Histone Methyltransferase G9a Promotes Invasion of Non-small Cell Lung Cancer Through Enhancing Focaladhesionkinase Activationvia NF-KB Signaling Pathway

Ting Sun
City of Hope National Medical Center
Keqiang Zhang
City of Hope National Medical Center
Rajendra P. Pangeni
City of Hope National Medical Center
Wendong Li
City of Hope National Medical Center
Yong Du
General Hospital of Ningxia Medical University
Shyambabu Chaurasiya
City of Hope National Medical Center
Leonidas Arvanitis
City of Hope National Medical Center
Dan J. Raz (draz@coh.org)
City of Hope National Medical Center

Research

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Abstract

Background

Overexpression of euchromatic histone methyltransferase 2 (EHMT2 or G9a) is frequently found in a number of human cancers. Potential roles of G9a in invasion and metastasis are not well understood in non-small cell lung cancer (NSCLC). Here we investigated the effect and underlying mechanisms of G9a, and therapeutic implications of targeting G9a in the invasion of NSCLC.

Methods

G9a-associated gene sets were identified by RNAseq analysis. Migration and invasion assays were applied to examine the impact of targeting G9a by siRNA knockdown orits specific inhibitor UNC0638 in NSCLC cells. Rescue experiments were designed to investigate the effect of focal adhesion kinase (FAK) and NF-κB inhibitors on the invasion of NSCLC cells overexpressing G9a. Correlation between the protein level of phosphorylated FAK and G9a was analyzed in NSCLC tissues.

Results

Knockdown and inhibition of G9a drastically suppressed in vitro cellular proliferation, migration, and invasion; while overexpression of G9a significantly enhanced proliferation, migration, and invasion of A549 and H1299 NSCLC cells. Targeting G9a led to a significant decrease in the expression of FAK protein and activation of FAK signaling pathway in both A549 and H1299 cells. Additionally, defactinib, a potent FAK inhibitor, partially abolished the enhanced migration and invasion by overexpression of G9a in these NSCLC cells. Furthermore, G9a was found to boost nuclear factor-kappa B (NF-κB) transcriptional activity in NSCLC cells through partially downregulating inhibitor of κB (IκBα), and an NF-κB inhibitor partially abolished the enhanced FAK activation by overexpressed G9a, which suggests that G9a-enhanced invasion and activation of FAK may be mediated by elevated NF-κB activity. Notably, a strong positive correlation between the immunohistochemical staining of G9a and phosphorylated FAK proteins was identified in H1299 xenografts and 159 cases of NSCLC tissues (R = 0.408).

Conclusions

These data strongly demonstrate that G9a may promote invasion and metastasis of NSCLC cells by enhancing FAK signaling pathway via elevating NF-κB transcriptional activity, indicating potential significance and therapeutic implications of these pathways in the invasion and metastasis of NSCLCs that overexpress G9a protein.

Background

Lung cancer is a major global health problem and is the leading cause of cancer-related mortality in both men and women[1]. Although advances including lung cancer screening, targeted therapies, and immunotherapyhave improved outcomes in patients with lung cancer[2], the overall 5-year survival of

lung cancer patients is still just 5% for patients with advanced lung cancer[3]. Therefore, novel therapeutic approaches for lung cancer are urgently needed.

As a histone methyltransferase encoded by the euchromatic histone-lysine N-methyltransferase 2 (EHMT2), G9a is responsible for catalyzing mono-methylation and di-methylation of Histone H3 Lysine 9(H3K9me1 and H3K9me2), and plays an important role in regulating gene expression[4]. G9a epigenetically blocks tumor suppressors and activates oncogenes leading to carcinogenesis and cancer cell growth. Previous studies have shown that G9a expression is elevated in many types of human cancers, including lung, breast, colorectal, pancreatic, and bladder cancers[5-7]. Overexpression of G9a isassociated with enhanced proliferation and metastasis in many cancer types. However, the underlying mechanisms about how G9a participates in lung cancer metastasis are stillpoorly understood.

Tumor metastasis is the major cause of cancer-related death, yet precise mechanisms of metastasis remain incompletely understood[8]. Focal Adhesion Kinase (FAK) has been shownto play a central role in metastasis. FAK is a non-receptor cytoplasmic tyrosine kinase which is transcriptionally regulated by P53 and NF-κB[9]. It contributes to almost every aspect of tumor metastasis, as well as cancer cell adhesion, growth, migration and invasion [10, 11]. Enhanced phosphorylation of FAK at specific sites, especially at Y397, has been reported in a number of cancer types[12]. Studies have shownthat FAK isup-regulated in non-small cell lung cancers (NSCLC)[13]and is associated with an aggressive clinical course [14].In patients with KRAS mutation, FAK is a potential druggable target as it is a downstream effector of KRAS signaling[15]. In KRAS-driven lung adenocarcinomas, FAK inhibitors showed potent antitumor effects in KRAS-G12V-INK4a/ARF-deficient lung cancers in mice[16]. Preclinical data in KRAS-mutated lung cancers showed that inhibition of FAK resulted in sustained DNA damage by suppressing DNA repair mechanisms and enhancing radiation sensitivity [17].In addition, FAKis considered to bea potential therapeutic target to prevent tumor metastasis in a number of solid tumors[18].

Recently, we reportedthatG9a was frequently overexpressed in NSCLC tissues and that targeting G9a potently suppressedthe growth of NSCLC cells, suggesting that G9a may be a therapeutic target for NSCLC[19]. In this study, we extended ourinvestigation into the role of G9a in NSCLC cellular migration, invasion, and metastasis. We studied potential mechanisms of G9a-mediated invasion and its impact onFAK signaling pathway in NSCLC. Additionally, we explored the effect of targeting G9a oninvasive potential andthe activation of FAK signaling pathway in NSCLC cells.

Materials And Methods

Cultured cell lines and treated inhibitors

Two human RAS mutated non-small cell lung adenocarcinoma cell lines were used in this study. Both cell lines were purchased from American Type Culture Collection (ATCC, USA). H1299 was cultured in RPMI 1640 (ATCC, USA) medium and A549 was cultured with DMEM/F12 (Corning, USA) medium. For all cell lines, growth medium was supplemented with 10% FBS, 100 U/mL penicillin and 100 mg/mL streptomycin. UNC0638, a selective inhibitor for G9a and GLP methyltransferase, was purchased from

Cayman Chemical Company (USA) and used at a concentration of 2.5 μM and 5 μM. Defactinib hydrochloride, a FAK inhibitor which inhibits FAK phosphorylation at Tyr397, was purchased from MedChemExpress (USA) and used at a concentration of 1μM. Parthenilide, NF-κB inhibitor, was purchased from Abcam and used at a concentration of 20 μM.

