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Original Article

Induction of epithelial-mesenchymal transition (EMT) by hypoxia-induced lncRNA *RP11-367G18.1* through regulating the histone 4 lysine 16 acetylation (H4K16Ac) mark

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Abstract: Hypoxia activates various long noncoding RNAs (lncRNAs) to induce the epithelial-mesenchymal transition (EMT) and tumor metastasis. The hypoxia/HIF-1α-regulated lncRNAs that also regulate a specific histone mark and promote EMT and metastasis have not been identified. We performed RNA-sequencing dataset analysis to search for such lncRNAs and lncRNA *RP11-367G18.1* was the hypoxia-induced lncRNA with the highest hazard ratio. High expression of lncRNA *RP11-367G18.1* is correlated with a worse survival of head and neck cancer patients. We further showed that lncRNA *RP11-367G18.1* is induced by hypoxia and directly regulated by HIF-1α in cell lines. Overexpression of lncRNA *RP11-367G18.1* induces the EMT and increases the *in vitro* migration and invasion and *in vivo* metastatic activity. Knockdown experiments showed that lncRNA *RP11-367G18.1* plays an essential role in hypoxia-induced EMT. LncRNA *RP11-367G18.1* specifically regulates the histone 4 lysine 16 acetylation (H4K16Ac) mark that is located on the promoters of two "core" EMT regulators, *Twist1* and *SLUG*, and *VEGF* genes. These results indicate that lncRNA *RP11-367G18.1* regulates the deposition of H4K16Ac on the promoters of target genes to activate their expression. This report identifies lncRNA *RP11-367G18.1* as a key player in regulating the histone mark H4K16Ac through which activates downstream target genes to mediate hypoxia-induced EMT.

Keywords: lncRNA, hypoxia, epithelial-mesenchymal transition, H4K16Ac

Introduction

Long noncoding RNAs (lncRNAs) are more than 200 nucleotides in length and do not have protein-coding sequences [1-4]. Many lncRNAs have been shown to regulate the processes of tumorigenesis and promote tumor progression in various tumor types [5-8]. Different mechanisms including chromatin interactions, protein interactions, and RNA interactions have been demonstrated to mediate the functions of different lncRNAs in the processes of metastasis and tumor progression [8, 9].

Tumor hypoxia has been demonstrated to be one of the key tumor microenvironmental factors that control metastasis and tumor progression [10, 11]. Hypoxia upregulates the hypoxia-inducible factor 1-alpha (HIF-1α) levels through the stabilization of its protein stability [10]. Increased HIF-1α levels contribute to the induction of tumor metastasis in various types of cancer [10, 11]. Among the mechanisms of hypoxia/HIF-1α-induced cancer metastasis, epithelial-mesenchymal transition (EMT) is one of the major mechanisms that control the initial phase of tumor metastasis [12-14]. Various EMT transcription regulators have been shown to regulate EMT [13-15]. Among the EMT regulators, Snail, Twist1, SIUG, ZEB1, and ZEB2 are considered the "core" EMT regulators [16]. Hypoxia/HIF-1α has also been shown to activate the "core" EMT regulators to induce EMT and tumor metastasis [13-15].

Hypoxia has been shown to activate numerous lncRNAs that promote EMT and tumor progression [6, 17-21]. Among the different mechanisms mediated by hypoxia-activated lncRNAs, epigenetic and chromatin regulations remain relatively unknown. Several examples working along this aspect have been shown. For example, HOTAIR simultaneously scaffolds PRC2 and LSD1 complexes to couple H3K27 methylation and H3K4 demethylation together and regulate downstream target gene expression [22]. WT1-AS modulates H3K4 and H3K9 methylation around the TSS of *WT1* gene to regulate its expression [23]. Hypoxia-induced *lncRNA-AK058003* resides upstream of *SNCG* to demethylate CpG islands of the *SNCG* promoter [24]. LncRNA *PVT1* scaffolds a transcription factor and a histone acetyltransferase (KAT2A) to activate the expression of *NF90* [25]. Hypoxia-activated lncRNA *MEG3* recruits DNMT3a, DNMT3b, and MBD1 to facilitate *TIMP2* promoter methylation [26]. LncRNA *GATA6-AS* interacts with LOXL2 to regulate the H3K4me3 histone modification [27]. However, these results mostly described the correlations between histone mark changes and lncRNAs without specifically delineating the molecular mechanisms. In addition, hypoxia-induced lncRNAs that regulate a specific histone mark remain to be identified.

In this discovery, a hypoxia-induced lncRNA *RP11-367G18.1* that activates the H4K16 acetylation (H4K16Ac) histone mark to regulates EMT regulators and target genes (*Twist1, SLUG, VEGF*) induced by hypoxia is identified. LncRNA *RP11-367G18.1* is crucial in the regulation of hypoxia-induced EMT and metastasis. LncRNA *RP11-367G18.1* most likely scaffolds a histone-modifying complex to regulate the crucial target genes induced by hypoxia to mediate EMT and metastasis.

Materials and methods

Cell culture and oxygen deprivation

Human lung cancer cell line H1299, head and neck cancer cell line OECM-1, and embryonic kidney 293 (HEK293) cell line were obtained from the American Type Culture Collection (ATCC, Gaithersburg, MD, USA). Human head and neck cancer cell line FADU, breast cancer cell lines MCF7 and MDA-MB-231, and cervical adenocarcinoma cell line HeLa were obtained from the Bioresource Collection and Research Center (BCRC, Hsinchu, Taiwan). The HEK293T cell line was obtained as described previously [14]. Human tongue squamous cell line SAS and bone osteosarcoma cell line U2-OS were obtained from Dr. Yung-Luen Yu's laboratory (Center for Molecular Medicine, China Medical University, Taichung, Taiwan). All cell lines were cultured in Dulbecco's Modified Eagle's Medium containing 10% fetal bovine serum at 37°C and 5% CO_2. These cell lines were not contaminated with mycoplasma and were analyzed by mycoplasma DNA PCR. To simulate oxygen deprivation conditions, cells were cultured in 1% O_2, 5% CO_2, and 94% N_2 for 18 h.

Public RNA-seq data analysis

Public hypoxia-related RNA-seq data of FADU was downloaded from GSE59990 [28]. After aligning sequenced data to hg19 with hisat2, the abundance of RNA fragments in each gene was counted by ht-seq with the annotation of Esemble v87. Raw counts of each gene were normalized by edgeR, a R package. With 1.5 fold-change cut off, we identified increased gene under hypoxia.

TCGA database analysis

Head and neck cancer TCGA database was analyzed by previous method with GDCRNAtools, a R package [29]. The expression of each tumor sample was normalized by edgeR to Log_2TMM, for downstream analysis or comparison. Significant harzrd ratio with cut off by 0.05 log rank *p*-value. Pan cancer survival analysis was analyzed by web based database GEPIA [30].

Plasmids

The expression constructs pHA-HIF-1α, pHA-HIF-1α (ΔODD), and pHA-HIF-1α (LCLL) containing cDNA encoding the wild-type and mutant HIF-1α with a hemagglutinin (HA) tag were obtained from L. E. Huang (University of Utah) [31]. The expression constructs containing the human lncRNA *RP11-367G18.1* V1 and *RP11-367G18.1* V2 sequences were synthesized by Biotools (Taiwan) and then subcloned into pcDNA3.1 (+) vector (Life Technologies). The expression constructs encoding human Twist1 and SLUG were purchased from GenScript (USA). The details of the construction

of these plasmids are provided in Tables S1 and S2.

Protein and histone extraction and Western blotting

The extraction of proteins and histones from cells and Western blotting were performed as described previously [32, 33]. The antibodies used for Western blotting are listed in Table S3.

RNA extraction and quantitative real-time PCR

RNA isolation, cDNA synthesis, and quantitative real-time PCR were performed as described previously [32, 33]. RNA was isolated using the TRIzol reagent (Invitrogen; Life Technologies, Carlsbad, CA, USA). RNA expression levels were analyzed by quantitative real-time PCR using Fast SYBR™ Green Master Mix (Applied Biosystems). Detailed information for the primers used in the quantitative real-time PCR experiment are shown in Table S4.

Transient transfection and luciferase assays

Lipofectamine 2000 (Invitrogen) and PolyJet (SignaGen) reagents were used for transfection according to the manufacturer's instructions. The reporter constructs containing the promoter region of human lncRNA *RP11-367G18.1* were cloned at the 5' end of the luciferase gene in the pGL3-basic vector (**Figure 2E** and Tables S1 and S2). For the luciferase reporter assay, FADU cells (5×10^5) were seeded onto 6-well plates and then co-transfected with the reporter constructs and different expression vectors under normoxic/hypoxic conditions. After 2 days, the luciferase activity was measured using Dual-Luciferase™ Reporter kit (Promega) and further normalized with the *Renilla* luciferase activity as previously described [14, 32, 34].

Quantitative chromatin immunoprecipitation (qChIP)

The qChIP assay was performed as described previously [32, 33]. Whole cells were cross-linked with 1% formaldehyde, sonicated using Bioruptor, and immunoprecipitated using the antibody. The DNA samples for qChIP assay were quantified using the SYBR Green assay via quantitative real-time PCR. Quantitative real-time PCR data were analyzed using the CT method, and the results are plotted as a percentage of the input DNA. The primers and antibodies used in the qChIP assay are listed in Tables S3 and S5, respectively.

Lentiviral siRNA knockdown experiments

Lentivirus was produced as described previously [32, 33]. The shRNA targeting lncRNA *RP11-367G18.1* (#1 and #2) and *RP11-367G18.1* V2 (#1 and #2) were designed and purchased from Biotools (Taiwan; Table S6) and subcloned into a pLV2-U6-Puro lentiviral vector. The sequence and clonal names of the pLKO-scrambled or pLKO plasmids targeting HIF-1α are listed in Table S6. The pLKO plasmid and the packaging plasmids pCMVΔR8.91 and pMD.G were provided by the National RNAi Core Facility, Academia Sinica, Taiwan. The virus was isolated 24-48 h after transfection into HEK293T cells. To generate lncRNA *RP11-367G18.1*, *RP11-367G18.1* V2, or HIF-1α-knockdown stable cell lines, OECM-1, SAS, H1299, MDA-MB-231, FADU, or MCF7 cells were infected with the virus for 24 h and were subsequently selected using antibiotics for 2 weeks.

Single-molecule RNA fluorescence in situ hybridization (RNA-FISH)

Single-molecule RNA-FISH was performed as described previously with minor modifications [35, 36]. Cells were fixed with 4% formaldehyde for 15 min at room temperature. After rinsing with PBS, the fixed cells were treated with 1% pepsin and dehydrated with a gradient of 70% to 100% ethanol. The cells were then incubated in a hybridization buffer [100 mg/ml dextran sulfate, 0.2 mg/ml bovine serum albumin, and 10% formamide in 2 × saline-sodium citrate buffer (SSC)] with RNA-FISH probes and incubated for 4 h at 55°C in the dark. Cells were washed three times with SSC and subsequently mounted using ProLong Gold Antifade Mountant with DAPI (Invitrogen). The RNA-FISH probes targeting *RP11-367G18.1* were designed and obtained from LGC Biosearch Technologies. The Stellaris RNA-FISH probes consisted of 20 fluorescein-conjugated probes to *RP11-367G18.1* and are listed in Table S7.

For RNA-FISH double staining, cells were incubated with an antibody against HIF-1α (1:200 dilution; Abcam) at 4°C overnight. After hybrid-

ization, cells were rinsed with TBST thrice and then incubated with the secondary antibody (1:500 dilution; goat anti-rabbit Alexa488, Abcam). The samples were then mounted using ProLong Gold Antifade Mountant with DAPI. Images taken using ImageXpress Micro Confocal System (Molecular Devices) were visualized and quantified by the MetaXpress High-Content Image Acquisition and Analysis software.

Migration and invasion assays

Millicell tissue culture plate well inserts (Millipore) and BD BioCoat Matrigel Invasion Chambers (Becton Dickson) were used for migration (3×10^4 cells/well) and invasion (5×10^4 cells/well) assays, which were performed using 24-well plates for 12 and 20 h, respectively, as described previously [14, 32, 34].

Soft agar colony formation assay

First, 1.5 mL of culture medium containing 0.5% agar was plated in a 6-well plate. Next, five thousand cells of each clone were suspended in 1 ml of culture medium containing 0.3% agar and seeded onto the bottom layer of agar and incubated at 37°C. To prevent desiccation, 200 µl of fresh medium was added to the plates every three days. After 14 days, these cells were stained with 0.0005% crystal violet. The colonies with diameters larger than 0.1 mm were counted under a light microscope.

