G9A promotes tumor cell growth and invasion by silencing CASP1 in non-small-cell lung cancer cells

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Non-small-cell lung cancer (NSCLC) is one of the leading causes of cancer-related death worldwide. Although epigenetic deregulation is known to be important for tumor progression, the molecular mechanisms in NSCLC remain unclear. Here, we found that G9A (known as EHMT2), a histone methyltransferase responsible for mono- or di-methylation of histone 3 (H3) lysine 9 (K9), is significantly upregulated in NSCLC. Knocking down G9A or pharmacological inhibition of its activity suppressed tumor cell growth, colony formation, invasion and migration. Furthermore, G9A exerts these functions by repressing CASP1 expression. Knocking down CASP1 in G9A-deficient cell restored capacities of tumor cell invasion and migration. Mechanistically, G9A silences the CASP1 promoter activity by increasing H3K9me2 around its promoter. Finally, high expression of G9A or low expression of CASP1 is correlated with poor overall survival in lung adenocarcinoma. Overall, our study uncovers a novel mechanism of G9A promoting tumor cell growth and invasion by silencing CASP1, and implies that G9A may serve as a therapeutic target in treating NSCLC.

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Lung cancer is a leading cause of death in all types of cancers. Non-small-cell lung cancer (NSCLC) is the major type of lung cancer. It is a heterogeneous disease; many different oncogenic mutations have been identified. Epigenetic deregulation is implicated in tumor development.1 Histone methylation is one of primary epigenetic modifications affecting gene expression, and is involved in many cellular processes.2 G9A/EHMT2 is a histone lysine methyltransferase that specifically mono- and dimethylates Lys9 of histone H3 (H3K9me1 and H3K9me2, respectively).3–5 It is overexpressed in many types of cancer.6–10 and its higher expression is associated with poor survival of cancer patients.6,9,11 Mechanistically, G9A acts as a transcriptional repressor to silence gene expression.12,13 For example, G9A interacts with Snail, a transcriptional factor, and is critical for Snail-mediated E-cadherin repression in human breast cancer.14 Moreover, hypoxic stress induced accumulation of G9A leads to increased H3K9me2 and repression of its target genes to promote cell survival.15 However, G9A also functions as a transcriptional activator depending on its interacting cofactors.16 For example, G9A can epigenetically activate the serine–glycine synthesis pathway to sustain cancer cell survival and proliferation.17 However, its role in NSCLC is not well understood. Identification of its key target genes or pathways will help to understand the molecular mechanism of tumorigenesis and metastasis in NSCLC.

CASP1, also known as caspase 1, belongs to the family of CASP proteins, which are cysteine proteases regulating many cellular processes, such as apoptosis, inflammation and necrosis, etc.18,19 Specifically, CASP1 mediates inflammation-related activation regulated immune response and disease pathogenesis.20 In addition, CASP1-induced pyroptosis is an innate immune effector mechanism against intracellular bacteria.21,22 However, the function and regulation of CASP1 in NSCLC is poorly understood.

In this study, we examined the biological function of G9A in NSCLC cells, and identified one of its key target genes, CASP1. We also uncovered the molecular mechanism of how G9A represses CASP1 to promote tumor cell growth and invasion. Finally, we analyzed whether G9A or CASP1 could serve as prognostic biomarkers in lung adenocarcinoma (LUAD). In addition, our study suggests that G9A may be a therapeutic target for NSCLC.

Results

G9A expression is aberrantly elevated in NSCLC patients. To examine whether G9A expression is dysregulated in NSCLC, we compared its expression between normal and cancer samples using the mRNA-Seq data of LUAD from the TCGA database. We found that G9A is significantly upregulated in tumor samples compared with the normal control (Figure 1a). In addition, G9A is upregulated in all stages of LUAD compared with the normal control (Figure 1b).

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We also examined the expression of G9A in lung cancer using the oncomine database, and found that G9A is upregulated in LUAD regardless of EGFR or KRAS mutation status (Figures 1c and d). Overall, this analysis indicates that G9A is abnormally elevated in LUAD of NSCLC compared with the normal lung tissues.

G9A promotes tumor cell growth and invasion in NSCLC.

