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ORIGINAL ARTICLE

E2F1-mediated repression of WNT5A expression promotes brain metastasis dependent on the ERK1/2 pathway in EGFR-mutant non-small cell lung cancer

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Abstract

Brain metastasis (BM) is associated with poor prognosis in patients with advanced non-small cell lung cancer (NSCLC). Epidermal growth factor receptor (EGFR) mutation reportedly enhances the development of BM. However, the exact mechanism of how EGFR-mutant NSCLC contributes to BM remains unknown. Herein, we found the protein WNT5A, was significantly downregulated in BM tissues and EGFR-mutant samples. In addition, the overexpression of WNT5A inhibited the growth, migration, and invasion of EGFR-mutant cells in vitro and retarded tumor growth and metastasis in vivo compared with the EGFR wide-type cells. We demonstrated a molecular mechanism whereby WNT5A be negatively regulated by transcription factor E2F1, and ERK1/2 inhibitor (U0126) suppressed E2F1's regulation of WNT5A expression in EGFR-mutant cells. Furthermore, WNT5A inhibited β-catenin activity and the transcriptional levels of its downstream genes in cancer progression. Our research revealed the role of WNT5A in NSCLC BM with EGFR mutation, and proved that E2F1-mediated repression of WNT5A was dependent on the ERK1/2 pathway, supporting the notion that targeting the ERK1/2-E2F1-WNT5A pathway could be an effective strategy for treating BM in EGFR-mutant NSCLC.

Keywords EGFR mutation · Brain metastasis · WNT5A · β-Catenin · Tumor suppressor

Abbreviations

BM	Brain metastasis
NSCLC	Non-small cell lung cancer
EGFR	Epidermal growth factor receptor
TKI	Tyrosine kinase inhibitor
ERK1/2	Extracellular signal-regulated kinase ½
WNT5A	Wnt family member 5A
ROR2	Receptor tyrosine kinase-like orphan receptor 2
β-Catenin	Catenin beta 1
E2F1	E2F transcription factor 1
PI3K	Phosphoinositide 3-kinase
HDAC	Histone deacetylase
cDNA	Complementary DNA
qRT-PCR	Quantitative real-time PCR
ChIP	Chromatin immunoprecipitation
HR	Hazard ratio
mut	Mutation
wt	Wild-type
GEO	Gene Expression Omnibus
TFs	Transcriptional factors
OS	Overall survival

Introduction

Lung cancer is the most commonly diagnosed cancer and the leading cause of cancer death worldwide [1]. Non-small cell lung carcinoma (NSCLC) constitutes approximately 85% of all lung cancer cases [2] and is frequently characterized by the presence of brain metastasis (BM), which is common in patients with advanced NSCLC with the incidence rate of more than 25% [3]. BM is associated with poor overall survival (OS) and causes significant peripheral

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neuropathy, cognitive effects, and emotional consequences [4].

Epidermal growth factor receptor (EGFR) mutations are observed in approximately 50% of patients with lung cancer [5]. EGFR mutation induces ligand-independent receptor dimerization and activation, resulting in the phosphorylation of EGFR and activation of the downstream signaling cascade, such as the PI3K/AKT or extracellular signal-regulated kinase 1/2 (ERK1/2) pathway [6, 7]. Emerging evidence suggests that EGFR-mutant advanced NSCLC is particularly prone to develop the BM, implying that EGFR mutation could be a risk factor for BM in NSCLC [5, 8]. However, the exact mechanism of how EGFR mutation in lung cancer contributes to brain metastasis remains largely unknown.

Unlike canonical Wnt/β-catenin signaling, WNT5A, as a member of non-canonical Wnt signaling, controls various aspects of cell migration, which are essential in normal and malignant development [9] and the aberrant expression of WNT5A induces distinct effects in different types of cancer [10–12]. Previous studies have shown that WNT5A has two protein isoforms, namely WNT5A-long (WNT5A-L) and WNT5A-short (WNT5A-S); the former inhibits the proliferation of tumor cell lines, while the latter promotes their growth [13]. In addition, accumulating evidences suggest that WNT5A exerts antagonistic and agonistic effects on the WNT/β-catenin pathway dependent on the receptor that binds to it [14]. For example, in ROR2 (receptor tyrosine kinase-like orphan receptor 2) or FZD4 (frizzled class receptor 4), WNT5A has been implicated in restrained canonical WNT/β-catenin signalling in the presence of ROR2, whereas, when bound to FZD4, WNT5A enhanced WNT/β-catenin pathway [15, 16]. Notably, WNT5A has a vital role in neuron development in association with the EGFR pathway [17]. However, the underlying mechanism for this correlation in BM with lung cancer has not yet been elucidated.