Xenograft tumorigenicity assay

In this study, all animal experiments were carried out with the approval of the Institutional Animal Care and Use Committee of China Medical University. Five-week-old BALB/c nu/nu mice (National Science Council Animal Center, Taipei, Taiwan) were used, and 2×10^6 cells were subcutaneously injected. After implantation at 30-35 days, the mice were euthanized and the tumor incidence was monitored [32]. At least 5 mice per experimental group were used in the study.

In vivo tail vein/orthotopic metastatic assays

Metastatic assays in male non-obese diabetic/severe combined immunodeficiency mice (NOD-SCID mice, National Science Council Animal Center, Taipei, Taiwan) were performed as described previously [14, 34], and each group of experiments contained at least five mice. Briefly, FADU cells were implanted into 6-week-old NOD-SCID mice via tail vein (1×10^6 cells) or orthotopic (1×10^5 cells) injection. Animals were humanely killed at 9-14 weeks after implantation, and the metastatic nodules in the lungs were analyzed using a dissecting microscope and quantified. All animal experiments in current study were performed under protocols by the Institutional Animal Care and Use Committee of China Medical University.

Statistical analysis

Except as otherwise indicated, all samples were triplicated. For the *in vitro* study, each experiment was conducted at least thrice. Error bars depict the standard deviation (SD) of data. Differences between two groups of independent samples were compared using Student's *t*-test. A *P* value less than 0.05 was defined as statistically significant differences.

Results

RNA-seq dataset analysis identifies a new lncRNA RP11-367G18.1

To identify hypoxia-regulated and expressed long-intergenic RNAs (lincRNAs) ($\log_2$TMM > 2), RNA-seq analysis of a public database GSE59990 was performed [28]. After the filtering of non-expressed lincRNAs, there were 187 expressed lincRNAs (cut off by $\log_2$TMM > 2). Most of expressed lincRNAs were regulated by hypoxia with the comparison of increased gene expression (n=78, cut off by $\log_2$ (1.5) fold change) and decreased gene expression (n=13, cut off by -$\log_2$ (1.5) fold change) (**Figure 1A**). We selected the top 10 lncRNAs with highest expression ratio (hypoxia vs. normoxia) (**Figure 1B**). To focus on a lncRNA that has clinical significance, we examined the hazard ratio of each lncRNA from TCGA datasets and the result showed that lncRNA *RP11-367G18.1* had the highest hazard ratio from the head and neck cancer dataset (**Figure 1C**). Real-time PCR analysis showed the induction of lncRNA *RP11-367G18.1* expression in eight cell lines by hypoxia (**Figure 1D**). We further performed survival analysis using the head and neck cancer TCGA dataset and indeed patients with high lncRNA *RP11-367G18.1* expression had a sig-

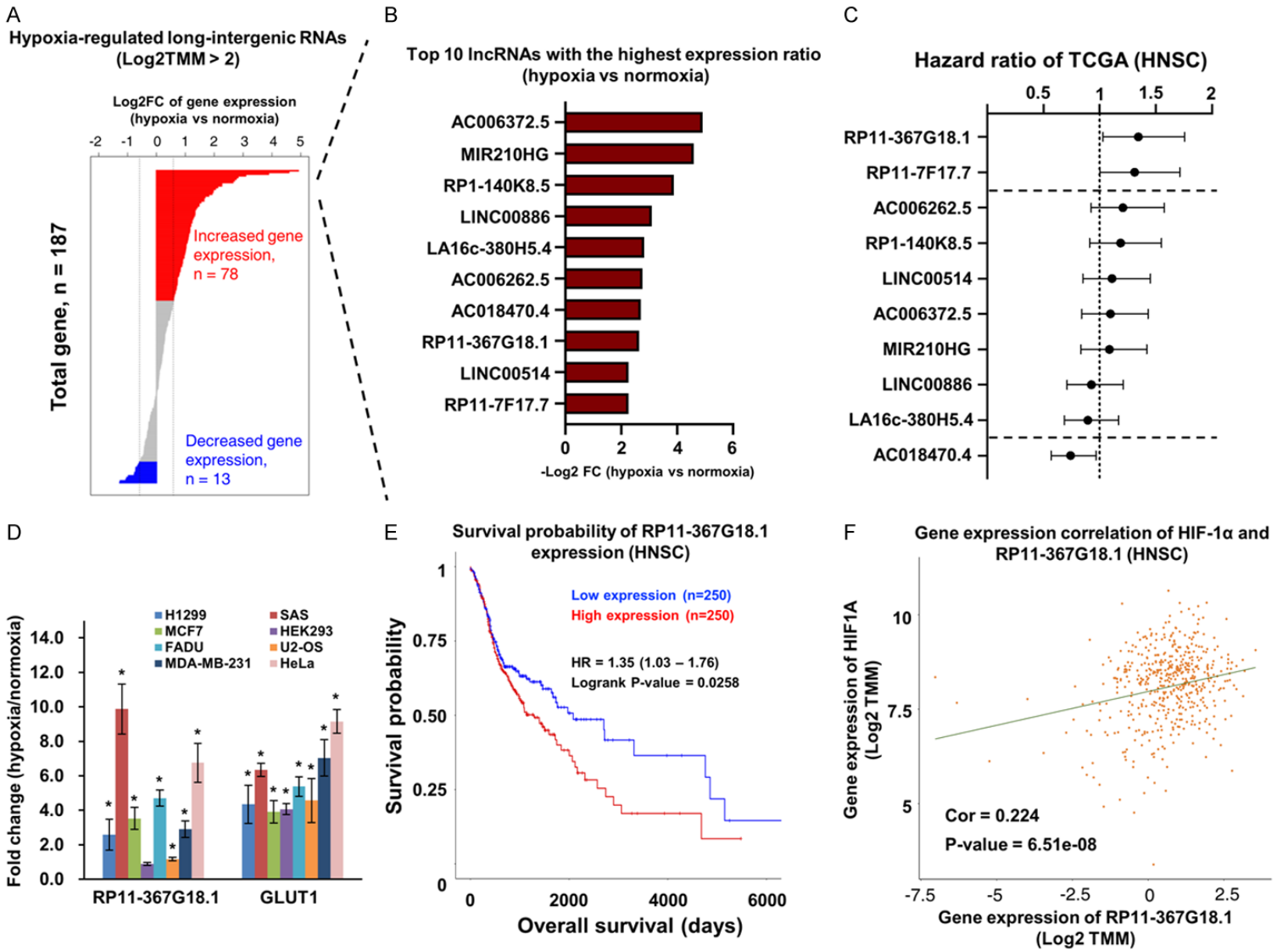

A
Hypoxia-regulated long-intergenic RNAs
(Log2TMM > 2)
Log2FC of gene expression
(hypoxia vs normoxia)
-2 -1 0 1 2 3 4 5
Total gene, n = 187
Increased gene expression, n = 78
Decreased gene expression, n = 13
B
Top 10 lncRNAs with the highest expression ratio
(hypoxia vs normoxia)
AC006372.5
MIR210HG
RP1-140K8.5
LINC00886
LA16c-380H5.4
AC006262.5
AC018470.4
RP11-367G18.1
LINC00514
RP11-7F17.7
0 2 4 6
-Log2 FC (hypoxia vs normoxia)
C
Hazard ratio of TCGA (HNSC)
0.5 1 1.5 2
RP11-367G18.1
RP11-7F17.7
AC006262.5
RP1-140K8.5
LINC00514
AC006372.5
MIR210HG
LINC00886
LA16c-380H5.4
AC018470.4
D
H1299
SAS
MCF7
HEK293
FADU
U2-OS
MDA-MB-231
HeLa
Fold change (hypoxia/normoxia)
14.0 12.0 10.0 8.0 6.0 4.0 2.0 0.0
RP11-367G18.1
GLUT1
E
Survival probability of RP11-367G18.1
expression (HNSC)
Low expression (n=250)
High expression (n=250)
HR = 1.35 (1.03 – 1.76)
Logrank P-value = 0.0258
Survival probability
1 0.75 0.50 0.25 0
0 2000 4000 6000
Overall survival (days)
F
Gene expression correlation of HIF-1α and
RP11-367G18.1 (HNSC)
Gene expression of HIF1A
(Log2 TMM)
10 7.5 5
Cor = 0.224
P-value = 6.51e-08
-7.5 -5 -2.5 0 2.5
Gene expression of RP11-367G18.1
(Log2 TMM)

Figure 1. Identification of the role of lncRNA *RP11-367G18.1* in tumorigenesis and its induction by hypoxia. A. Analysis of RNA-seq database (GSE59990) showed that 187 lncRNAs were highly expressed in FADU cells under hypoxic condition. B. Ten lncRNAs with the highest expression ratio (hypoxia vs. normoxia) were selected for further study. C. Comparing the hazard ratio of the top ten lncRNAs showed that lncRNA *RP11-367G18.1* had the highest hazard ratio. D. Real-time PCR analysis showed that hypoxia induced the expression of lncRNA *RP11-367G18.1* in eight different cell lines. The asterisk (*) indicated the fold of increase above two fold ($P<0.05$) in cell lines induced by hypoxia compared to non-induced cell lines. E. Analysis of the survival from the head and neck cancer TCGA dataset showed that patients with high lncRNA *RP11-367G18.1* expression had a worse overall survival. F. A positive significant correlation between HIF-1α and lncRNA *RP11-367G18.1*l expression was shown from TCGA dataset. HNSCC: head and neck squamous cell carcinoma.

nificantly worse overall survival (**Figure 1E**). TCGA analysis of aggregates of all cancers also showed a significantly worse survival of patient with high expression of lncRNA *RP11-367G18.1* (Figure S1). In addition, there was a positive significant correlation between the gene expression of HIF-1α and lncRNA *RP11-367G18.1* by analyzing the gene expression levels from the head and neck cancer TCGA dataset (**Figure 1F**). From the above results, we further focused our studies on lncRNA *RP11-367G18.1.*

Hypoxia activates the expression of lncRNA RP11-367G18.1

Due to the implication of lncRNA *RP11-367G18.1* in tumorigenesis (**Figures 1C**, **1E**, S1) and the role of hypoxia in mediating tumor progression in various tumor types [10, 11, 14], the possible regulation of lncRNA *RP11-367G18.1* by hypoxia was tested. Real-time PCR analysis showed that hypoxia activated the expression of lncRNA *RP11-367G18.1* in two different cell lines (**Figure 2A**). Knocking down HIF-1α in two different cell lines with high HIF-1α expression decreased the expression of lncRNA *RP11-367G18.1* using real-time PCR analysis (**Figure 2B**). Overexpressing a HIF-1α or HIF-1α (ΔODD) (a HIF-1α expression vector containing deletion of the oxygen-dependent degradation domain (ODD)) [31] activated the expression of lncRNA *RP11-367G18.1* (**Figure 2C**). In two hypoxia-induced cell lines undergoing HIF-1α knockdown, hypoxia did not induce the expression of lncRNA *RP11-367G18.1*, indicating that HIF-1α was the major transcription factor regulating lncRNA *RP11-367G18.1* expression (**Figure 2D**).

Activation of lncRNA RP11-367G18.1 by hypoxia is directly regulated by HIF-1α

To examine whether the expression of lncRNA *RP11-367G18.1* was directly activated by HIF-1α, different luciferase reporter constructs that contained the promoter of lncRNA *RP11-367G18.1* were generated (**Figure 2E**) [14, 32]. In the proximal promoter of lncRNA *RP11-367G18.1,* a putative hypoxia response element (HRE) (-416 to -412 bp upstream of the transcription start site of the lncRNA *RP11-367G18.1* gene; sequence: ACGTG) was identified (**Figure 2E**). The HRE mutant (change from ACGTG to ACTGT) of the reporter construct was also generated. Luciferase reporter gene assays were performed by transfecting the wild-type vs. mutant reporter construct together with a HIF-1α expression vector demonstrated the response of wild-type HRE, but not the mutated HRE, to HIF-1α activation (**Figure 2E**). Only the reporter construct containing the wild-type HRE responded to the activation by a constitutively active HIF-1α (ΔODD) (**Figure 2E**). The presence of HRE identified in the proximal promoter of lncRNA *RP11-367G18.1* was also confirmed by quantitative chromatin immunoprecipitation (qChIP) assays in two different cell lines (**Figure 2F**). All the results described above indicate that HIF-1α directly activated the expression of lncRNA *RP11-367G18.1*.