To investigate the function of G9A in NSCLC cells, we knocked down the level of G9A protein significantly in PC9 and A549 cells by selecting cells stably expressing G9A shRNA (Figure 2a), and found that cell invasion and migration capacities were reduced considerably in these cells (Figures 2c and d). In addition, these cells also had a slower cell proliferation rate (Figure 2e). Transient depletion of G9A by siRNA transfection in these cells also showed the similar phenotypes as cells with stable knockdown of G9A (Supplementary Figures S1A–E).

Similarly, colony formation and sphere formation abilities were reduced upon stable G9A knockdown in both PC9 and A549 cells (Figures 2h and i). Furthermore, cell cycle progression was delayed due to the prolonged G1 phase and shortened the G2 and M phases (Figure 2k), but the apoptosis rate was not significantly affected in cells with stable G9A knockdown, demonstrated by the lack of cleavage products of CASP3 and PARP1 proteins (two markers for apoptosis) in WB assays (Figure 2j).

To study whether G9A knockdown affects tumor cell growth in vivo, we carried out xenograft assays in nude mice, and found that tumors derived from cells stably expressing G9A shRNA showed significantly slower growth rates and smaller tumor sizes, compared with those derived from cells expressing the control shRNA (Figure 2l). We also carried out immunohistochemistry analysis, and found that the intensity for cell proliferation marker (Ki67) was considerably reduced in xenograft tumor depleted with G9A (Supplementary Figure S2A), which indicates that G9A knockdown reduced cell proliferation in vivo. Altogether, we conclude that G9A promotes tumor cell growth and invasion in NSCLC cells.
CASP1 expression is repressed by G9A. To study the molecular mechanism of G9A-mediated tumor growth and invasion, first, we aimed to identify target genes of G9A in NSCLC cells, by carrying out gene expression correlation analysis in LUAD using the data downloaded from the cbioPortal website, and found that CASP1 expression is negatively correlated with G9A expression in NSCLC (Figure 3a). In addition, the expression of CASP1 is significantly downregulated in every T stage in LUAD compared with the normal tissue control (Figure 3b). Furthermore, we examined the correlation of G9A and CASP1 expression in different stages of LUAD, and found that they are also significantly negatively correlated in T1 and T2 stages (Figure 3c).

To study whether G9A represses CASP1 expression in NSCLC cells, we overexpressed G9A in PC9 and A549 cells, and found that CASP1 expression was repressed (Figure 3d). Conversely, depletion of G9A by shRNA reactivated CASP1 expression in these cells, validated by RT-qPCR (Figure 3e), indicating G9A represses CASP1 expression in these cells. Therefore, our results indicate that G9A suppresses CASP1 gene expression in NSCLC cells, and their expressions are significantly negatively correlated between each other.

CASP1 suppresses G9A-mediated cell invasion and migration. To investigate whether repression of CASP1 expression is necessary for G9A-mediated cell invasion and migration, we transiently overexpressed CASP1 in PC9 and A549 cells (Figure 4a), and found that CASP1 overexpression significantly inhibited tumor cell invasion and migration abilities in these cells (Figures 4c–e). By contrast, when
CASP1 was knocked down by siRNA in G9A-depleted tumor cells (Figure 4b), the invasion and migration capabilities of these cells were rescued (Figures 4f–h).

To investigate the biological function of CASP1 in tumorigenesis, we carried out bioinformatics analysis to examine the cellular pathways either positively or negatively correlated with the CASP1 expression in four different cancer types, including LUAD, breast cancer (BRCA), colon adenocarcinoma (COAD) and lung squamous cell carcinoma (LUSC) from the TCGA database (Supplementary Figure S3). We found that CASP1 expression is positively associated with pathways including cytokine–cytokine receptor interaction in LUAD, BRCA and LUSC, and metabolic pathways in COAD, but negatively associated with pathways, such as tight junction in LUAD, endocytosis, AMPK and mTOR signaling in BRCA and COAD (Supplementary Figure S3).

In conclusion, our results indicate that CASP1 overexpression suppresses G9A-mediated cell invasion and migration. The expression of CASP1 is either positively or negatively associated with distinctive pathways in cancer.