Plasmids, Cell transfection, and qRT-PCR

G9a mRNA (isoform a) was cloned into pcDNA3.1 vector with Hind III and EcoR I (New England BioLabs, USA). Two independent G9a siRNAs:Cat No.10630318 (5'-GGCAUCUCAGGAGGAUGCCAAUGAA-3') and Cat No. 10620319 (5'-UUCAUUGGCAUCCUCCUGAGAUGCC-3') purchased from Thermo Fisher Scientific Corporation (Carlsbad, USA) were used to silence G9a expression by using RNAi maxi reagent (Invitrogen, USA) as describe previously [20]. Cells were transfected with G9a-pcDNA3.1 plasmid and pcDNA3.1 plasmid as control by using Lipofectamine 3000 reagent (Invitrogen, USA) according to the manufacturer's protocol. Total RNAs from cells were extracted with RNeasy Mini Kit (Cat#: 74104) and cDNA was synthesized from 1.0μg RNA with QuantiTech@ Reverse Transcription Kit (Cat#:205313) which were purchased from Qiagen Company. G9a (Forward: GGACATCATCACTCATGCGGAAA, Reverse: GCAAAACCATGTCCAAACCAGG), FAK (Forward: GCAATGGAGCGAGTATTAAAGGTC, Reverse: TGGCCACATGCTTTACTTTGTGAC) and GAPDH (Forward: GGGAAGGTGAAGGTCGGAGTC, Reverse: GAGTTAAAAGCAGCCCTGGTGAC) primers were used in the qRT-PCR assay to quantitatively measure the mRNA expression. Power SYBR@ Green PCR Master Mix was purchased from Life Technology LTD (Cat#: 4367695). Data was presented as ratio to control to show the relative expression of target, normalized to internal control and relative to the calibration sample.

Cell proliferation, Migration and Invasion Assays

For cell proliferation, cells were seeded as 1×10^5 per well in 6 well plates. After 72 hours, cells were harvested and counted with Cello-meter Spectrum (Nexcelom Bioscience, USA). For migration assay, after transfected with siRNA, plasmids or treated with inhibitors for 48 hours, cells were detached from tissue culture plate by using 0.25% Trypsin-EDTA solution, and pelleted by centrifugation. The cells were resuspended in serum free culture medium and seeded as 5×10^5 per well in a 6-well plate. After 24 hours, cells were scratched with the tip of 20ul pipette and washed twice with PBS. Cells were then cultured with serum free medium andtreated with inhibitors. Images were captured immediately after scratching (0 hour), and then at 24h for H1299 and 36h for A549. Five locations were imaged for each well. Migration distances were measured by image J software. Three measurements were performed for each image and average migratory distance was determined. Cell invasion assay was performed using 8 μm Matrigel invasion chambers (Corning, USA). Cells were detached after being transfected or treated with inhibitors for 48 hours as previously described [21]. Then 2.5×10^4 cells were seeded on top of the insert with serum-free medium. Lower chambers were filled with growth medium with 10% FBS. The culture medium was continuously supplemented with respective inhibitor in the inhibitors treated group. After 22 hours, media containing the remaining cells that did not migrate from the top of the membrane was carefully removed. Cells were fixed by placing the insert in 500 μl 10% formalin for 15 min, air-drying for 10 min at room

temperature, and staining with 1% crystal violet solution for 10 minutes. The fixed inserts were then washed with distilled water. Trans-wells were imaged by microscope and cells were counted using Qu path software[22]. Data were analyzed and presented as the ratio of the average of total number in treated group divided by the average of total number in the paired control group.

Western Blot

Total cellular protein samples were extracted with SDS lysis buffer and heated in 95°C for 7 minutes. Proteins were separated by electrophoresis and transferred onto Polyvinylidenefluoride (PVDF) membranes (Thermo Fisher, USA). Membrane was blocked with 5% nonfat milk in PBST for 40min in room temperature and blotted with primary and secondary antibodies, respectively. A primary antibody against β-actin (1:3000) was purchased from Santa Cruz Biotechnology. FAK (1:1000), pFAK (Tyr397) (1:1000), IκBα (1:1500), H3K9-me2 (1:2000) antibodies were purchased from Cell Signaling Technology. Antibody against G9a (1:1000) was purchased from GeneTex Inc (USA).

Luciferase Reporter Assay of NF-κB

pSI-Check2-hRluc-NFkB-firefly was a gift from Qing Deng (Addgene plasmid # 106979; http://n2t.net/addgene:106979; RRID:Addgene_106979) [23]. For each well, 50 ng NF-κB reporter plasmids were transfected into cells at a concentration of 3×10^3 per well for H1299 and 5×10^3 per wellfor A549 in 96-well platesusing lipofectamine 3000 reagent, with either siRNAs orthe overexpression plasmids. For inhibitor experiments, cells were treated with UNC0638 12 hours after transfection with the NF-κB plasmid. At 72hours post transfection, cells were washed with PBS and lysed with 40μl 1× passive lysis buffer. *Firefly* and *renilla* luciferase activity were detected by using Dual-Luciferase® Reporter Assay System (Promega, USA). For each group, five replicate experiments were performed. *Firefly* and *renilla* luciferase activities were determined and calculated as described previously (24).

In vivo Xenograft Model

All animal protocols wereapproved by the institutional animal care and use committee (IACUC,16005)of City of Hope and performed in the animal facility at City of Hope in accordance with federal, local, and institutional guidelines. H1299 cell line was transfected with G9a-shRNA-GFP plasmid and transfected cells were selected with puromycin. NOD/SCID/IL2R gamma null mice (NSG, 24-27g, 8-10 weeks of age, 6 mice per group) from Jackson Labs (Bar Harbor, USA) were used for xenograft experiment. A suspension of 5×10^6 tumor cells (H1299 shRNA-control and H1299 G9a-shRNA) in 0.1 ml RPMI 1640 was injected into the subcutaneous dorsa of mice at the proximal midline [25]. About 3 weeks after injection, mice were euthanized by CO_2 inhalation, and tumors were excised and fixed for IHC staining.

Immunohistochemistry Analysis

This study was reviewed and approved by the Institutional Review Board (IRB,13240) of City of Hope National Medical Center. All subjects gave written informed consent. A total of 250 patients with lung

adenocarcinoma and squamous carcinoma who underwent surgical resection for curative intent between 2002 and 2014 without preoperative chemotherapy or radiation therapy were included, as previously reported[19]. Formaldehyde-fixed paraffin embedded (FFPE) tumor samples were sectioned and stained for G9a, H3K9me2 and pFAK (Tyr397) at the pathology core laboratory, as previously described [26]. Antibody against pFAK (Tyr397) (1:2000) was purchased from Invitrogen (Cat#: 700255). Antibody against G9a (1:300) was purchased from GeneTex (Cat#: 129153). Antibody against H3K9me2 (1:200) was purchased from Abcam (Cat#: 5327). G9a and pFAK (Tyr397) IHC staining was scored according to different expression positive percentage and intensity as 0 negative, +minimal, ++ moderate and +++ strong, as we reported previously.

Results

G9a regulates PTK2 gene expression in NSCLC cells

Through gene expression profilingby RNA-Seq analysis, of which the original data is saved on NCBI GEO website with accessionnumber GSE113493, and gene set enrichment analysis, we found that knockdown of G9a significantlysuppressed gene sets of cell motility and cell adhesion signaling pathways of NSCLC cells, which are critical for cancer invasion and metastasis; notably, PTK2 that encodes FAK protein is among these significantly downregulated genes in the gene sets, suggesting thatPTK2may represent an important G9a target [19]. Tofurther validate the finding and explore the association between G9a and FAK expression, we first knocked down G9a in H1299 and A549 cells with two independent siRNAs. qRT-PCR showed that G9a mRNA expression level was significantly silenced in both H1299 and A549 cell lines transfected with specific G9a siRNAs (**Figure 1a**P < 0.01). Simultaneously, mRNA expression of PTK2 gene was down regulated in these two cells (**Figure 1b,** P < 0.05). As shown in **Figure 1c**, in both A549 and H1299 cell lines, upon knockdown of G9a, the level of H3K9me2 was decreased, and FAK proteinwas also dramatically decreased in both H1299 and A549 cell lines. These data indicate that FAK expression is associated with G9a expression in NSCLC.