Knockdown of lncRNA RP11-367G18.1 decreases tumorigenesis and in vitro migration/invasion activity

To test the ability of lncRNA *RP11-367G18.1* in enhancing tumorigenesis, the tumor volume was decreased in OECM-1 cells undergoing knockdown of lncRNA *RP11-367G18.1* using nude mice xenograft assays (**Figure 3A**, **3B**). The *in vitro* migration/invasion activity in OECM-1 and SAS cell lines was decreased through knocking down lncRNA *RP11-367G18.1* (**Figure 3A**, **3C**). Finally, the *in vitro* migration/invasion activity of FADU cells overexpressing a constitutively active HIF-1α (ΔODD) construct was decreased through knocking down lncRNA *RP11-367G18.1* (**Figure 3D**). These above results indicate the role of lncRNA *RP11-*

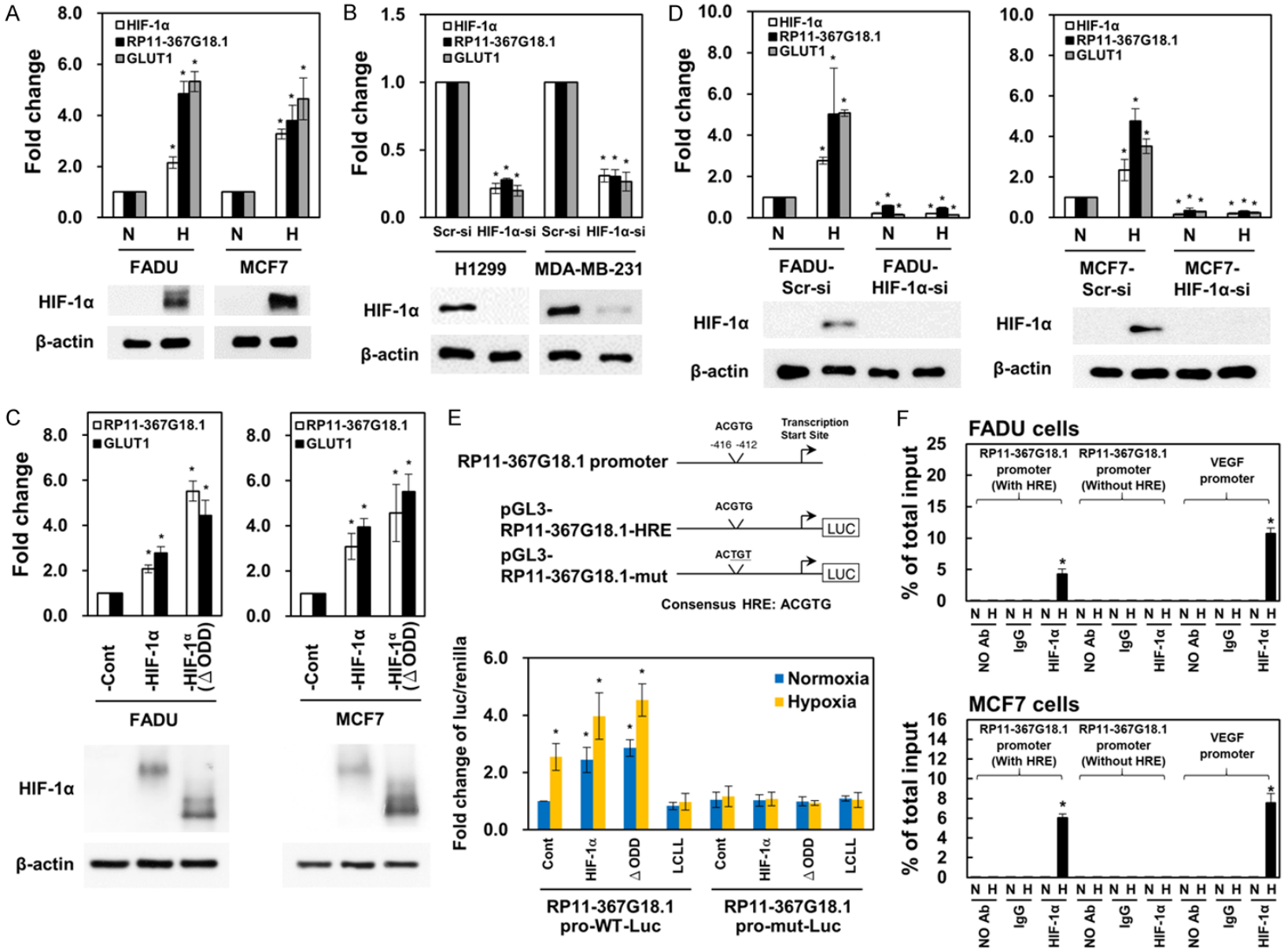
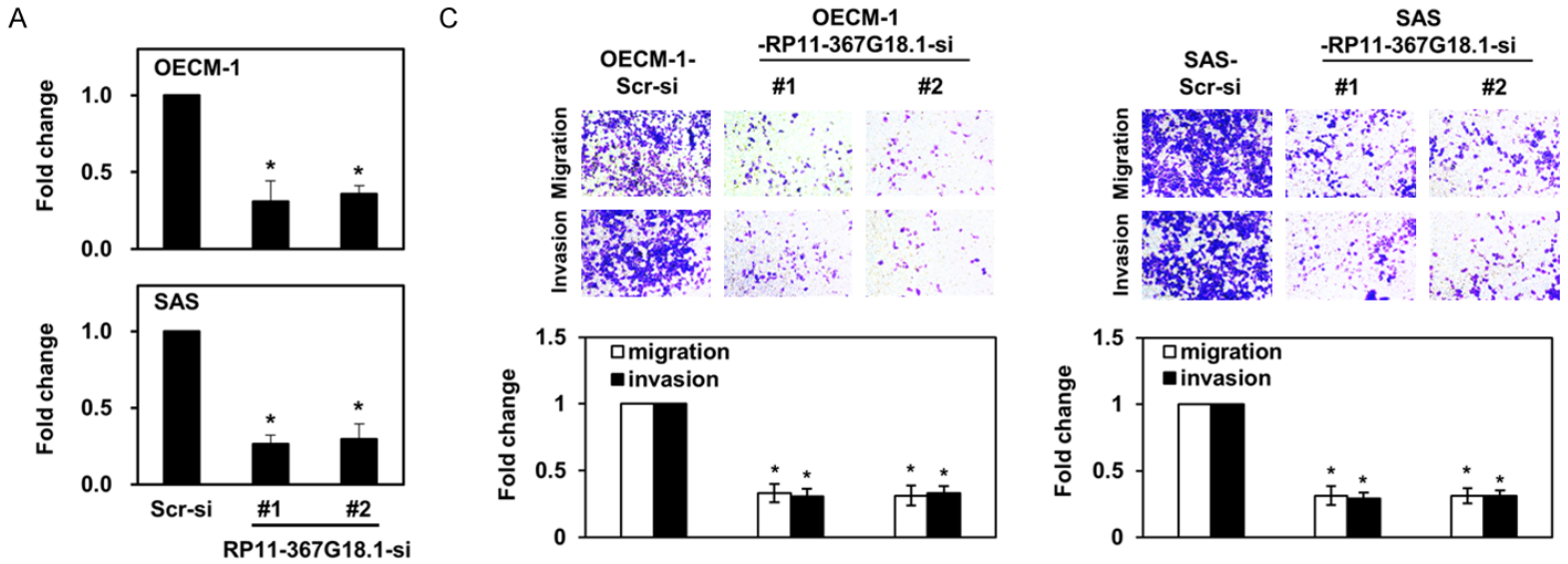

A
Fold change
HIF-1α
RP11-367G18.1
GLUT1
N H N H
FADU MCF7
HIF-1α
β-actin
B
Fold change
Scr-si HIF-1α-si Scr-si HIF-1α-si
H1299 MDA-MB-231
D
FADU-Scr-si FADU-HIF-1α-si
MCF7-Scr-si MCF7-HIF-1α-si
C
-Cont -HIF-1α -HIF-1α (ΔODD)
FADU MCF7
E
RP11-367G18.1 promoter
ACGTG
-416 -412
Transcription Start Site
pGL3-RP11-367G18.1-HRE
pGL3-RP11-367G18.1-mut
ACTGT
LUC
Consensus HRE: ACGTG
Fold change of luc/renilla
Normoxia
Hypoxia
Cont HIF-1α ΔODD LCLL Cont HIF-1α ΔODD LCLL
RP11-367G18.1 pro-WT-Luc
RP11-367G18.1 pro-mut-Luc
F
FADU cells
% of total input
RP11-367G18.1 promoter (With HRE)
RP11-367G18.1 promoter (Without HRE)
VEGF promoter
NO Ab IgG HIF-1α
MCF7 cells

Figure 2. Direct regulation of lncRNA *RP11-367G18.1* expression by hypoxia/HIF-1α. A. Real-time PCR analysis showed that hypoxia activated the expression of lncRNA *RP11-367G18.1* expression in two different cell lines. B. Real-time PCR analysis showed that knockdown of HIF-1α decreased expression of lncRNA *RP11-367G18.1* in two different cell lines. C. Real-time PCR analysis showed that overexpression of a wild-type HIF-1α or a constitutively active HIF-1α (ΔODD) vector activated lncRNA *RP11-367G18.1* expression in two different cell lines. D. Real-time PCR analysis showed that knockdown of HIF-1α abolished the activation of lncRNA *RP11-367G18.1* induced by hypoxia in two different cell lines. E. Reporter gene assays identified the HRE site responsive to hypoxia in the lncRNA *RP11-367G18.1* promoter through constructing reporter gene constructs containing wild type and HRE-mutated promoter of lncRNA *RP11-367G18.1*. F. Direct binding of HIF-1α to the HRE located in the proximal promoter of lncRNA *RP11-367G18.1 was confirmed by qChIP assays* in two different cell lines. The asterisk (*) indicated statistical significance ($P<0.05$) between experimental and control clones/transfections (between hypoxia and normoxia-A, D; between control and HIF-1α knockdown-B, D; between control and HIF-1α (ΔODD) expression-C; between transfections of different expression vectors-E; between qChIP assays-F). N, normoxia; H, hypoxia.



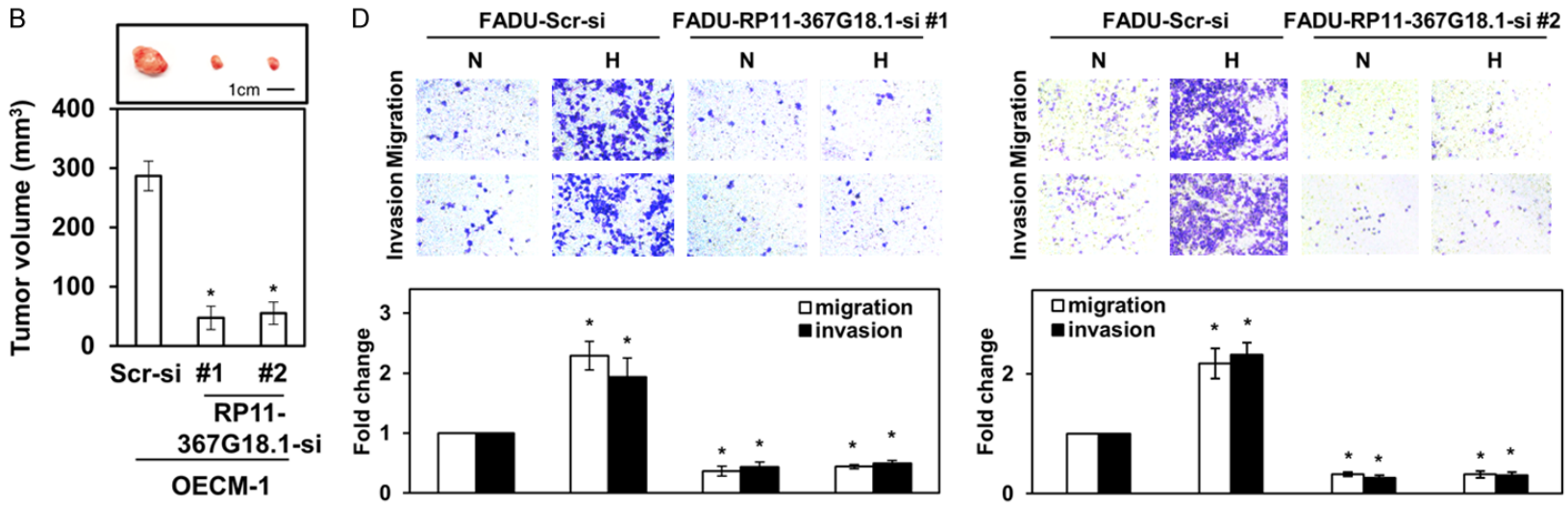


Figure 3. Knocking down lncRNA *RP11-367G18.1* decreased the tumor volume as well as the i*n vitro* migration/invasion activity in various cell lines. A, B. Knockdown of lncRNA *RP11-367G18.1* decreased the tumor volume in xenografted OECM-1 cells. C. Knocking down lncRNA *RP11-367G18.1* decreased the *in vitro* migration and invasion activity in two different cell lines. D. Hypoxia induced the *in vitro* migration and invasion activity in FADU cells, which was abolished through knockdown of lncRNA *RP11-367G18.1.* Migration and invasion assays were performed in Transwell inserts for 12 or 20 h, respectively. N, normoxia; H, hypoxia. The asterisk (*) indicated statistical significance ($P<0.05$) between experimental and control clones.