G9A knockdown reduces the level of H3K9me2 at the CASP1 promoter and its overexpression represses the CASP1 promoter activity. G9A is a histone
methyltransferase that increases the level of H3K9me2 around the promoter region of its target genes, and interacts with other co-repressors to silence target gene expression.5,12,23,24

To investigate whether depletion of G9A reduced the level of H3K9me2 around the CASP1 gene promoter, we carried out chromatin immunoprecipitation assay using anti-G9A and anti-H3K9me2 antibodies, followed by qPCR to examine the relative enrichment of H3K9me2 around the CASP1 promoter in these cells as well (Figure 5b, lower panel).

To study whether G9A represses the CASP1 promoter activity, we cloned the CASP1 gene promoter into pGL3 vector, carried out luciferase reporter assays and found that G9A overexpression significantly suppressed the promoter activity of CASP1 promoter, and this effect was dosage dependent (Figures 5c and d). Overall, our results reveal that G9A silences the CASP1 promoter activity by inducing H3K9me2 around its promoter.

Pharmacological inhibition of G9A inhibits cell proliferation, invasion and migration in NSCLC cells. To study whether pharmacological inhibition of G9A activity can inhibit tumor cell growth, invasion and migration, we treated PC9 and A549 cells with various concentrations (1, 5, 10 μM) of BIX-01294, which is a small molecular inhibitor of G9A widely used in other cancer studies.25

We found that 1 or 5 μM BIX-01294 treatment significantly inhibited the proliferation rate of PC9 or A549 cells, respectively (Figure 6a), and 5 μM BIX-01294 suppressed cell invasion and migration in both cells (Figures 6b and c). In addition, as low as 1 μM BIX-01294 treatment significantly inhibited colony formation of these cells (Figure 6d).

To study whether these changes were due to enhanced apoptosis induced by BIX-01294, we treated these cells with 1, 5 or 10 μM of the inhibitor, and found that 5 or 10 μM BIX-01294 treatment caused significantly increased apoptosis rate in A549 or PC9 cells, respectively, compared with the control treatment (Figure 6e). It seems that A549 cells are more sensitive to BIX-01294-mediated apoptosis than PC9 cells.

To determine whether inhibiting the enzymatic activity of G9A can reactivate CASP1 expression, we treated these cells with 0, 1 and 5 μM BIX-01294, and found that 5 μM BIX-01294...
in PC9 cells, but as low as 1 μM BIX-01294 in A549 cells significantly reactivated CASP1 expression (Figure 6f).

Interestingly, we found that as low as 1 μM BIX-01294 treatment could reduce the total level of H3K9me2 in both PC9 and A549 cells, and the level of G9A was decreased at 5 μM BIX-01294 treatment (Figure 6g). To assess the effect of this inhibitor on the signaling pathway, we treated these cells with 1, 5 and 10 μM BIX-01294, and found that 5 μM BIX-01294 significantly suppressed phosphorylation of the ERK kinase in both cells (Figure 6g).

Taken together, these results indicate that inhibition of G9A enzymatic activity can suppress G9A-mediated tumor cell growth, invasion and migration, which is consistent with our data using G9A-depleted NSCLC cells.

High expression of G9A and low expression of CASP1 are significantly associated with poor overall survival in NSCLC patients. To investigate whether the expression of G9A or CASP1 can serve as prognostic markers for NSCLC, we downloaded the clinical data of 488 patients with LUAD from a public database (http://kmplot.com/),26 and carried out Kaplan–Meier survival analysis. We found that high expression of G9A is associated with poor overall survival in LUAD (Figure 7a), while high expression of CASP1 is associated with better overall survival in LUAD (Figure 7b), with log-rank (Mantel–Cox) P-values of 0.008 for G9A and 1.2e-09 for CASP1, respectively.

To further study the effect of the expressions of both genes on patient overall survival, we divided the LUAD patients into four groups: high or low expressions of both G9A and CASP1, low expression of G9A and high expression of CASP1, and high expression of G9A and low expression of CASP1. Using the log-rank test, we found that the most significant difference in patient survival is between the group carrying high expression of G9A and low expression of CASP1 and the group carrying low expression of G9A and high expression of CASP1, with log-rank P-value of 3.14E-09 (Figure 7c and Supplementary Table S1).
Therefore, we conclude that high expression of G9A or low expression of CASP1 may be used as a poor prognostic marker for LUAD patients.