Knockdown and inhibition of G9a suppresses cell migratory and invasive potentialof NSCLC cells

To investigate the potential roles of G9a in cell migration and invasion of NSCLCs, cancer cells were first transfected withtwo different G9a-specific siRNAs for *in vitro* migration and invasion assays. We observed that cell proliferation was also significantly suppressed (P < 0.01) upon G9a knockdown in these two lung cancer cells (**Figure S1a&b**). As shown in **Figure 2a**, compared to the control group, cell migrationpotential was significantly suppressedupon knockdown of G9a in both H1299 (upper panel) and A549 (lower panel) cell lines. A significantlydecreased migrationdistance (P<0.001) was observed in both H1299 and A549 cells upon G9a knockdown **(Figure 2b)**.

Invasionassay wascarried out in G9a-silenced cells. As shown in **Figure 2c**, cells transfected with G9a siRNA showed lower invasive potentialcompared to cell transfected with the control siRNA in both H1299 (upper panel) and A549 (lower panel) cells.Statistics analysis showed that compared to the controls, the invasive potentialof the G9a-silenced groupsdecreased significantly (P<0.05) in the two NSCLC cells

(**Figure 2d**). To examine if pharmacological inhibition on G9a activity will also suppress the invasive potential of lung cancer, UNC0638, a selected G9a inhibitor, was used to suppress the G9a methyltransferase activity. As shown in **Figure 2e**, a drastically decrease of H3K9me2protein was found in cancer cells treated with UNC0638, indicating the methyltransferase activity of G9a was inhibited significantly. Similar to the data of G9a knockdown experiment, cell migration in these two cells was also suppressed by UNC0638 treatment (**Figure S2a**). Compared to the control group, a significant decrease was observed in UNC0638 treated cells (**Figure 2f, left panel)** in these two cells ($P<0.05$). Besides, the number of invasive cells in A549 and H1299 cell lines was also significantly decreased by UNC0638 treatment (**FigureS2b**). Quantitative analysis of invasive data was presented in **Figure 2f, right panel**($P<0.05$). Therefore, above data suggests G9aplays an important role in migratory and invasive potential of NSCLC cell lines.

Inhibition of G9a suppresses the activation of FAK signal pathway in NCSLC

Considering the critical role of FAKin cancer migration and invasion, we hypothesize that G9a may regulate cell invasion and migration through FAK signal pathway. To investigate this underlying mechanism, total protein was extracted from cells either transfected with G9a siRNA or treated with UNC0638. Western blot analysis showed that, compared to control siRNA group, FAK and phosphorylation of FAK (**Figure 3a**) were drastically decreased in both A549 and H1299 cells upon G9a knockdown. Similarly, after being treated with UNC0638 for 72 hours, total FAK andphospho-FAK (pFAKat Tyr397, an autophosphorylation site on the activatedFAK that is used as an indicator for FAK activation) proteinswere decreasedin the both two cell lines (**Figure3b**). In contrast, overexpression ofG9a significantly elevated the levels of FAK and pFAK (Tyr397) (**Figure 3c**). These data indicated that G9a positively regulates the expression of PTK2 gene and activation of FAK signal pathway in NSCLC cells.

Overexpression of G9a enhancescell migratoryand invasive potentialof NCSLC cells

To further validate the role of G9a in migration,invasionand activation of FAK signal pathway of NSCLC cells,we inserted G9a gene into the pcDNA3.1 vector (**Figure S2**) andexamined if G9a overexpression will enhance the invasion and activation of FAK signaling pathway.Consistently, compared to control cells, we observed overexpression of G9A significantly increased lung cancer cell proliferation (**Figure 4Sa&b**, $P < 0.05$). Meanwhile,migration of NSCLC cells (**Figure 4a**) was significantly increasedwith overexpressed G9a protein ($P<0.001$,**Figure 4b&c**). Furthermore, compared to control group, the invasion of NSCLC cells was also increased significantlywithoverexpression of G9a protein (**Figure d&e**, $P < 0.05$). These data further demonstrated a key role of G9a in cell invasion, migration as well as activation of FAK signal pathway in NSCLC.

G9a regulates cell migration and invasion through FAKsignal pathway

To investigate whether G9a promotes cell invasion and migration directly or indirectly through activating FAK signal pathway, arescue experiment was designed. In this experiment we investigate if the FAK inhibitor (defactinib) can suppress the G9a enhanced FAK activation and cell invasion in NSCLC cells. As

shown in **Figure 5a**, overexpression of G9a enhanced the phosphorylation of FAK, while FAK inhibitordefactinib attenuated or even completelyabolished the elevated phosphorylated FAK. While cell migration (**Figure 5b&c**) and invasion (**Figure 5d&e**) were boosted by overexpression of G9a,similar to the change in phosphorylated FAK, the enhanced migration and invasion was reversed by FAK inhibitorin these two cell lines. Therefore, the above data suggests that the elevated cell invasion by overexpression of G9awas partially abolished by FAK inhibitor, and G9a promoted cell invasion and migration via activating of FAK signal pathway.

G9a activates FAK signal pathway by elevatingNF-κB transcriptional activity

Studies have alreadydemonstrated that FAK was transcriptionally regulated by P53 and NF-κB transcription factors(9). We observed the regulation of FAK expression by G9a in both p53-wildtype A549 and p53-null H1299 cells, therefore, we hypothesized that G9a might activate FAK expression through NF-κB signaling pathway. Therefore, we investigated the effect of knockdown and overexpression of G9a on transcriptional activity ofthe NF-κB-controlled luciferase reporter. Dual-luciferase assays showed that silencing of G9a significantly suppressed NF-κBluciferase activity (**Figure 6a**, $P<0.05$), while overexpression ofG9a significantly increased NF-κB activity(**Figure 6b**, $P<0.01$) in these two NSCLC cell lines.UNC0638 also significantly suppressed NF-κB luciferase activity in both the cell lines (**Figure 6c,** $P < 0.05$). Interestingly, the level of IκBα protein,an inhibitor of NF-κB signaling pathway,was foundto beupregulated by knockdown of G9a anddownregulated by overexpression of G9a (**Figure 6d)**, indicating thatsuppression of G9a on IκBα expression may contribute to the over-activation of NF-κB signaling pathway.

To further investigate whether G9a activates FAK signal pathway through NF-κB, we treated the G9a-overexpressedH1299 cellswith a NF-κB inhibitor,Parthenilide.Western blot analysis showed that NF-κB inhibitor could partially abolish the elevatedphosphorylation of FAK (Tyr397) that wascausedby overexpression of G9a (**Figure 6e**). Taken together, these data indicatethat G9a activates FAK signal pathway partially through NF-κB signal pathway.

pFAK (Tyr397) level is correlated with G9a *in vivo* and in NSCLC tissues.