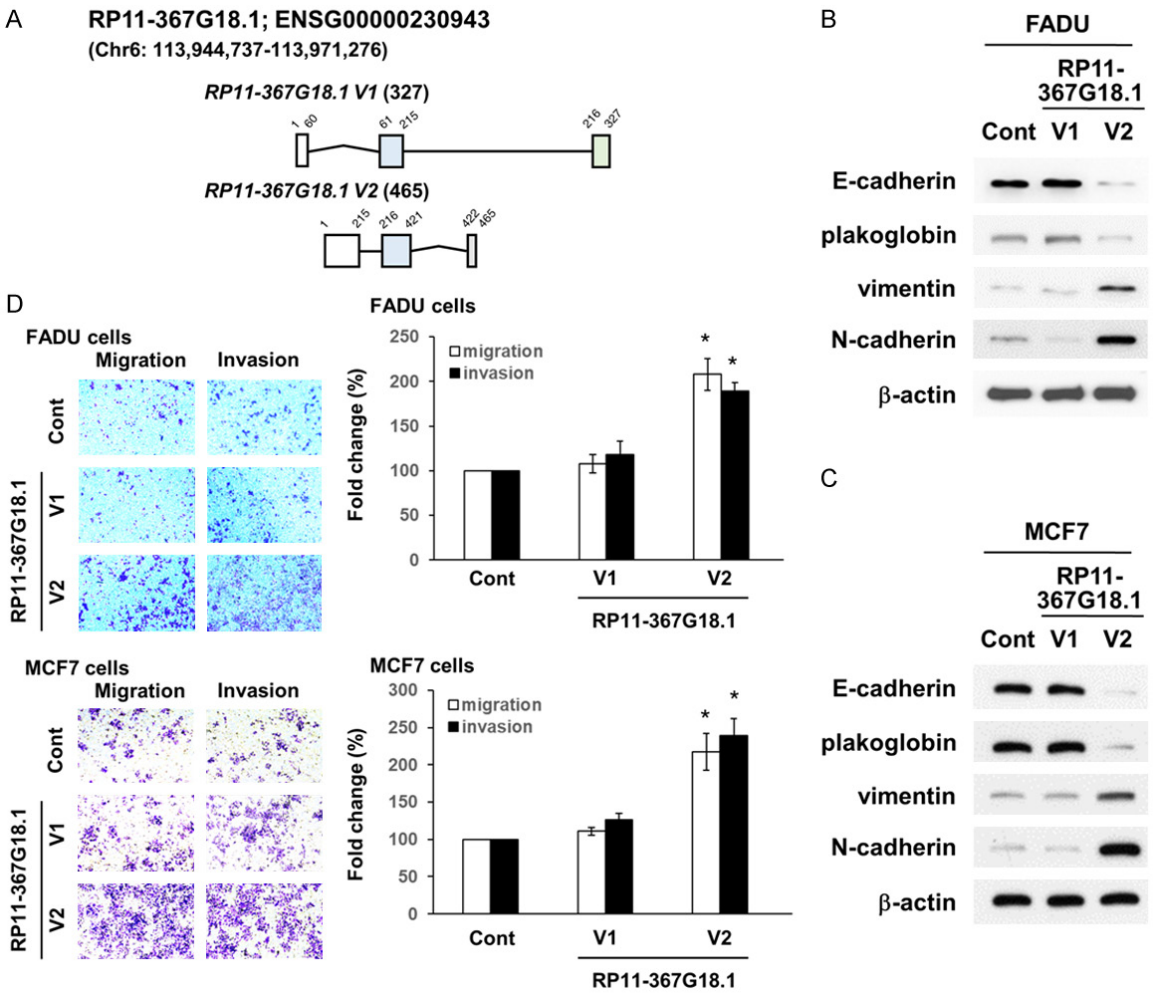


Figure 4. Annotation of lncRNA *RP11-367G18.1* and overexpression of lncRNA *RP11-367G18.1* V2 version induced the epithelial-mesenchymal transition (EMT) and increased the *in vitro* migration/invasion activity. (A) Annotation of lncRNA *RP11-367G18.1* showed that there are two versions. (B, C) Overexpression of lncRNA *RP11-367G18.1* V2 version in FADU (B) or MCF7 (C) cells induced the EMT. (D) Overexpression of lncRNA *RP11-367G18.1* V2 version increased the *in vitro* migration/invasion activity in two different cell lines. Migration and invasion assays were performed in Transwell inserts for 12 or 20 h, respectively. The asterisk (*) indicated statistical significance ($P<0.05$) between experimental and control clones.

367G18.1 in enhancing tumorigenic activity and *in vitro* metastatic activity.

The crucial role of the lncRNA RP11-367G18.1 V2 version in regulating the epithelial-mesenchymal transition (EMT)

Since the *in vitro* migration/invasion activity may be linked to the epithelial-mesenchymal transition (EMT), we tested the ability of lncRNA *RP11-367G18.1* in inducing EMT. From the annotation of lncRNA *RP11-367G18.1,* there are two versions (**Figure 4A**). We generated expression vectors from the different versions of lncRNA *RP11-367G18.1* and transfected them individually into FADU and MCF7 cells. Western blot analysis showed the induction of EMT in these two cell lines by overexpression of the V2 version of lncRNA *RP11-367G18.1* (**Figure 4B**, **4C**). Overexpression of the V2 version of lncRNA *RP11-367G18.1* also increased the *in vitro* migration/invasion activity in these two cell lines, consistent with its ability to induced EMT (**Figure 4D**). To further confirm the crucial role of lncRNA *RP11-367G18.1* in regulating EMT, we knockdown lncRNA *RP11-367G18.1* in two head and neck cancer cell lines (OECM-1 and SAS) and the results showed the reversion of EMT marker expression (**Figure 5A-C**) and a significant decrease in the *in vitro* migration and invasion activity of these two cell lines (**Figure 5D**, **5E**). All the above results sup-

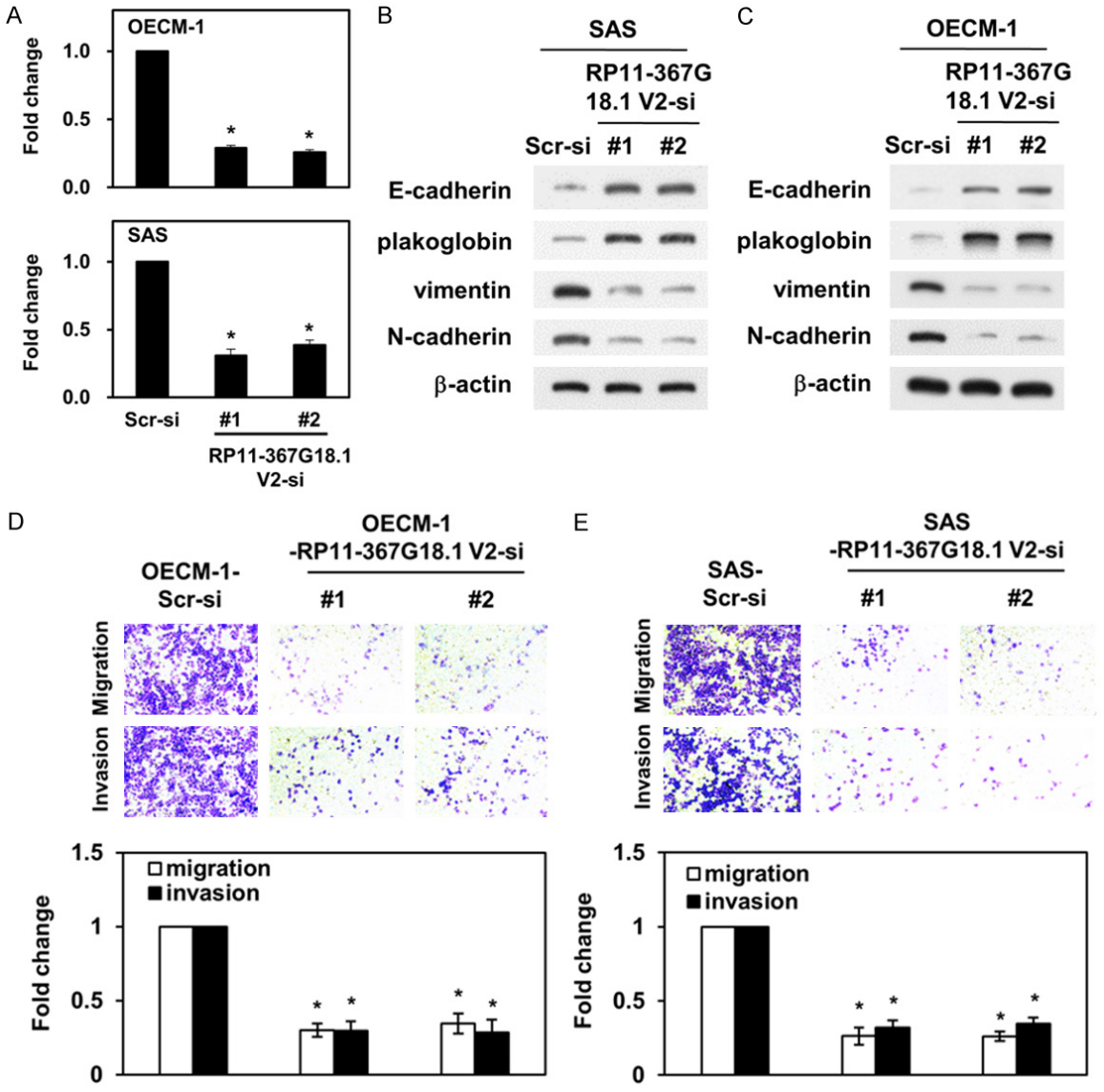


Figure 5. Knockdown of lncRNA *RP11-367G18.1* reversed the EMT phenotypes of OECM-1 and SAS cells and decreased the *in vitro* migration and invasion activity. A. Real-time PCR assays showed the decreased mRNA levels of lncRNA *RP11-367G18.1* through knockdown using two different shRNAs. B, C. Knockdown of lncRNA *RP11-367G18.1* reversed the mesenchymal gene expression of OECM1 and SAS cells. D, E. Knockdown of lncRNA *RP11-367G18.1 significantly* decreased the *in vitro* migration/invasion activity of OECM-1 and SAS cells. Migration and invasion assays were performed in Transwell inserts for 12 or 20 h, respectively. The asterisk (*) indicated statistical significance ($P<0.05$) between experimental and control clones.

port the crucial role of lncRNA *RP11-367G18.1* in the regulation of EMT.