Discussion

In this study, we found that histone methyltransferase G9A is aberrantly upregulated in NSCLC, and it promotes cancer cell growth, colony formation, invasion and migration, as well as enhances tumor growth \textit{in vivo}. In addition, G9A mediates these effects through silencing expression of CASP1.

Pharmacological inhibition of G9A has the strong antitumor effect in NSCLC cells. Finally, high expression of G9A and low expression of CASP1 indicate poor prognosis in LUAD patients.

Based on these findings, we proposed a mechanistic model here (Figure 7d): G9A silences CASP1 expression by increasing the level of H3K9me2 around its promoter region. Decreased CASP1 expression is associated with changes in cytokine–cytokine receptor interaction and tight junction pathway possibly, which may lead to enhanced cell proliferation, migration and invasion in NSCLC cells.
It is possible that G9A regulates other genes and pathways in NSCLC; for example, G9A was found to mediate tumor metastasis by epigenetically repressing the cell adhesion molecule EPCAM in lung cancer cells. In addition, G9A can suppress the transcription of the SIAH1 gene, which encodes a member of E3 ubiquitin ligases and functions as a tumor suppressor, in lung cancer cells. Here, we demonstrate that CASP1 is one of the important target genes of G9A in NSCLC. How G9A is recruited to the promoter of CASP1 remains to be determined, it may require additional factors. For example, G9A is recruited by LSH, a member of SNF2 family of ATP-dependent chromatin enzymes, to induce DNA methylation and stable gene silencing during development. However, G9A recruitment by GR, Runx2 and NF-E2/p45 leads to activation of target genes in a SET-domain-independent manner. The Air noncoding RNA may also recruit G9A to chromatin and epigenetically silences the transcription of target genes. It would be interesting to identify such recruiting factor(s).

Our study also indicates that CASP1 suppresses tumor cell invasion and migration in NSCLC cells. CASP1 is one of the components of the inflammasome complex, which also includes PYCARD, NALP and caspase 5 (also known as ICH-3). They function in the innate immune system, and can activate inflammatory process and induce cell pyroptosis. Interestingly, several NALP proteins are also differentially expressed in NSCLC. How various components in the inflammasome affect tumorigenesis in NSCLC may need further investigation.

Overall, our results identify a novel mechanism by which G9A enhances tumor cell proliferation and invasion by silencing CASP1 expression, and suggests that G9A may serve as a therapeutic target in NSCLC.

Materials and Methods

Ethics. All animal experiments were performed using male BALB/C nude mice (4–5 weeks old). The mice were purchased from the SLAC Laboratory Animal Center (Shanghai, China) and cared for in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. All animal experimental
protocols performed in this study were approved by the Institutional Animal Care and Use Committee at Tongji University.

**Cell culture.** Human NSCLC cell lines, PC9 and A549, were grown in DMEM medium (HyClone, Logan, Utah, USA). Culture media contain 10% FBS (Carlsbad, CA, USA) supplemented with penicillin (100 U/ml) and streptomycin (100 mg/ml) (Life Technologies, Carlsbad, CA, USA). The cells were incubated at 37 °C in a humidified atmosphere of 5% CO2.

**siRNA or overexpression plasmid transfection.** G9A siRNAs were synthesized by Ribobio Inc. (Guangzhou, China). Transfections were performed with Lipofectamine 2000 (11668019; Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. Total RNA or cell lysates were prepared 48 h after transfection and were used for real-time RT-PCR or western blotting (WB).

Sequences for siRNAs targeting G9A were as follows: #1: 5'-CCAUGCUUGUC AACUAACAUUdTdT-3' (sense) and 5'-AUGUAAUGUUAGCAUCGUAdTdT-3' (antisense); and #2: 5'-GAUAUUUGCUUAAUACCACUdTdT-3' (sense) and 5'-CCUGGCGAUGGUACCUAdTdT-3' (antisense); and #3: 5'-GGAUUGAGACUCUUGCTGdTdT-3' (sense) and 5'-UCUGCUAGGUAGCCUAAUGdTdT-3' (antisense).