We further investigated the correlation betweenG9a expression and FAK activation inxenografts usingstable G9a-KD H1299 cells. As shown in **Figure 7a**, tumor tissue was stained with H&E staining. Compared to the xenograft tissues of the controls, the IHC intensities of G9a and H3K9me2 were strongly decreased in G9a-attenuated xenograft tissues. Consistent with the *in vitro* data, pFAK (Tyr397) levelsweredown-regulated dramatically in G9a-attenuated xenograft tissues, suggesting the activation of FAK signaling pathway was suppressed.

Furthermore, we analyzed the correlation between G9a and FAK expression afterIHC staining of pFAK (Tyr397). IHC analysisdemonstrated nuclear G9a staining**(Figure 7b)**, and quantitative analysis of G9a onIHC slideswas conducted as previously reported(19). IHC analysis showed cytoplasmicpFAK (Tyr397)stainingin tumors and staining was absentinadjacent normal cells. pFAK (Tyr397) IHC staining

in the same tissue arrays werequantitatively scored using the same scoring method, andrepresentative images of IHC scoringare shown in **Figure 7c**. Pearson correlation analysis was used to examine the correlation between G9a and pFAK (Tyr397)IHC staining in these tumor tissues,and the analysis revealed that pFAK (Tyr397)staining was significantly correlated with G9a staining (**Figure 7d**; R =0.408, P<0.001), indicating overexpression of G9a may enhance activation of FAK signaling pathway and then invasion and metastasis in NSCLC.

Discussion

This study demonstratedfor the first time that G9a may promote migration and invasion of NSCLC by activating FAK signaling pathway. Our data suggest that in NSCLC, G9ainhibitsIκBα protein to activateNF-κB, which in turn promotes FAK expression, promoting cell migration and invasion. Given the important role of both G9a and FAK in cancer cell growth and metastasis, this study underscores the potential role of G9a inhibition as a therapeutic target for lung cancer.

Withknockdown of G9aor inhibition of G9a methyltransferase activity, cell migration and invasion were inhibited while FAK and phosphorylation of FAK (Tyr397) were downregulated in lung cancer cells. Through rescue experiments we confirmed that G9a promotes cell growth, migration and invasion by activating FAK signaling.Functionally, G9a is responsible for mono or di-methylation of H3K9 and its activity leads to silencing ofa number of tumor suppressor genes including P53, CDH1, DUSP5 and SPRY4[27, 28]. G9a expression isassociated with poor prognosis. For example, G9a promotes cancer invasion and metastasis by silencing Ep-CAM in lung cancer [29]. Additionally, it is considered a marker of aggressive ovarian cancer and correlated with peritoneal metastasis [30]. It is also responsible for liver cancer development and metastasis by silencing tumor suppressor RARRES2 [31]. Recently, G9a has been demonstrated to promote tumor metastasis by upregulating ITGB3 in gastric cancer [32]. G9a is also involved in hypoxia-induced epigenetic regulationin ovarian cancer [33].Furthermore, G9a is known to activate hypoxia signaling pathway leading to suppression of apoptosis.Under normoxia,G9a ishydroxylated and inhibited by factor inhibiting HIF (FIH). However,under hypoxia, it is associated with hypoxia induced signal pathway in angiogenesis, including MMP2, VEGFR-2 and FAK in HUVECs [34]. G9awas also found tosuppresshypoxia-dependent gene type-II cadherin CDH10, which leads to advanced cellular movements in breast cancer cell lines[35].

In addition, G9a expression is closely associated with tumorigenesis, metastasis and drug resistance in many cancers [36]. We previously reported that G9a promotes tumor growth via enhancing HP1α and silencing APC2 expression (19).Researchers have shown that G9ais transcriptionally regulated by special AT-rich sequence-binding protein 2 (SATB2). Decreased expression of SATB2 leads to increased invasiveness of NSCLC through upregulation of G9a expression [37]. G9a expression in endometrial cancersisnegatively correlated with E-cadherin expression and enhanced the depth of myometrial invasion[38]. Others have reported that the G9a inhibitor UNC0638 reduced growth and metastasisof breast cancer and also significantly suppressed epithelial-mesenchymal transition-mediated cellular migration and invasion [39]. Another G9a methyltransferase activity inhibitor, UNC0646,was shownto

suppresshepatocellular tumor growth butactivate cellular apoptosis and metastasis [40]. Although G9a inhibitors hold promise as a therapy for solid tumors, none have successfully been advanced to clinical trials.

Meanwhile, we have found that FAK signaling pathway is a downstream target of G9a.FAK plays a pivotal role in transducing signals from the plasma membrane to the nucleus, andphosphorylation of FAK promotescancer cell growth, invasion, and angiogenesis[41]. High expression of pFAK (Tyr397) is associated with aggressive behavior of lung cancer, including rapid growth, frequent metastasis and poor overall survival[42].A phase I study of the FAK inhibitor BI 853520 showed that FAK inhibitor produced promising results in patients with advanced or metastatic solid tumor [43].Severalother phase I and phase II clinical trials are currently undergoing for FAK inhibitors in cancer[44]. FAK could be a potential combination therapy target for patients with G9a overexpressinglung cancers.

In addition, this study demonstratedfor the first time that G9a regulates FAK through NF-κB signaling pathway. NF-κBis activated by various stimuli, including hypoxia, cytokines (including TNF-α and IL-1β), growth factors, and DNA damage in tumor tissues. These stimuli lead to the activation of the inhibitor of κB (IκBα) kinase (IKK) complex. The activated IKK complex promotes the phosphorylation of IκBα and its degradation. IκBαforms a complex with P50 and P65. With the degradation of IκBα,P50 and P65 translocate to the nucleus to regulate downstream gene expression[45]. Previous study reported an association between G9a activity and NF-κB signaling [46]. In our study, we revealed that elevated expression of G9a suppressed IκBαprotein and increased NF-κB luciferase activity, activating FAK signaling and promoting migration and invasion in NSCLCs. Similarly, recent study has demonstrated that NF-κB signaling pathway playsa very important role in oncogenesis and metastasis[47].NF-κBsignaling pathway has previously been shown to be associated with FAK activation and increased cell proliferation in lung cancer [48]. Tetraspanin 15 (TSPAN15) promoted esophageal cancer metastasis via activating NF-κB through regulating IκBαphosphorylation[49].In NSCLC, researchers have found that HMGB1mediated cancer cell motility is mediated in part by NF-κB-activated FAK signaling pathway[50].These and other studies demonstrate the importantrole of NF-κB in FAK signaling pathway and cancer metastasis and suggest thatNF-κB can also be considered as a potential druggable target in patients with G9a overexpressing lung cancers.

Conclusions

In summary, we have revealed a novel mechanism by whichG9a mediatesinvasion and metastasis in NSCLC.Wehave demonstratedthatthe role of G9ain lung cancer invasion and metastasisis mediated in part by enhanced activation of FAK that appears to be mediated byNF-κB(**Figure 7e**). These findings suggest that the interaction between G9a, FAK, and NF-κBmay be druggable targets for NSCLCsthat overexpress G9a.