In this study, we revealed the anti-tumor effects of WNT5A in EGFR-mutant NSCLC and identified ERK1/2-E2F1-WNT5A pathway which may be critical for progression of BM in EGFR-mutant NSCLC.

Materials and methods

Patients' samples

Peripheral blood samples were collected from 94 NSCLC patients enrolled in the Union Hospital of Tongji Medical College, HUST. The demographic and clinicopathological details of patients showed in Supplementary Table 1. Among the cases, 68 were sequenced by next-generation sequencing (NGS) to identify the mutation status of EGFR, including 35 BM + and 33 BM-. The Institutional Review Board of Huazhong University of Science and Technology approved this study. Written informed consent was obtained from all legal guardians of the patients.

Cell lines

A549 and HCC827 cell lines were acquired from the Institute of Biochemistry and Cell Biology of the Chinese Academy of Sciences, Shanghai, China. H1299, H1975, and 293 T cell lines were procured from the American Type Culture Collection (ATCC). HCC827 cells harbored an activating in-frame deletion in exon 19 (Ex19del) of EGFR, whereas H1975 cells harbored activating L858R and T790M mutations in exon 20 of EGFR. A549 and H1299 were EGFR wide-type (wt) cells. All cells were cultured in an RPMI 1640 medium (Gibico, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (10% FBS). Cells were grown at 37 °C in a humidified atmosphere of 5% CO_2. The cell identification report is shown in the attached data.

Real-time quantitative RT-PCR (qRT-PCR) and PCR

Total RNA was extracted from plasma samples using a BIOG RNA IsoQuick Kit (#61011), and the RNA from cells was isolated using a TRIzol reagent (Invitrogen) and then subjected to reverse transcription with the PrimeScript RT Reagent Kit (Takara, China). The qRT-PCR was performed by means of the SYBR Premix Ex Taq II kit (Takara, China). The data were analyzed using the $2^{-\Delta\Delta C_T}$ method. The primer design and sequence are shown in Supplementary Table 2.

Overexpression and knockdown of genes

Human E2F1 overexpression vector and shRNA were purchased from GenePharma (Shanghai, China). Human ROR2 siRNA were purchased from Qijing_biology (Wuhan, China). An empty vector (Mock) and scramble shRNA (sh-Scb) were used as controls. Lentiviral WNT5A (NM_003392), the long isoform, overexpression and shRNA, which carry the EF1 promoter-driven firefly luciferase and puromycin-resistance gene, and the corresponding virus were purchased from GenePharma (Shanghai, China).

Establishment of stable lung cancer cell lines

WNT5A overexpression and knockdown viral vectors were transfected into HCC827 and H1299 cells according to the operating manual, and after screening with puromycin (Invitrogen), stable cancer cells were established. The E2F1 overexpression vector and shRNA were also transfected, and selected with puromycin (5 μg/mL), stable cell lines HCC827 and H1975 were obtained.

Drugs

Icotinib (99.9% purity) was kindly supplied by Zhejiang Beta PharmaInc (Zhejiang, China) and dissolved in dimethyl sulfoxide (DMSO, Sigma, St. Louis, MO, USA) to 50 mM of stock solution and stored at − 20 °C and then diluted with culture medium before use.

Cell counting kit-8 assay

The cell viability assay was conducted following the instructions for the use of the CCK-8 kit (Dojindo Laboratories, Kumamoto, Japan). Tumor cells (5×10^3 cells/well) were seeded in 96-well plates, and cell viability was determined by measuring the absorbance at 450 nm. All experiments were replicated three times.

