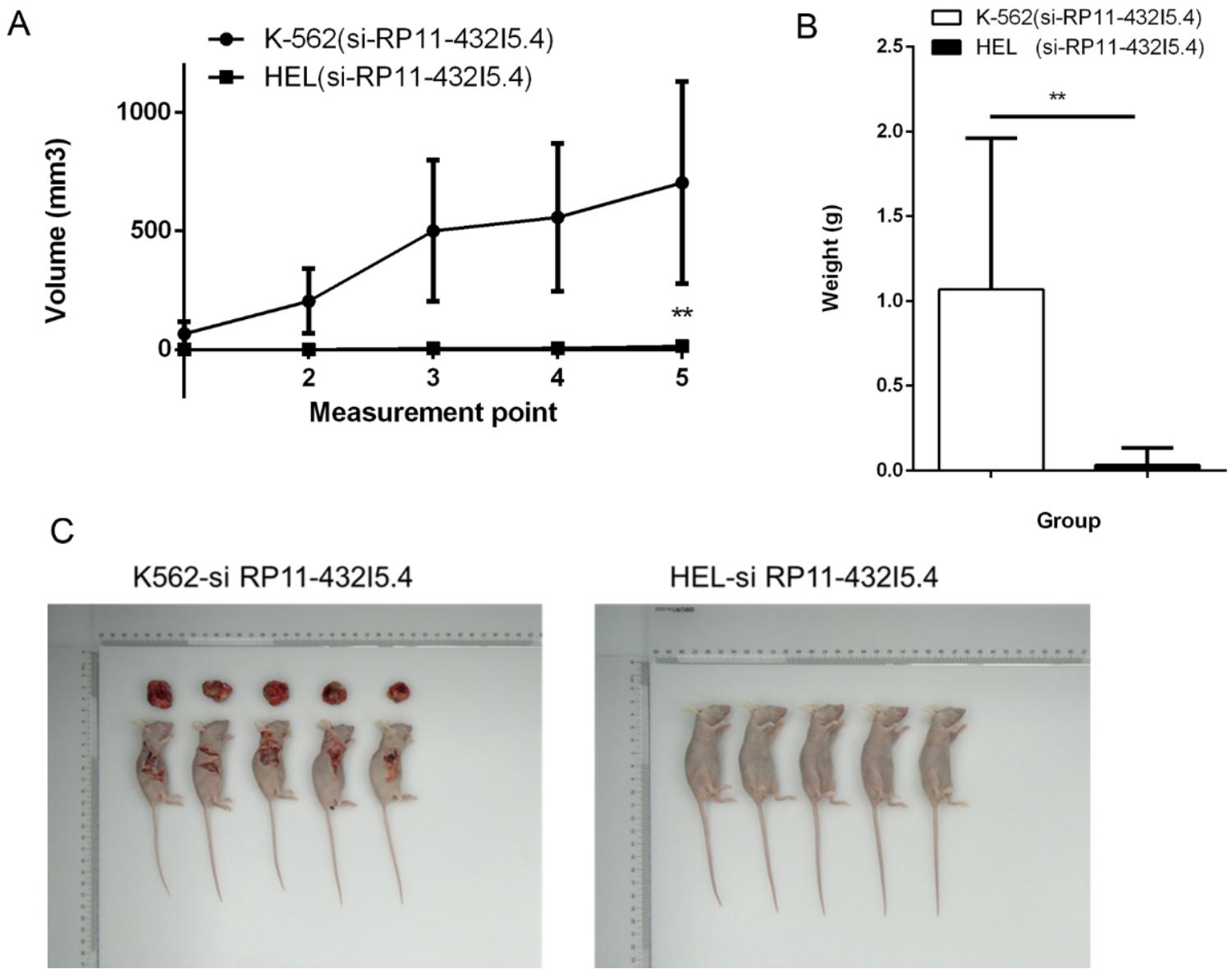


Figure 5

The silence of lncRNARP11-432I5.4 suppresses tumorigenesis in vivo. (A) LncRNA RP11-432I5.4-knockdowned HEL or K562 cells were subcutaneously injected into nude mice and monitoring tumor growth after inoculation. The volumes were calculated by using the following formula: volume = $0.5 \times \text{length} \times \text{width}^2$. (B), (C) The mice were sacrificed on day 34 (bar = 10 mm, **P < 0.01 compared to control group).