**Plasmid cloning and luciferase reporter assay.** The proximal promoter region (1.5 kb) of CASP1 gene was cloned into the pGL3 vector using the following PCR primers for genomic DNA amplification: the forward primer, 5'-CTAGGGTACCCAGCACGACTGGATGAGGAGGAAG-3' and the reverse primer, 5'-GGGCTTCAATGCTCGATGGTGTG-3'. The updated protocol can be found at http://research.hudsonalpha.org/Myers/. Anti-EHMT2/G9A antibody (Abcam; ab40542) and anti-Histone H3 dimethyl Lys9 (H3K9me2) antibody (Active motif, OR, USA).

**RNA isolation and real-time RT-PCR.** Total RNA extraction from cells and cell lysates was prepared 48 h after transfection and were used for real-time RT-PCR or western blotting (WB).

**Establishment of stable G9A knockdown cell lines.** PC9 and A549 cells were infected with the lentiviral supernatant containing the lentiviral construct for G9A shRNA or the control shRNA (prepared by Target Inc., Shanghai, China). Puromycin was added to the cells for killing uninfected cells. Multiple single colonies were selected and expanded. Total RNA and cell lysates from these colonies were prepared and used for real-time RT-PCR or WB to validate the G9A knockdown effect in these cells. The detailed procedure was described as previously described.33

**Cell cycle analysis.** Cells were centrifuged, washed twice with PBS and incubated with cold 70% ethanol at 4 °C overnight. Cells were mixed with PI/RNase staining buffer (BD Pharmingen, San Jose, CA, USA; #560825) according to the manufacturer's instructions. Stained cells were analyzed using a BD Accuri C6 Flow Cytometer (San Jose, CA, USA). Results were plotted using FlowJo, LLC, Ashland, OR, USA.

**Phenotypic analysis.** The xenograft tumor growth assay was a standard method using the following PCR primers for genomic DNA amplification: the forward primer, 5'-CTAGGGTACCCAGCACGACTGGATGAGGAGGAAG-3' and the reverse primer, 5'-GGGCTTCAATGCTCGATGGTGTG-3'. The updated protocol can be found at http://research.hudsonalpha.org/Myers/. Anti-EHMT2/G9A antibody (Abcam; ab40542) and anti-Histone H3 dimethyl Lys9 (H3K9me2) antibody (Active motif, OR, USA).

**Statistical analysis.** The RNA-Seq data of LUAD were obtained from the TCGA project (http://cancergenome.nih.gov) and the firebrowse (http://firebrowse.org). Differentially expressed genes were analyzed as previously described.33 Pearson's correlation was applied to analyze the correlation coefficient between two genes in the normal or cancer samples.
Comparison of pathway analysis was carried out in R (https://www.bioconductor.org/packages/devel/bioc/manuals/clusterProfiler/man/clusterProfiler.pdf) using gene expression correlation data downloaded from cBioportal (http://www.cbioportal.org).

Gene expression and survival information for NSCLC patients were from the released database (2015 version) downloaded from http://kmplot.com/. Kaplan-Meier survival analysis was carried out using the statistics package for IBM SPSS version 22 (Armonk, NY, USA). A comparison is made between the outcomes of the control condition and one of the treatment conditions unless stated otherwise. For comparisons between two groups, Student’s t-test was used. For comparisons among multiple groups, one-way ANOVA was used. For all analyses, a P-value of <0.05 was considered statistically significant. \( P<0.05, \quad \text{**} P<0.01, \quad \text{***} P<0.001, \quad \text{****} P<0.0001.\)

Conflict of Interest
The authors declare no conflict of interest.

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Author contributions
THH, WL, TZ, ZZX and YY performed the experiments. SJC and FZ performed the bioinformatics analyses. THH, FZ, YHF, FL, XSL, LZ, GNJ and FZ analyzed and interpreted the data. FZ conceived the project, designed the experiments and wrote the manuscript with contributions from all the authors.

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