Data Analysis

All experiments were performed in duplicates or triplicates and repeated at least two times. For group-group statistics analysis, data were analyzed for variation and significance using Student's T test. All data are shown as mean ± SD. Statistical significance was set at $P < 0.05$. Pearson's correlation coefficient was used to measure correlation of G9a and pFAK (Tyr397) gene expression.

List Of Abbreviations

G9a, Euchromatic histone-lysine N-methyltransferase 2 (EHMT2); FAK, *focal adhesion kinase; NF-κB,* Nuclear Factor kappa-light-chain-enhancer of activated B cells; NSCLC, non-small cell lung cancer; H3K9me2, di-methylation at the 9th lysine residue of the histone H3 protein; Ep-CAM, *Ep*ithelial cell adhesion molecule; qRT-PCR, quantitative reverse transcription polymerase chain reaction; CDH1, cadherin 1; DUSP5, Dual-specificity phosphatase 5;SPRY4, sprout RTK Signaling Antagonist 4;RARRES2, Retinoic Acid Receptor Responder 2; ITGB3, Integrin beta-3; KRAS, Kirsten rat sarcoma 2 viral oncogene homolog; ATCC, American Type Culture Collection; GLP, G9a-like protein; pFAK, phosphorylation of FAK; IκBα, inhibitor of κBα; IKK, IκB kinase. HIF, hypoxia induced factor; MMP2, matrix metallopeptidase 2; VEGF-2, vascular endothelial growth factor C; HUVECs, human umbilical vein endothelial cell; HP1α, heterochromatin protein 1α; APC2, adenomatous polyposis coli protein 2; SATB2, special AT-rich sequence-binding protein 2; HMGB1, high mobility group box 1.

Declarations

Ethic approval and consent to participate

For animal xenograft experiments, all animal protocols wereapproved by the institutional animal care and use committee (IACUC,16005)of City of Hope and performed in the animal facility at City of Hope in accordance with federal, local, and institutional guidelines. For tissue assay patients' sample collection, this study was reviewed and approved by the Institutional Review Board (IRB,13240) of City of Hope National Medical Center. All subjects gave written informed consent.

Consent for publication

All authors agreed on the publication of this manuscript.

Availability of data and materials

Figure S1-S3 was shown in additional file1.

Competing interests

The authors declare no conflict of interest.

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Authors contributions

TS performed experiments and analyzed data, wrote the manuscript. KZ conceived and supervised all the studies; and revised the manuscript. RP and WL provided material and technicalsupport on xenograft experiments. YD provided suggestions on experiments and revision. SC provided technical support on IHC staining. LA did the IHC tissue array scoring. DR conceived the study and revised the manuscript. All authors read and approved the final version of the manuscript.

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Figures

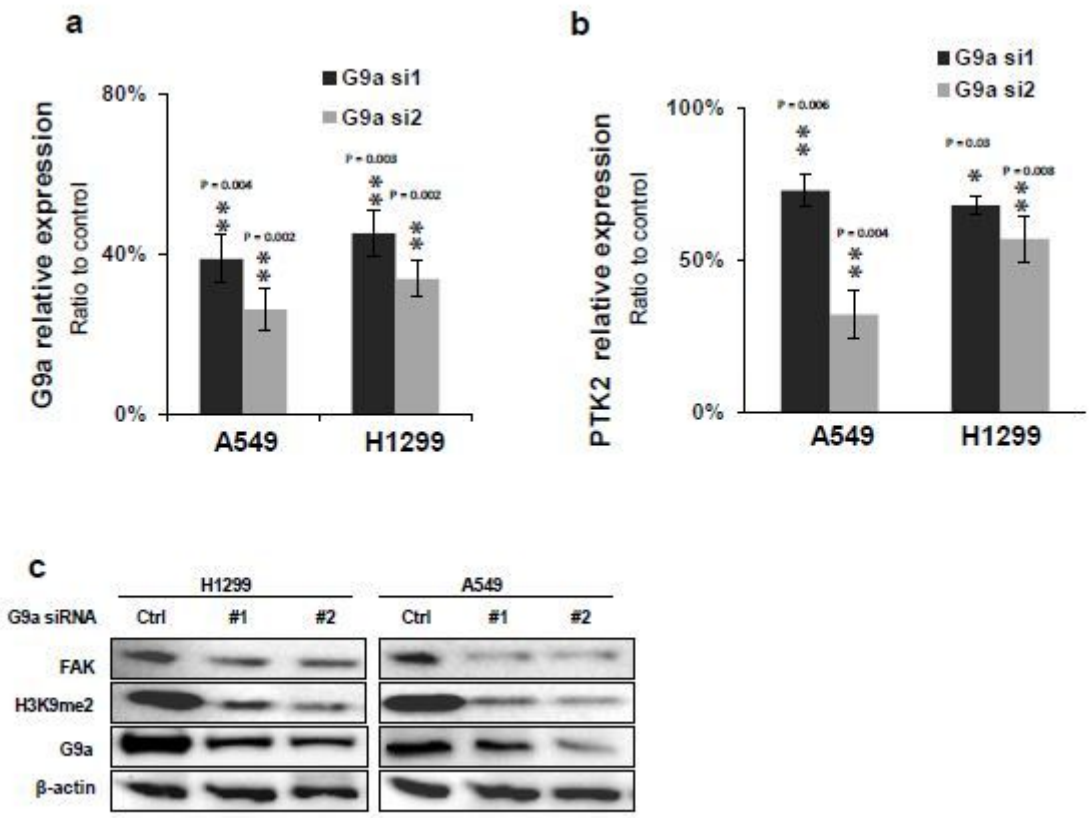

Figure 1

G9a regulates PTK2 gene expression in NSCLC cells a.qRT-PCR showed that G9a gene expression was down-regulated in NSCLC transfected with G9a siRNAs. Data was present as the ratio to control (**P < 0.01, compared to the controls). b. qRT-PCR showed that PTK2 gene expression was down-regulated upon silence of G9a in NSCLC(**P < 0.01). c.Western blot showed that H3K9-Me2 and FAK was downregulated upon silence of G9a in NSCLC. Data are presented as means ± standard division (SD). Three independent experiments were performed.