The crucial role of lncRNA RP11-367G18.1 in HIF-1α or hypoxia-induced EMT and metastatic phenotypes

To test the role of lncRNA *RP11-367G18.1* in hypoxia or HIF-1α-induced EMT, Western blot analysis was performed and the results showed that knockdown of lncRNA *RP11-367G18.1* V2 version reversed the EMT phenotypes in FADU cells that overexpressed a constitutively active HIF-1α (ΔODD) vector (**Figures 6A**, S2A). The *in vitro* migration and invasion activity in FADU cells that overexpressed a constitutively active HIF-1α (ΔODD) vector was also significantly decreased through knockdown of the V2 version of lncRNA *RP11-367G18.1* (**Figures 6B**, S2B). We further tested the role of lncRNA

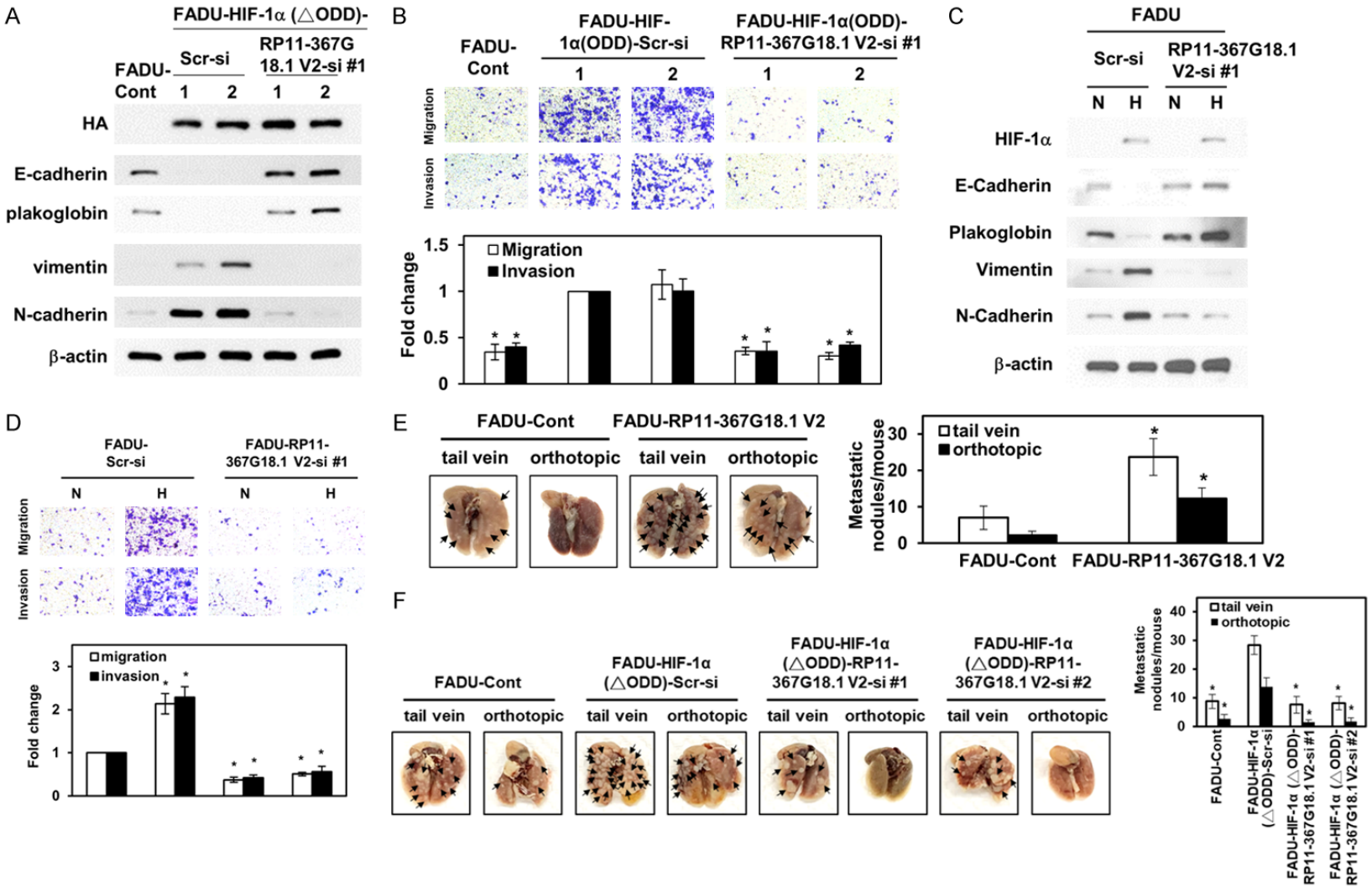


Figure 6. Knockdown of lncRNA *RP11-367G18.1* reversed the EMT phenotypes of FADU cells overexpressing a constitutively active HIF-1α mutant or under hypoxic treatment and decreased the *in vitro* migration and invasion and *in vivo* metastatic activity. A. Knockdown of lncRNA *RP11-367G18.1* reversed the EMT marker gene expression of FADU cells overexpressing a constitutively active HIF-1α mutant. B. Knockdown of lncRNA *RP11-367G18.1* decreased the in vitro migration and invasion activity of FADU cells overexpressing a constitutively active HIF-1α mutant. C. Knockdown of lncRNA *RP11-367G18.1* reversed the EMT marker gene expression of FADU cells undergoing hypoxia. D. Knockdown of lncRNA *RP11-367G18.1* decreased the in vitro migration and invasion activity of FADU cells undergoing hypoxia. E. Overexpression of lncRNA *RP11-367G18.1* in FADU cells increased the in vivo metastatic activity. F. Knockdown of lncRNA *RP11-367G18.1* decreased the *in vivo* metastatic activity of FADU cells overexpressing a constitutively active HIF-1α mutant. Migration and invasion assays were performed in Transwell inserts for 12 or 20 h, respectively. The asterisk (*) indicated statistical significance ($P<0.05$) between experimental and control clones.

RP11-367G18.1 V2 version in hypoxia-induced EMT and the results showed knocking down lncRNA *RP11-367G18.1* V2 version in hypoxic FADU cells abolished hypoxia-induced EMT (**Figures 6C**, S2C). The *in vitro* migration and invasion activity of hypoxic FADU cells were also decreased through knockdown of lncRNA *RP11-367G18.1* V2 version (**Figures 6D**, S2D). Similar results (regulation of EMT markers and *in vitro* migration/invasion activity) were obtained when knockdown of lncRNA *RP11-367G18.1* V2 version was performed in hypoxic MCF7 cells (Figure S2E, S2F). To test the ability of lncRNA *RP11-367G18.1* V2 version to induce *in vivo* metastatic activity, we further overexpressed lncRNA *RP11-367G18.1* V2 version in FADU cells and showed the significant increase in *in vivo* metastatic activity by tail vein injection and orthtopic implantation assays (**Figure 6E**). Knocking down lncRNA *RP11-367G18.1* V2 version significantly decreased the *in vivo* metastatic activity in FADU cells that overexpressed a constitutively active HIF-1α (ΔODD) vector (**Figure 6F**). These results indicate that lncRNA *RP11-367G18.1* plays a crucial role in hypoxia-induced EMT and *in vitro/in vivo* metastatic activity.

Enhancement of tumorigenicity activity by LncRNA RP11-367G18.1

To test the ability of lncRNA *RP11-367G18.1* V2 version to increase tumorigenicity activity, we overexpressed lncRNA *RP11-367G18.1* V2 version in FADU cells and indeed it was able to increase the soft agar colony formation activity (**Figure 7A**). Knockdown of lncRNA *RP11-367G18.1* V2 version significantly decreased the soft agar colony formation activity in FADU cells that overexpressed a constitutively active HIF-1α (ΔODD) vector (**Figure 7B**). Tumor xenograft experiments in nude mice using the same approaches described above also showed the ability of lncRNA *RP11-367G18.1* V2 version to induce the tumor volume of xenografted FADU cells (**Figure 7C**), whereas knockdown of lncRNA *RP11-367G18.1* V2 version in FADU cells that overexpressed a constitutively active HIF-1α (ΔODD) vector significantly decreased the tumor volume (**Figure 7D**). To examine the colocalization of HIF-1α and lncRNA *RP11-367G18.1* V2 version in xenografted tumors, immunofluorescence staining showed the colocalization of these two molecules (**Figure 7E**). We further checked the putative molecules (c-Myc, cyclin D1) [37-39] that contribute to the increased tumor volume induced by lncRNA *RP11-367G18.1* V2 version. Western blot analysis showed that knockdown of lncRNA *RP11-367G18.1* V2 version in OECM-1 and SAS cells decreased the protein levels of c-Myc and cyclin D1 (**Figure 7F**). We further examined the mRNA levels of *c-Myc* and *cyclin D1* from xenografted tumors and real-time PCR analysis showed the decreased *c-Myc* and *cyclin D1* mRNA levels in tumors from xenografted OCEM-1 and SAS cells undergoing knockdown of lncRNA *RP11-367G18.1* V2 version (**Figure 7G**, **7H**), indicating that modulation of the c-Myc and cyclin D1 levels by lncRNA *RP11-367G18.1* V2 version regulates the tumor volume.

LncRNA RP11-367G18.1 regulates the histone 4 lysine 16 acetylation (H4K16Ac) mark and certain hypoxia-induced target genes

Since lncRNAs are able to scaffold a chromatin modifying complex to regulate chromatin [4, 8, 9, 22], knockdown of lncRNA *RP11-367G18.1* V2 version was performed followed by examination of the protein levels of various histone marks. Western blot analysis showed that the levels of H4K16 acetylation (H4K16Ac) histone mark were decreased through knockdown of lncRNA *RP11-367G18.1* V2 version in two different cell lines (**Figure 8A**). This result was confirmed by overexpression of lncRNA *RP11-367G18.1* V2 version that led to the activation of H4K16Ac histone mark (**Figure 8B**). In addition, knockdown of lncRNA *RP11-367G18.1* V2 version in hypoxic FADU and MCF7 cells significantly abolished the activation of H4K16Ac induced by hypoxia (**Figures 8C**, S3A). We further tested the downstream target genes induced by hypoxia that could be regulated by lncRNA *RP11-367G18.1* V2 version. Real-time PCR analysis showed that knockdown of lncRNA *RP11-367G18.1* V2 version significantly decreased the mRNA levels of *Twist1, SLUG,* and *VEGF*, indicating that these genes were regulated by lncRNA *RP11-367G18.1* V2 version (**Figure 8D**). Knockdown of lncRNA *RP11-367G18.1* V2 version also abolished the increased Twist1 and SLUG protein levels induced by hypoxia in two cell lines (**Figures 8E**, S3B). Overexpression of lncRNA *RP11-367G18.1* V2 version activated the Twist1 and SLUG protein levels by Western blot analysis (**Figure 8F**). In