Western blotting

Tumor cell or tissue protein was prepared using 1 × cell lysis RIPA buffer (Beyotime) supplemented with a protease-inhibitor cocktail (Thermo Scientific, Waltham, MA, USA) and PMSF. Western blotting was performed as described previously [18, 19]. Anti-EGFR (#4267), anti-p-EGFR (#3777), anti-AKT (#4691), anti-pAKT (#4060), anti-ERK1/2 (#4695), anti-pERK1/2 (#4370), and anti-E2F1 (#3742) antibodies were purchased from Cell Signaling Technology (CST, Danvers, MA). Anti-WNT5A (A12744), Anti-ROR2 (A5620), anti-b-catenin (A11512), and anti-GAPDH (AC033) were acquired from ABclonal (USA), and anti-Histone H3 (ab176842) was procured from Abcam (USA).

Chromatin immunoprecipitation (ChIP)

ChIP assay was carried out using the ChIP-IT® Express kit (Cat. No. 53008; Active Motif, Carlsbad, CA, USA) following the manufacturer's protocol. Antibodies used for this assay included control IgG (ab150081, Abcam) and antibody specific to E2F1 (#3742, CST). DNA was extracted and sonicated into a 200 bp size. For qRT-PCR, the gene promoter-specific primers (human WNT5A promoter bearing E2F1 site [− 823/− 806]) (Figure S3A) were used, as explicitly described in a previous report [20].

Luciferase reporter assay

TOP-FLASH and FOP-FLASH reporters were obtained from Millipore. A human WNT5A luciferase reporter was established by inserting oligonucleotides containing E2F1-binding sites into pGL3-Basic (TsingKE biological technology). The pRL-TK vector was used as an internal control for luciferase activity (Promega). Relative β-catenin activation was determined using the TOP-FLASH/FOP-FLASH ratio. Dual-luciferase assay was undertaken, as reported previously [21].

Transwell invasion assays

The invasion assay was conducted in transwell chambers (Costar, Corning Inc., NY, USA), starved tumor cells (1.5×10^6) were transferred onto the upper chamber coated with Matrigel (BD Biosciences, San Jose, CA, USA). After 24 h of incubation, invaded cells were stained with 0.1% crystal violet (Sigma) and counted under the microscope (Olympus, Tokyo, Japan).

Wound-healing assay

For the wound-healing assay, cells were seeded into 6-well plates (1×10^5 cells/well), and the monolayer was scratched with a 10 µL plastic pipette tip to create a uniform wound. The wound margin distance between the two edges of the migrating cell sheets was photographed under a phase-contrast microscope. All experiments were performed in triplicate.

In vivo xenograft assay

Tumor cells were prepared by suspending 6×10^6 HCC827 and H1299 cells in 100 µL of serum-free media and injecting into the right rear flanks of 4-week-old female BALB/c nude mice (Beijing Huafukang Bioscience Company, Beijing, China). Tumor growth was monitored and recorded every week after the inoculation. Tumor volume was calculated as follows: 0.5 × tumor length × tumor width^2. Four weeks later, tumor burden was evaluated and tumors were excised and dissected for characterization and further studies. All animal experiments were performed under the protocol approved by the Institutional Animal Care and Use Committee of Huazhong University of Science and Technology.

Brain metastatic xenografts

The mouse model of brain metastasis was performed as described previously. Female nude mice (5–6 weeks of age) were purchased from Beijing Hua Fukang Bioscience Company (Beijing, China). H1299 and HCC827 cell suspensions were prepared by stably transfecting the cells with WNT5A lentivirus (1×10^7 cells/mL in PBS). 50 µL of cell suspension was slowly injected into the intracarotid artery of nude mice. The in vivo Optical Imaging System (In Vivo FX PRO, Bruker Corporation) was used to acquire fluorescent images of each mouse after an intraperitoneal injection of 150 mg/kg luciferin at a final concentration of 15 mg/