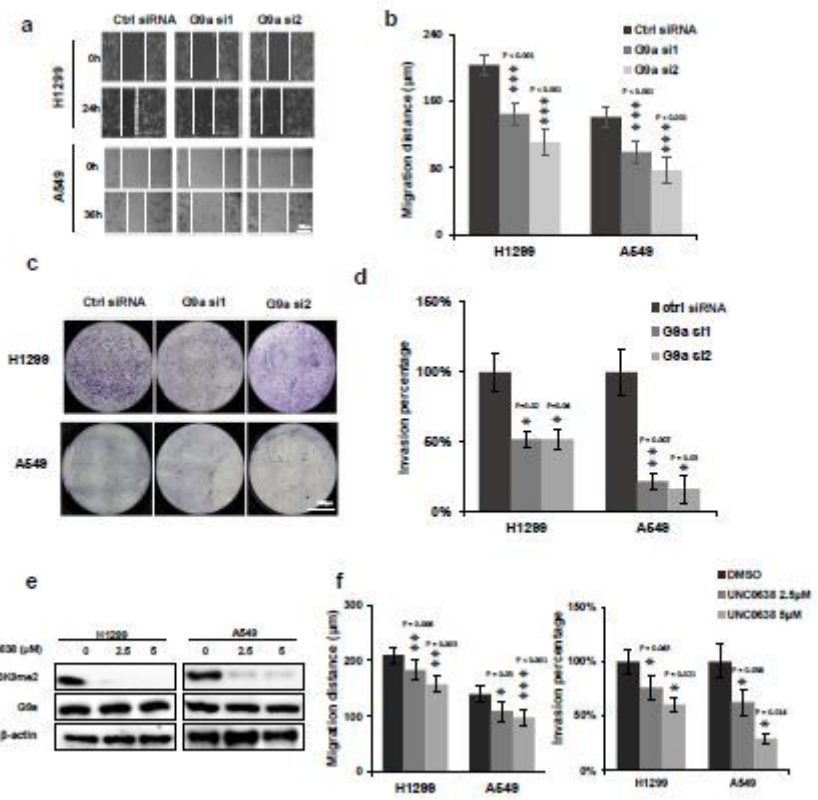

Figure 2

Knockdown and inhibition of G9a suppresses cell migratory and invasive potential of NSCLC cells a.Representative image of scratch experiments showed that knockdown of G9a suppressed cell migration of NSCLCs. Scale bar refersto 200μm. b.Quantitative analysis of migration distances in G9a-silenced NSCLC cells.***P < 0.001. c.Representative image of invasive cells showed that knockdown of G9a suppressed cell invasive potential of NSCLCs, scale bar refersto 2000μm. d. Quantitative analysis for invasive cells in G9a-silenced NSCLC cells, *P < 0.05, **P < 0.01. e. Western blots showed that H3K9-Me2 was decreased by G9a inhibitor UNC0638.f. Inhibition of G9a suppressed cell migration and invasion of NSCLC. Left panel: Quantitative analysis of migration distances of H1299 and A549 cells treated with UNC0638. ** p < 0.01. Right panel: Quantitative analysis of invasive cells for H1299 and A549 cell lines treated with UNC0638. Data are presented as the normalized percentage to the control. *P < 0.05, **P < 0.01, ***P < 0.001.Data are presented as means ± standard division (SD). Three independent experiments were performed.

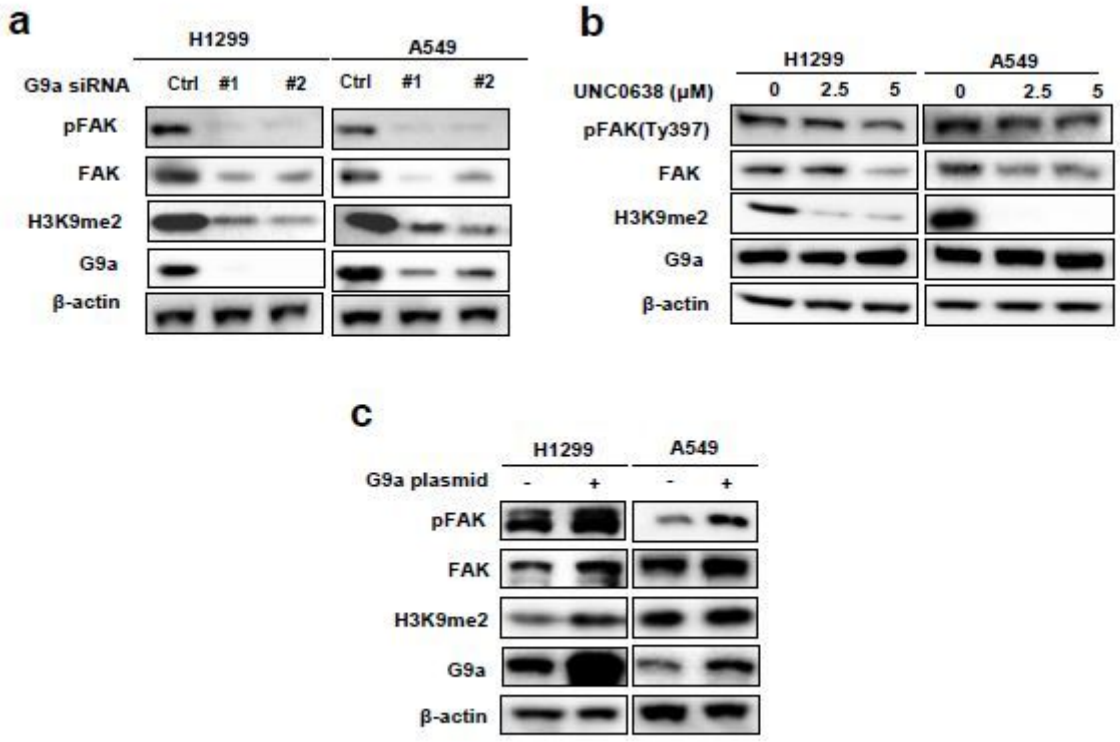

Figure 3

Inhibition of G9a suppresses the activation of FAK signal pathway in NCSLC a. Western blot analysis showing that the total FAK and pFAK (Tyr397) was decreased in G9a-silenced NSCLC cell lines. b. Western blots showing G9a inhibitor UNC0638 decreased the FAK and pFAK (Tyr397) in NSCLC cell lines. c. Western blot analysis showing that overexpression of G9a elevated FAK and pFAK(Tyr397) in NSCLC cell lines. Three independent experiments were performed.