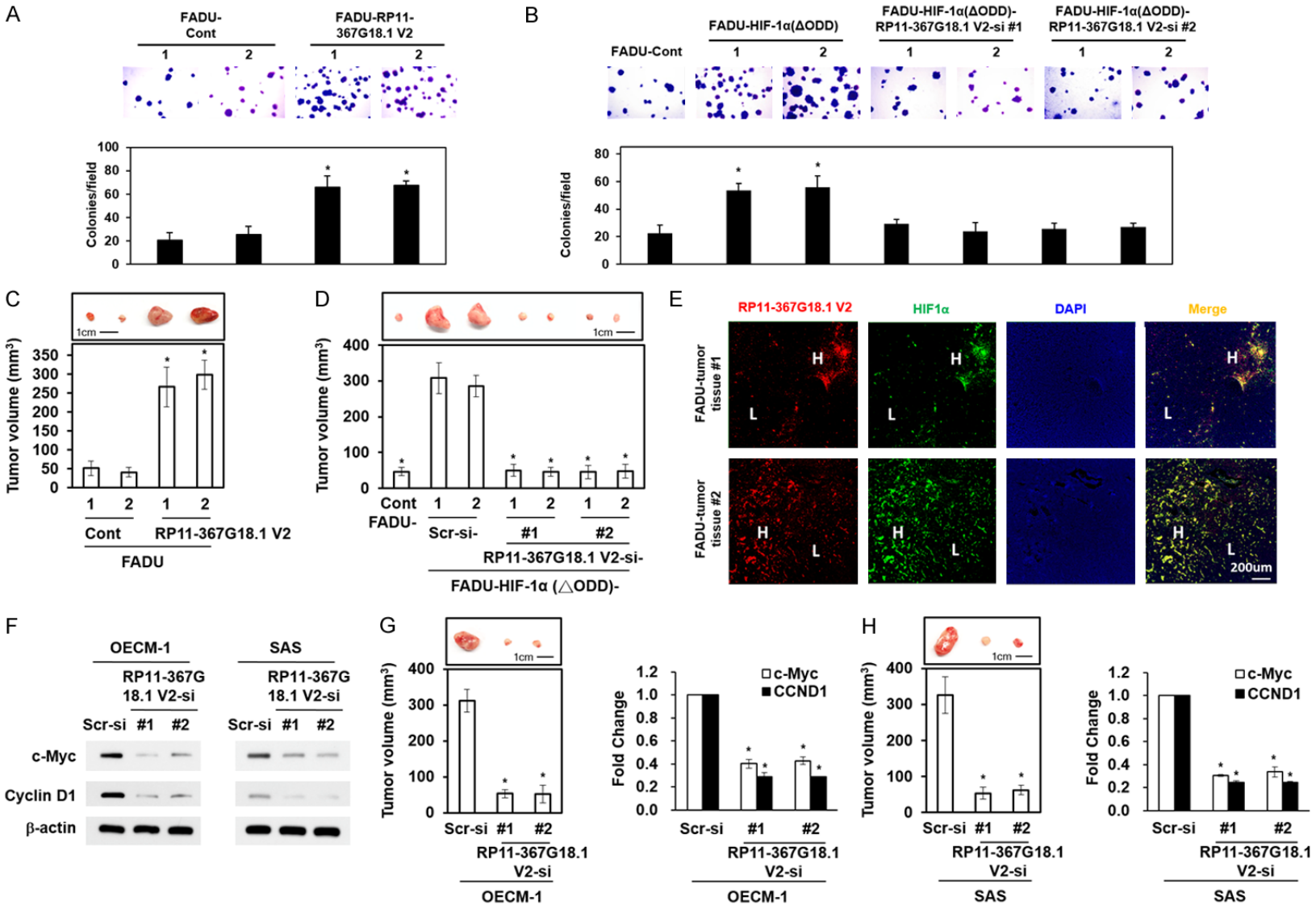


Figure 7. Enhancement of tumorigenicity of FADU cells by lncRNA *RP11-367G18.1* V2 version, co-localization of lncRNA *RP11-367G18.1* V2 version and HIF-1α, and the regulation of c-Myc and cyclin D1 expression by lncRNA *RP11-367G18.1* V2 version. (A) Increase in soft agar colony formation activity by overexpression of lncRNA *RP11-367G18.1* V2 version in FADU cells. (B) Knockdown of lncRNA *RP11-367G18.1* decreased the soft agar colony formation activity of FADU cells overexpressing a constitutively active HIF-1α mutant. (C) Overexpression of lncRNA *RP11-367G18.1* V2 version in FADU cells increased the tumor volume using xenograft assays. (D) Knockdown of lncRNA *RP11-367G18.1* V2 version in FADU cells overexpressing HIF-1α (ΔODD) significantly decreased the tumor volume using xenograft assays. (E) Immunofluorescence staining assay showed the co-localization of lncRNA *RP11-367G18.1* V2 version and HIF-1α in xenografted tumors. H: high density of co-localization; L: low density of co-localization. (F) Western blot analysis showed the decrease in the protein levels of c-Myc and cyclin D1 in OECM-

1 cells undergoing knockdown of lncRNA *RP11-367G18.1* V2 version. (G, H) Real-time PCR analysis showed the decreased mRNA levels of c-Myc and cyclin D1 in xenografted OECM-1 (G) or SAS (H) cells undergoing knockdown of lncRNA *RP11-367G18.1* V2 version. The asterisk (*) indicated statistical significance (P<0.05) between experimental and control clones.

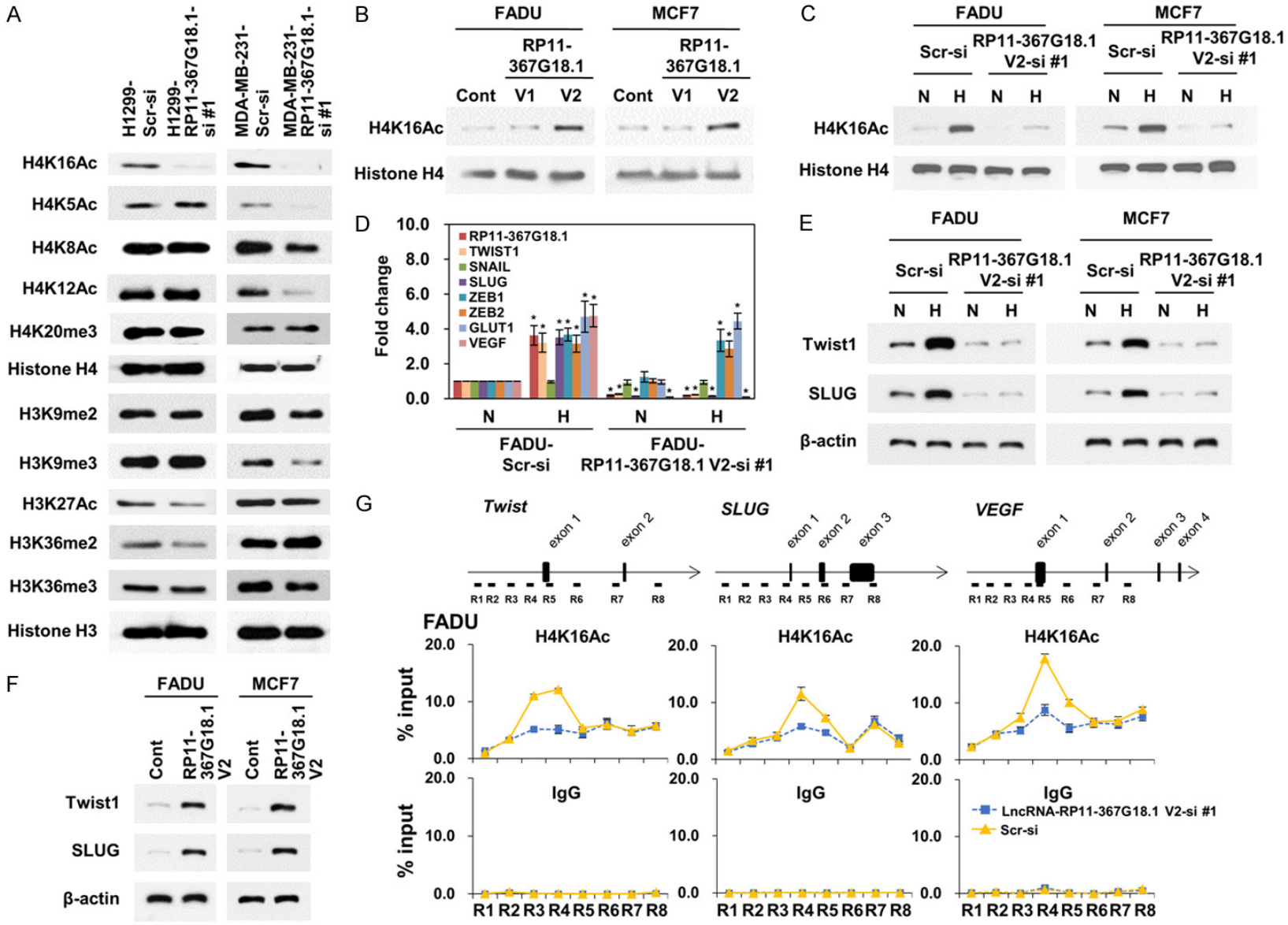


Figure 8. LncRNA *RP11-367G18.1* V2 version regulated the H4K16Ac mark. A. Western blot analysis showed that knockdown of lncRNA *RP11-367G18.1* V2 version significantly decreased the levels of H4K16Ac mark in two cell lines. B. Western blot analysis showed that overexpression of lncRNA *RP11-367G18.1* V2 version increased the H4K16Ac levels in two different cell lines. C. Knockdown of lncRNA *RP11-367G18.1* V2 version abolished the H4K16Ac levels induced by hypoxia in two different cell lines. D. Real-time PCR analysis showed that the downstream target genes (*Twist1, SLUG, VEGF*) induced by hypoxia could be significantly decreased by knockdown of lncRNA *RP11-367G18.1* V2 version. E. Knockdown of lncRNA *RP11-367G18.1* V2 version abolished the activated protein levels of Twist1 and SLUG induced by hypoxia in two cell lines using Western blot analysis. F. Overexpression of lncRNA *RP11-367G18.1* V2 version activated the protein levels of Twist1 and SLUG using Western blot analysis. G. qChIP assays showed the decrease in H4K16Ac levels on the promoters of *Twist1*, *SLUG*, and *VEGF* genes through knockdown of lncRNA *RP11-367G18.1* V2 version in hypoxic FADU cells. N, normoxia; H, hypoxia. The asterisk (*) indicated statistical significance ($P<0.05$) between experimental and control clones.

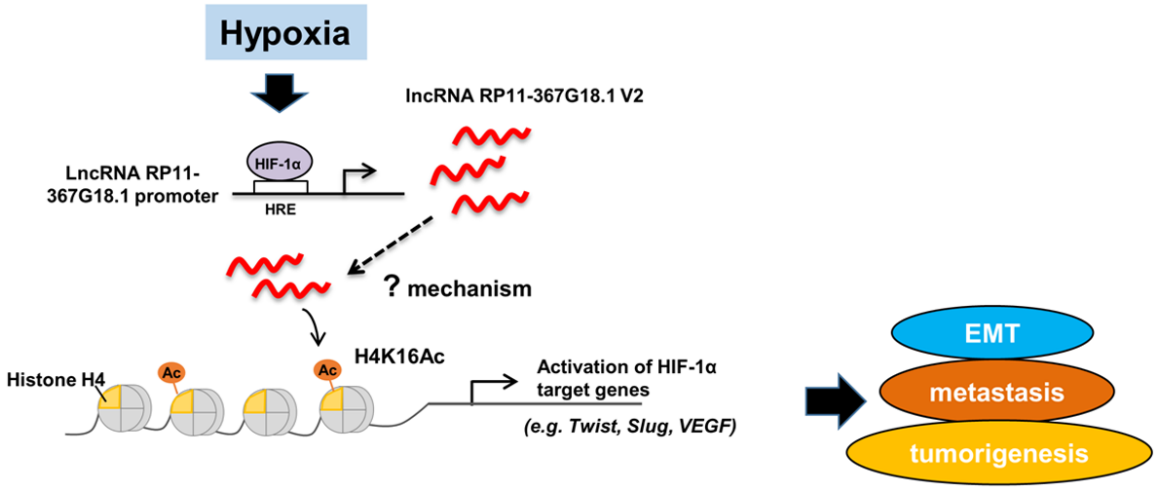


Figure 9. A model to depict the induction of lncRNA *RP11-367G18.1* by hypoxia activated the H4K16Ac mark to further regulate the downstream target genes, including *Twist1*, *SLUG*, and *VEGF*, leading to EMT and tumor metastasis.

addition, we tested the ability of Twist1 or SLUG to rescue the *in vitro* invasive activity mitigated by knockdown of lncRNA *RP11-367G18.1* V2 version. In three different conditions (FADU cells that overexpressed a constitutively active HIF-1α (ΔODD) vector, hypoxic FADU cells, OECM-1 cells), knockdown of lncRNA *RP11-367G18.1* V2 version significantly decreased the *in vitro* invasive activity (Figure S3C-E), whereas reconstitution with Twist1 or SLUG restored the *in vitro* invasive activity (Figure S3C-E), supporting the signaling connection between lncRNA *RP11-367G18.1* V2 version and Twist1/SLUG. We further performed qChIP assays to examine the levels of H4K16Ac on the promoters of *Twist1, SLUG,* and *VEGF* genes. The results confirmed the decreased H4K16Ac levels on the promoters of these genes in hypoxic FADU cells undergoing knockdown of lncRNA *RP11-367G18.1* V2 version (**Figure 8G**). All the above results are consistent with the ability of lncRNA *RP11-367G18.1* V2 version to scaffold a H4K16Ac histone-modifying complex to specifically activate the levels of H4K16Ac, leading to the activation of downstream target genes (Twist1, SLUG, VEGF).

Discussion

Hypoxia induces tumor metastasis through different mechanisms, and the epithelial-mesenchymal transition (EMT) represents one of the early mechanisms induced by hypoxia/HIF-1α [10-14]. Hypoxia also activates many lncRNAs that contribute to tumor metastasis [6, 17-21]. Many different mechanisms controlled by hypoxia-induced lncRNAs have been shown to regulate hypoxia-induced metastasis [6, 19, 20, 23-27]. However, hypoxia-induced lncRNAs that can regulate a specific histone mark have not been identified. Here we show that lncRNA *RP11-367G18.1* V2 version specifically activates the H4K16Ac histone mark. Certain hypoxia-induced target genes, including *Twist1*,

SLUG, and *VEGF* are regulated by lncRNA *RP11-367G18.1* V2 version through knockdown experiments. Since Twist1 and SLUG represent two of the "core" EMT transcription regulators [16], the role of lncRNA *RP11-367G18.1* V2 version should be important in hypoxia-induced EMT, which is confirmed by the crucial role of lncRNA *RP11-367G18.1* V2 version in hypoxia-induced EMT from various experiments. Although other hypoxia-induced lncRNAs were shown to scaffold a chromatin modifying complex [22], our results represent an advancement since we identified the specific histone mark that is regulated by lncRNA *RP11-367G18.1* V2 version. Further proteomics approach will be required in order to identify the protein complex participating in regulating the formation of H4K16Ac histone mark. Since lncRNA *RP11-367G18.1* obviously plays a crucial role in hypoxia-induced EMT, it will be interesting to see what other target genes that could be regulated by this lncRNA through scaffolding the H4K16Ac histone-modifying complex.