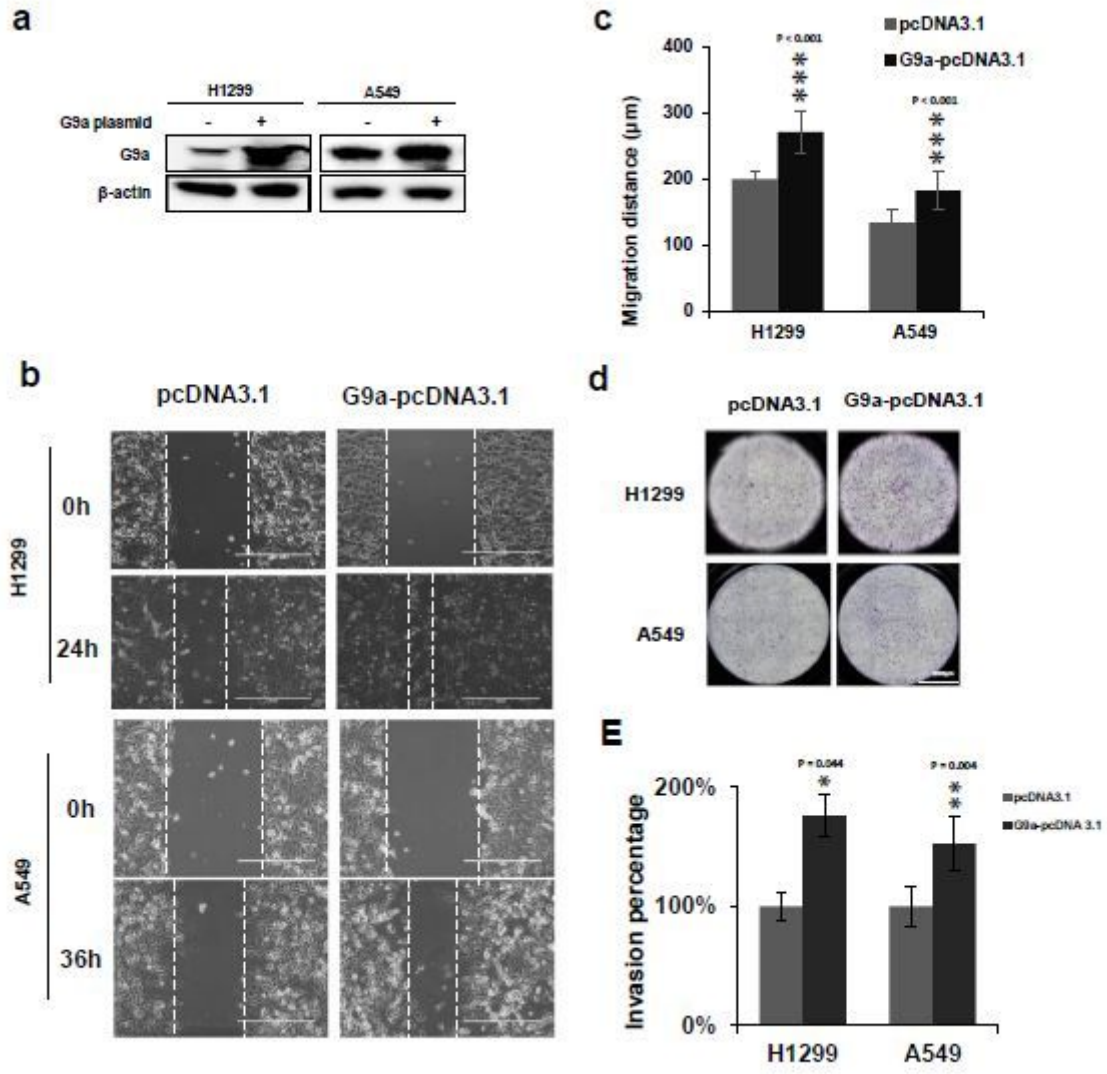

Figure 4

Overexpression of G9a enhances cell migratory and invasive potential of NCSLC cells a. Western blot analysis of G9a overexpression in H1299 and A549 cell lines. b. Representative images of scratch experiments at 0h and 24h for H1299, 0h and 36h for A549 in NSCLC cell lines with overexpressed G9a. Scale barrefersto400μm. c. Representative images of invasive cells in G9a-overexpressed NSCLC cell lines. ***P < 0.001. d. Representative images of invasive cells in G9a-overexpressed NSCLC cell lines. Scale barrefersto2000μm. e. Quantitative analysis for invasive cells in G9a-overexpressed NSCLC cell lines. Data are presented as percentage of control, *P < 0.05, **P < 0.01. Data are presented as means ± standard division (SD). Three independent experiments were performed.

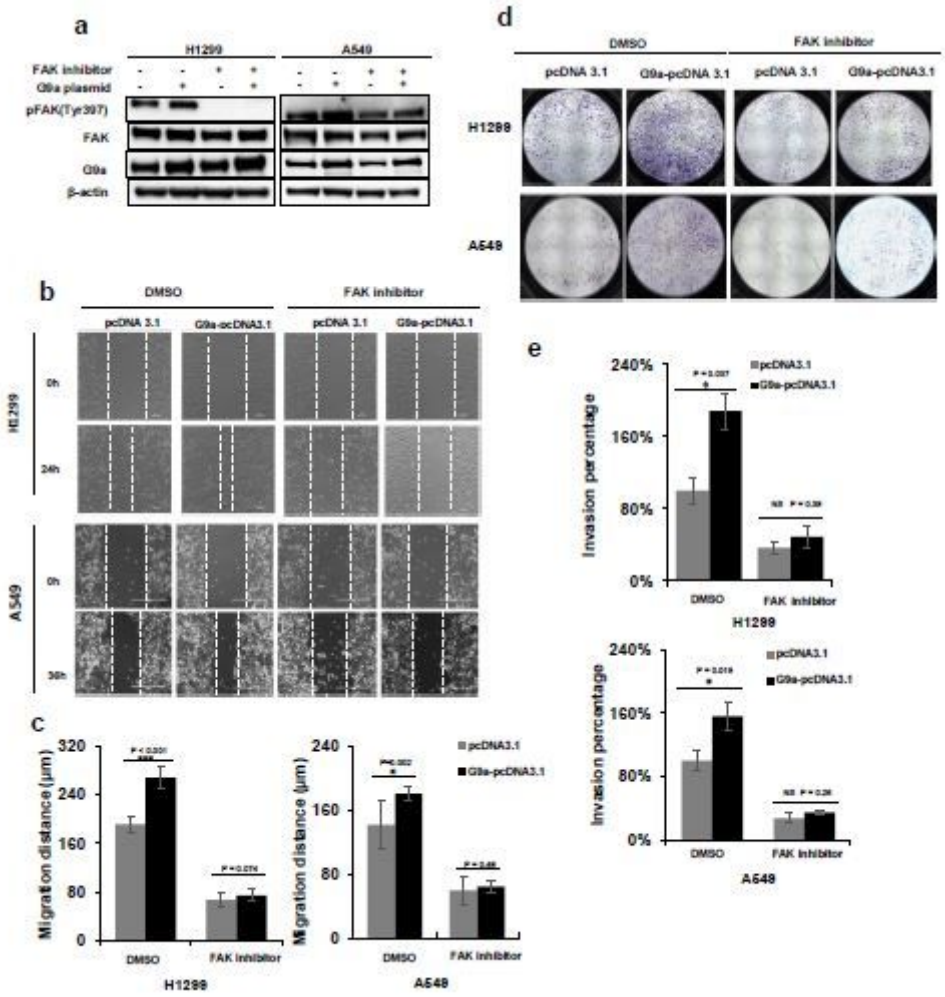

Figure 5

FAK inhibitor attenuates the enhanced cell migration and invasion by G9a overexpression a. Western blots showing that activation of FAK signal pathway was attenuated by FAK inhibitor in G9a overexpressed NSCLC cells. b. Representative images of scratch experiments at 0h and 24h for H1299, 0h and 36h for A549 shows the enhanced migration was reversed by FAK inhibitor in NSCLC cell lines. Scale bar=400μm. c. Quantitative analysis of migration distances in A549 and H1299 cells treated with FAK inhibitor. **P < 0.01, ***P < 0.001, NS, no significant difference. d. The enhanced invasive ability was attenuated by FAK inhibitor in A549 and H1299 cell lines. Representative image of invasive cells in NSCLC cells treated with FAK inhibitor. Scale bar refersto 2000μm. e. Quantitative analysis for invasive cells in G9a-overexpressed A549 and H1299 cancer cells treated with FAK inhibitor. Data are presented as percentage of control, *P < 0.05; NS, no significant difference. Data are presented as means ± standard division (SD). Three independent experiments were performed.

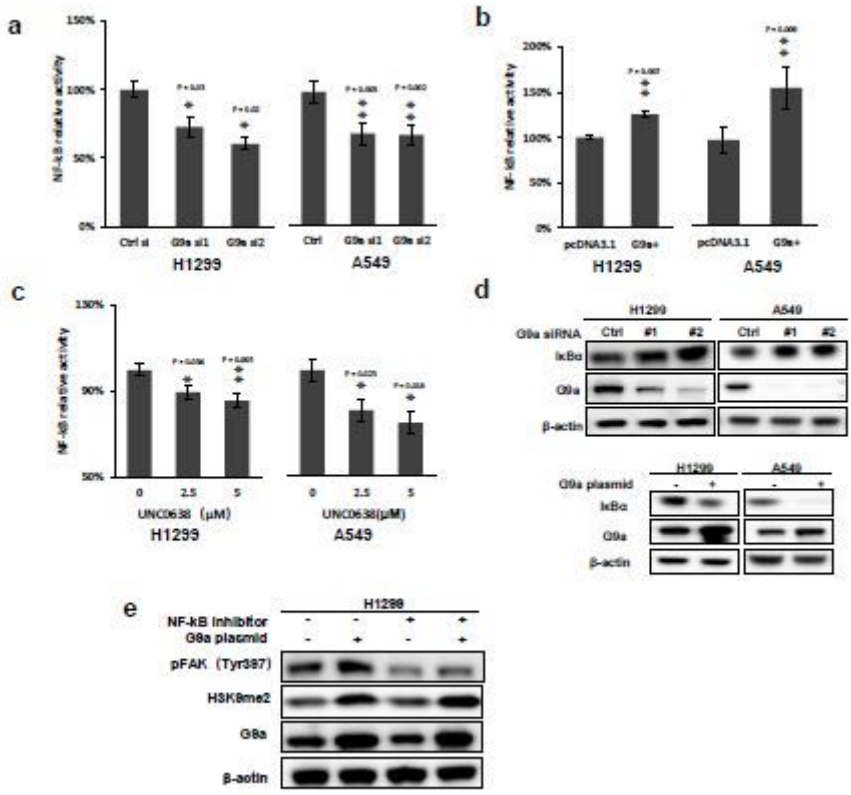

Figure 6

G9a activates FAK signal pathway by elevating NF-kB transcriptional activity a. Knockdown of G9a suppressed NF-κB transcriptional activity in H1299 and A549 cells; *P < 0.05, **P < 0.01 (compared to control siRNA). b. Overexpression of G9a enhanced NF-κB transcriptional activity in A549 and H1299 cells; **P < 0.01(compared to pcDNA 3.1).c. NF-κB activity was suppressed in UNC0638 treated NSCLCs; *P < 0.05, **P < 0.01 (compared to DMSO treated cell). d. Western blot analysis showed that the expression of IκBα was up-regulated upon overexpression of G9a in A549 and H1299 cells. e. Western blots showed that NF-κB inhibitor partially abolished the elevated phosphorylation of FAK (Tyr397) that was enhanced by overexpression of G9a. All of data are presented as means ± standard division (SD). For each group, experiments were performed as triplicates and experiments were repeated three times.

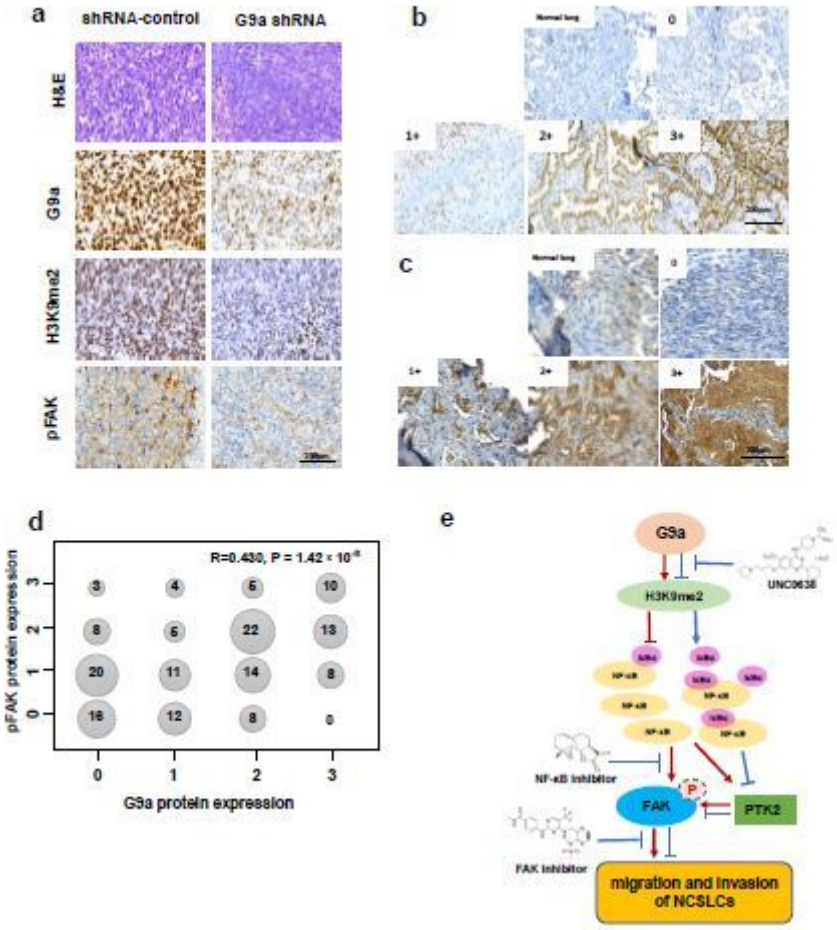

Figure 7

pFAK (Tyr397) level is correlated with G9a in vivo and in NSCLC tissues. a. Representative images of H&E and proteins IHC stained in these xenografts showed that G9a, H3K9me2 and pFAK (Tyr397) were strongly decreased in G9a-attenuated xenograft tissues. Scale barrefersto200μm. b. Representative image scored as 0, 1+, 2+, 3+ for IHC staining of G9a in NSCLC tissue arrays. Scale barrefers to 200μm. c. Representative image scored as 0, 1+, 2+, 3+ for IHC staining of pFAK(Tyr397) G9a in NSCLC tissues. Scale bar refers to 200 μm. d. IHC staining of pFAK (Tyr397) was significantly positively correlated with that of G9a in NSCLC tissues. The numbers in the bubble shows the case for each scored stage. R = 0.408, p < 0.001.e.Schematic diagram of the potential mechanisms forG9a-enhanced invasion and related therapeutic implicationsin NSCLC. G9a di-methylates H3K9, inhibits IκBα expression, thus releases and activates NF-κB signal pathway, finally promotes PTK expression and phosphorylation of FAK at tyrosine 397,eventually promotes NSCLC cell migration and invasion. G9a specific siRNAs and inhibitors down-regulate global H3K9me2 and upregulate IκBα protein, inhibits NF-κB activity, thus suppresses PTK expression and phosphorylation of FAK, eventually suppress migration and invasion of NSCLC. NF-κB inhibitor partially abolished elevated phosphorylation of FAK enhanced by G9a, and FAK inhibitor partially abolished the elevated migratory and invasive potential enhanced by G9a.

Supplementary Files

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