Hypoxia induced the expression of lncRNA *RP11-367G18.1* in all the cell lines tested (**Figure 1D**), supporting its *bona fide* role as a HIF-1α target. High expression of lncRNA *RP11-367G18.1* is correlated with a worse survival of head and neck cancer patients (**Figure 1E**), indicating its role in tumorigenesis and tumor progression. Knocking down lncRNA *RP11-367G18.1* decreased the tumor volume of xenografted tumors (**Figure 3B**), consistent with its role in inducing tumorigenicity. The contribution of lncRNA *RP11-367G18.1* to tumor growth could be attributed at least to its regulation of c-Myc and cyclin D1 (**Figure 7F-H**). A model is depicted that provides the summary of the results in this report (**Figure 9**).

This report identifies a hypoxia-induced lncRNA *RP11-367G18.1* that regulates the H4K16Ac histone mark to regulate hypoxia-induced target genes (*Twist1, SLUG, VEGF*), leading to EMT, metastasis, and tumorigenicity. LncRNA *RP11-367G18.1* may be considered a good therapeutic target for future management of certain types of cancers (e.g., head and neck cancer).

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Disclosure of conflict of interest

None.

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Table S1. Sequence of the oligonucleotides and restriction enzymes used for plasmid construction

Constructions	Sequence (5'→3')	Restriction Enzyme site
RP11-367G18.1 promoter (-585 to +15)	F: ATAGGTACCATCAGAAGGCTGTTAG	*KpnI*
	R: ATACTCGAGGTCTGTTCCTAACTCC	*XhoI*
RP11-367G18.1 promoter mut (-585 to +15)	F: CTCTAGACCAACTCATGGCAACTGTTAAAAGGAGGTGACTCATTCCC	*Recombination*
	R: GGGAATGAGTCACCTCCTTTTAACAGTTGCCATGAGTTGGTCTAGAG	*Recombination*

Table S2. Schemes of plasmid constructions for constructs used

Plasmid	Template	Vector	Restriction enzyme site (F/R)
RP11-367G18.1 promoter (-585 to +15)	gDNA	pGL3	*KpnI/XhoI*
RP11-367G18.1 promoter mut (-585 to +15)	pGL3-RP11-367G18.1 promoter	pGL3	*KpnI/XhoI*
pcDNA3.1 (+)-RP11-367G18.1 V1	synthesized by Biotools (Taiwan)	pcDNA3.1 (+)	*KpnI/EcoR*I
pcDNA3.1 (+)-RP11-367G18.1 V2	synthesized by Biotools (Taiwan)	pcDNA3.1 (+)	*KpnI/EcoR*I

Table S3. List of proteins tested by and characteristics of the corresponding antibodies

Protein	Assay	Origin	Dilution	Incubation period
HIF-1α	WB	#610959, BD Biosciences	1:500	4°C, Overnight
HIF-1α	ChIP	C15410234, diagenode	5 μl	4°C, Overnight
HIF-1α	IF	ab1, abcam	1:200	4°C, Overnight
E-cadherin	WB	#3195, Cell signaling Technology, Inc.	1:500	4°C, Overnight
vimentin	WB	V-6630, Sigma-Aldrich Corp.	1:1000	4°C, Overnight
N-cadherin	WB	#610921, BD Biosciences	1:500	4°C, Overnight
plakoglobin	WB	ab12083, abcam	1:1000	4°C, Overnight
Twist1	WB	sc-15393, Santa cruz Biotechnology, Inc.	1:500	4°C, Overnight
SLUG	WB	#9585, Cell signaling Technology, Inc.	1:1000	4°C, Overnight
c-Myc	WB	#5605, Cell signaling Technology, Inc.	1:1000	4°C, Overnight
Cyclin D1	WB	#55506, Cell signaling Technology, Inc.	1:1000	4°C, Overnight
β-actin	WB	GTX629630, Genetex	1:10000	4°C, Overnight
HA	WB	#2367, Cell signaling Technology, Inc.	1:1000	4°C, 1 hour
H3K9me2	WB	ab1220, abcam	1:1000	4°C, Overnight
H3K9me3	WB	ab8898, abcam	1:1000	4°C, Overnight
H3K36me2	WB	GTX60372, Genetex	1:1000	4°C, Overnight
H3K36me3	WB	ab9050, abcam	1:1000	4°C, Overnight
H3K27Ac	WB	ab4729, abcam	1:1000	4°C, Overnight
H4K5Ac	WB	ab51997, abcam	1:1000	4°C, Overnight
H4K8Ac	WB	ab15823, abcam	1:1000	4°C, Overnight
H4K12Ac	WB	ab46983, abcam	1:1000	4°C, Overnight
H4K16Ac	WB	#17-10101, Millipore	1:1000	4°C, Overnight
H4K16Ac	ChIP	#17-10101, Millipore	5 μl	4°C, Overnight
H4K20me3	WB	ab9053, abcam	1:1000	4°C, Overnight
Histone H3	WB	#9715, Cell signaling Technology, Inc.	1:1000	4°C, Overnight
Histone H4	WB	ab10158, abcam	1:1000	4°C, Overnight

Abbreviations: ChIP, Chromatin Immunoprecipitation; WB, Western blot; IF, Immunofluorescence.

Table S4. Sequence of the oligonucleotides for real-time PCR

Target	Sequence (5'→3')
RP11-367G18.1	F: GGGCCCTTGTGAATTGATGA
	R: GCCATATGTCTTCTTGCAGAGAGTT
RP11-367G18.1 V2	F: TACGGCTCGATCAGCTTTCTGT
	R: GAAAGACTGAAAAGCTGGGGAG
HIF-1α	F: TTTTTCAAGCAGTAGGAATTGGA
	R: GTGATGTAGTAGCTGCATGATCG
GLUT1	F: CGGGCCAAGAGTGTGCTAAA
	R: TGACGATACCGGAGCCAATG
Twist1	F: AGCTACGCCTTCTCGGTCT
	R: CCTTCTCTGGAAACAATGACATC
Snail	F: CTTCCAGCAGCCCTACGAC
	R: CGGTGGGGTTGAGGATCT
SLUG	F: GACCCTGGTTGCTTCAAGGA
	R: TGTTGCAGTGAGGGCAAGAA
ZEB1	F: ACTGCTGGGAGGATGACAGA
	R: ATCCTGCTTCATCTGCCTGA
ZEB2	F: AAAACCATGGCGTGGGTA
	R: CAATAGCCGAGGCATCAA C
VEGF	F: CGCAAGAAATCCCGGTATAA
	R: TCTCCGCTCTGAGCAAGG
c-Myc	F: TCCCTCCACTCGGAAGGAC
	R: CTGGTGCATTTTCGGTTGTTG
CCND1	F: GCTGCGAAGTGGAAACCATC
	R: CCTCCTTCTGCACACATTTGAA
18s	F: GGCGGCGTTATTCCCATGA
	R: GAGGTTTCCCGTGTTGAG

Table S5. Sequence of the oligonucleotides for qChIP assay

Target	Sequence (5'→3')
RP11-367G18.1 (with HRE)	F: TCCATGGTCACCACAGTACCC
	R: CTCCAGAGGTGTAACTTCCCTATCC
RP11-367G18.1 (without HRE)	F: AGGAACATTCGTCTTGTAGAGGTAAGA
	R: ACATAGTCTCTTGTAAGGAGTAGGGCA
VEGF (with HRE)	F: ACAGACGTTCCTTAGTGCTGG
	R: AGCTGAGAACGGGAAGCTGTG
Twist1 R1 (-1136~-1024)	F: CCTCTTTGGGGCTCTTCGTT
	R: TCTAGGGCATCCAGTGGACA
Twist1 R2 (-891~-767)	F: TGAAATGGCCACAGGGTCTC
	R: TTCGGTGGAAGGAAACCCAG
Twist1 R3 (-509~-399)	F: CACTTTTCTTGGCATGCCCC
	R: GGTGATGTCTCATCTCGCCC
Twist1 R4 (-98~-29)	F: GGGACTGGAAAGCGGAAACT
	R: TGTCATTGGCCTGACGTGAG
Twist1 R5 (647~800)	F: GTCCGCAGTCTTACGAGGAG
	R: TTGAGGGTCTGAATCTTGCTC
Twist1 R6 (1483~1677)	F: AACAGCCGCAGAGACCTAAA
	R: CACGCCCTGTTTCTTTGAAT

Twist1 R7 (3536~3640)	F: GGAAGGCGATTATGTGTTGG
	R: ATGCTTTCCCTCATCCTCCT
Twist1 R8 (4770~4897)	F: CATGGTTTGGCAAAGTCAGA
	R: ATCATGAGAGCGGGACAAAC
SLUG R1 (-3092~-2924)	F: AACCTCACGGATCTAATTTATTCCC
	R: TGAAGAAGCTGACCAAGATAACTTGT
SLUG R2 (-2090~-1938)	F: AGCACGCGGATCTGTGTAATG
	R: ATCACAGAGGCATAATCCAGCC
SLUG R3 (-991~-790)	F: CAGGAAACTGGTAGATACTGAGATGG
	R: CCACCGGACATTCTCTCACA
SLUG R4 (-159~4)	F: ACAGCCCATTTTGAACCAGAA
	R: AACTGAGCCCGTTTTGGCT
SLUG R5 (462~591)	F: ATCTCGCAGCTTCGTTTGCT
	R: ATCAAGCCGCCTTCTAAAGGA
SLUG R6 (1495~1628)	F: AGACCCTGGTTGCTTCAAGGA
	R: AAGCACTATGTCACAACTTCATGCA
SLUG R7 (2388~2586)	F: GTTTCCTCTAAAAACACTGACTGTC
	R: TGCAGGAGAGACATTCTGGA
SLUG R8 (3578~3720)	F: CAACCTGAAGACTTGTGAAATCAAT
	R: GTGGTTTGGTACTAATCATGAAGCA
VEGF R1 (-992~-900)	F: GCCAGACTCCACAGTGCATA
	R: CTGAGAACGGGAAGCTGTGT
VEGF R2 (-889~-787)	F: TTGGTGCCAAATTCTTCTCC
	R: GCCTGCAGACATCAAAGTGA
VEGF R3 (-526~-470)	F: GCGTCTTCGAGAGTGAGGAC
	R: CACACGCACACACTCACTCA
VEGF R4 (-340~-285)	F: AAAGAGGGAACGGCTCTCAG
	R: AGGGAGCAGGAAAGTGAGGT
VEGF R5 (407~517)	F: ACAGGGGCAAAGTGAGTGAC
	R: CTGTCTGTCTGTCCGTCAGC
VEGF R6 (1578~1649)	F: GCTTGCTGTCACTGCCACT
	R: AGCAATCCACCCCAAAACTT
VEGF R7 (3063~3146)	F: CTAGCAGGGTCTGGTGTTCC
	R: CTCCAGCTCTCACCAACTCC
VEGF R8 (4803~4935)	F: TGGATCCTCCCATTTCTCTG
	R: CCACGCCAGATTTAGGTCAG

Table S6. Lentivirus used in RNAi experiments

Target	Sequence
Scrambled control	CCTAAGGTTAAGTCGCCCTCG
HIF-1α	GTGATGAAAGAATTACCGAAT
RP11-367G18.1 #1	GGGCCCTTGTGAATTGATGAA
RP11-367G18.1 #2	GCAGAAATAGAACTGAGAAGA
RP11-367G18.1 V2 #1	GGTTCTACTTCCTGGCAAGTA
RP11-367G18.1 V2 #2	GTCGAGGACAACAGTATAATG

Table S7. Sequence of the probes for RP11-367G18.1 RNA-FISH assay

Probe Name	Sequence (5'→3')
RP11-367G18.1-FISH1	AACAGAAAGCTGATCGAGCC
RP11-367G18.1-FISH2	CATGGCATCGTAAGGCAATG
RP11-367G18.1-FISH3	CTGATTTGACAATAGTTCCA
RP11-367G18.1-FISH4	ACTGAAAAGCTGGGGAGTGA
RP11-367G18.1-FISH5	CCTCTGATAGAGCAAGAATT
RP11-367G18.1-FISH6	ATCATTATACTGTTGTCCTC
RP11-367G18.1-FISH7	TGCCAGGAAGTAGAACCTAT
RP11-367G18.1-FISH8	GGCCCGTGGGAAAATGATTA
RP11-367G18.1-FISH9	AGGTAGAGACAGTTATTTCA
RP11-367G18.1-FISH10	CTGTCTTTCATTCTTTCCAT
RP11-367G18.1-FISH11	TTCTTGCAGAGAGTTGCTTA
RP11-367G18.1-FISH12	AGCAGAACCTAAGCCATATG
RP11-367G18.1-FISH13	TGCATGGAATCAAATCACCA
RP11-367G18.1-FISH14	TGTCAAGTCCCGGTGCTG
RP11-367G18.1-FISH15	CCCATCTCAGTAGTGAATAC
RP11-367G18.1-FISH16	GTCTTCATATTCATCAATTC
RP11-367G18.1-FISH17	GTTCTATTTCTGCTTTCTTA
RP11-367G18.1-FISH18	TAGACAATTCGAGAAAGGTA
RP11-367G18.1-FISH19	CCACATAGCAAGATCCCATC
RP11-367G18.1-FISH20	AGGAGTTTGAGGCCAGCCTG

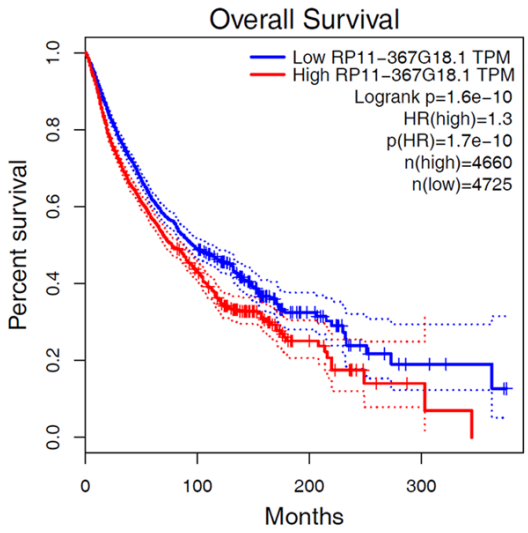

Figure S1. Median survival analysis of pan-cancer showed a poor survival of patients with high expression of lncRNA *RP11-367G18.1* across different cancer types.

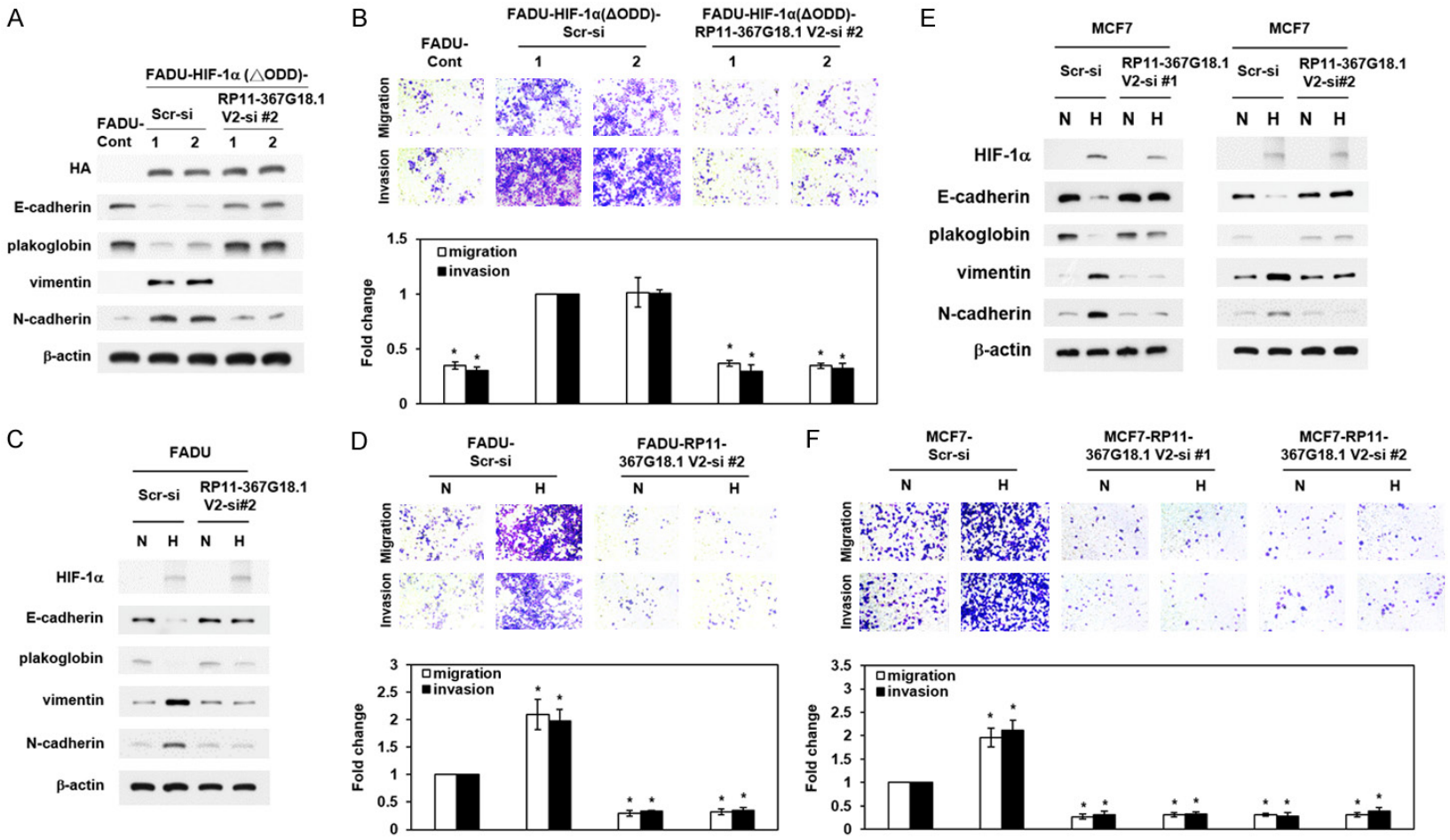

Figure S2. Knockdown of lncRNA *RP11-367G18.1* reversed the EMT phenotypes of FADU or MCF7 cells overexpressing a constitutively active HIF-1α mutant or under hypoxic treatment and decreased the *in vitro* migration and invasion activity. A. Knockdown of lncRNA *RP11-367G18.1* reversed the EMT marker gene expression of FADU cells overexpressing a constitutively active HIF-1α mutant. B. Knockdown of lncRNA *RP11-367G18.1* decreased the in vitro migration and invasion activity of FADU cells overexpressing a constitutively active HIF-1α mutant. C. Knockdown of lncRNA *RP11-367G18.1* reversed the EMT marker gene expression of FADU cells undergoing hypoxia. D. Knockdown of lncRNA *RP11-367G18.1* decreased the in vitro migration and invasion activity of FADU cells undergoing hypoxia. E. Knockdown of lncRNA *RP11-367G18.1* reversed the EMT marker gene expression of MCF7 cells undergoing hypoxia. F. Knockdown of lncRNA *RP11-367G18.1* decreased the in vitro migration and invasion activity of MCF7 cells undergoing hypoxia. Migration and invasion assays were performed in Transwell inserts for 12 or 20 h, respectively. The asterisk (*) indicated statistical significance ($P<0.05$) between experimental and control clones.

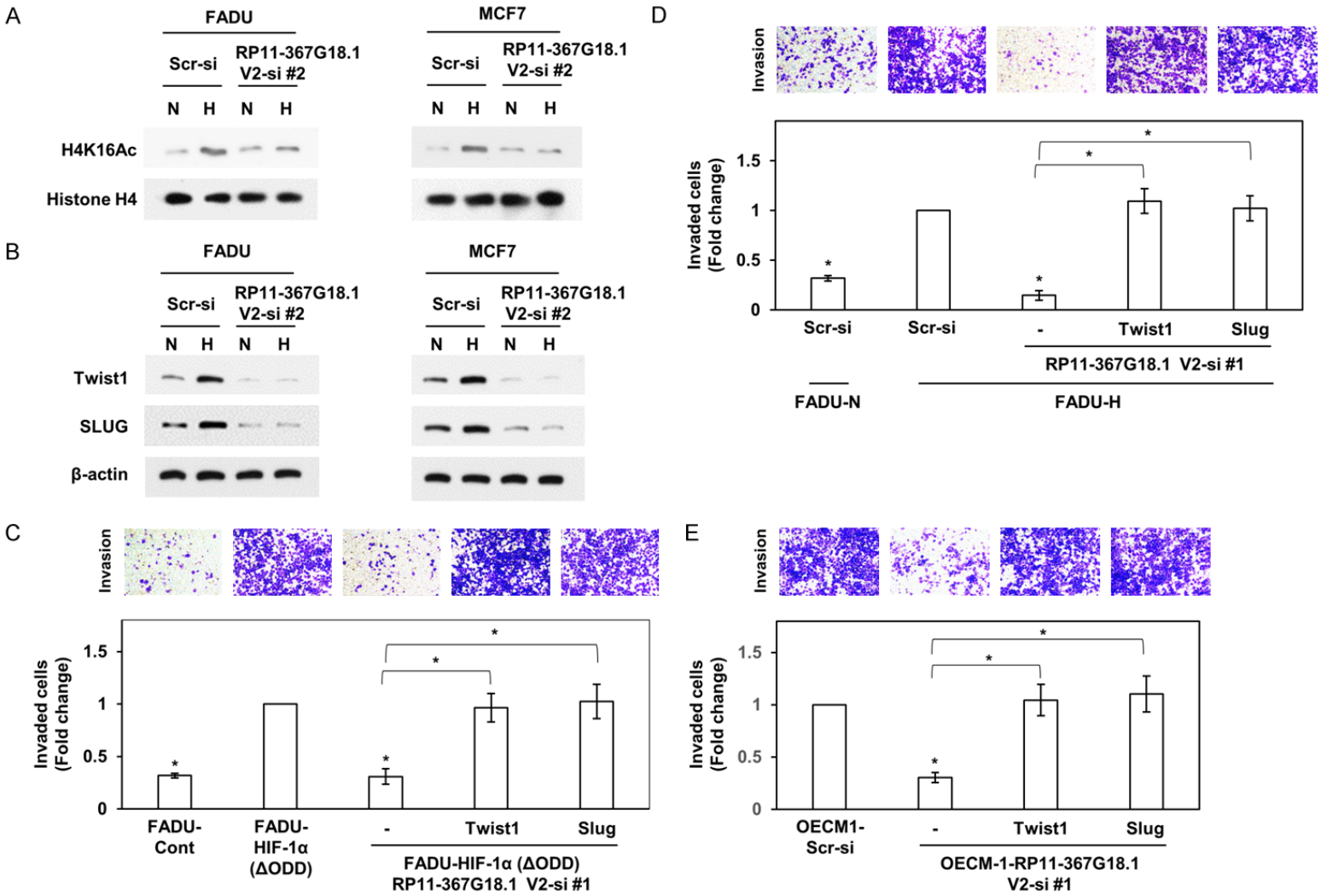

Figure S3. Regulation of the H4K16Ac histone mark by LncRNA *RP11-367G18.1* V2 version and reconstitution by Twist1 or SLUG restored the in vitro invasive activity. A. Knockdown of lncRNA *RP11-367G18.1* V2 version abolished the H4K16Ac levels induced by hypoxia in two different cell lines. B. Knockdown of lncRNA *RP11-367G18.1* V2 version abolished the activated protein levels of Twist1 and SLUG induced by hypoxia in two cell lines using Western blot analysis. C. Reconstitution by Twist1 or SLUG restored the *in vitro* invasive activity in hypoxic FADU cells undergoing knockdown of lncRNA *RP11-367G18.1* V2 version. D. Reconstitution by Twist1 or SLUG restored the *in vitro* invasive activity in FADU cells overexpressing a constitutively active HIF-1α mutant that underwent knockdown of lncRNA *RP11-367G18.1* V2 version. E. Reconstitution by Twist1 or SLUG restored the *in vitro* invasive activity in OECM-1 cells undergoing knockdown of lncRNA *RP11-367G18.1* V2 version. Invasion assays were performed in Transwell inserts for 20 h. The asterisk (*) indicated statistical significance ($P<0.05$) between experimental and control clones.