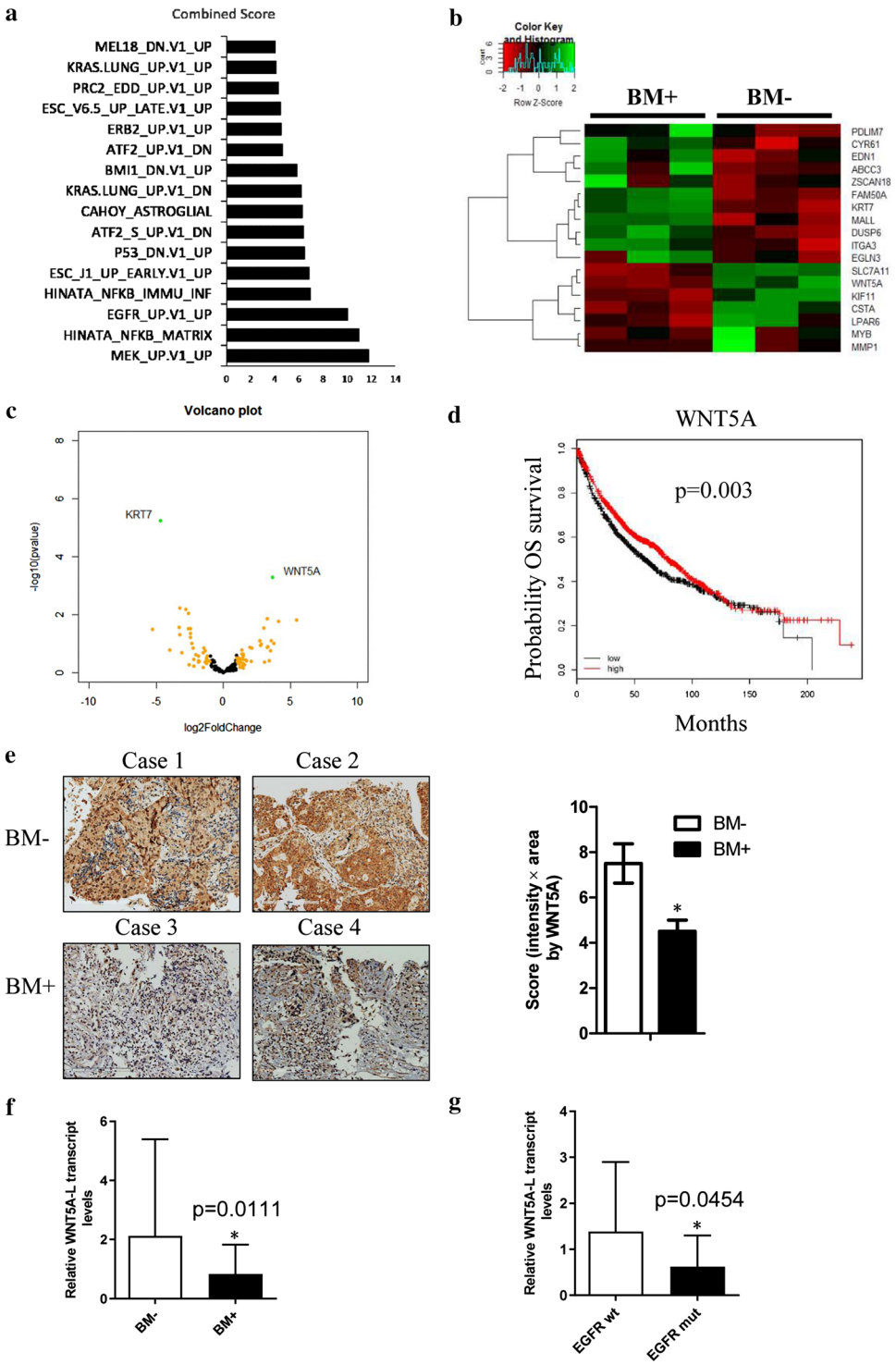


Fig. 1 Identification of WNT5A associated with brain metastasis of NSCLC. **a** MSigDB Oncogenic Signatures enriched by RNA sequencing data, 566 differentially expressed genes identified and all oncogenic signatures with p-value less than 0.05 were indicated. **b** Heatmap for the differentially expressed genes enriched in EGPR signature with BM+ or BM−. **c** Volcano plot for RNA profiling of 182 EGPR signature genes in RNA sequencing dataset, compared to BM + group, according to the value of $\log_2$Foldchange ($\log_2$FC) and padj, Orange dots ($\log_2$FC > 1, padj > 0.1), Green dots ($\log_2$FC > 1, padj < 0.1), Black dots ($\log_2$FC < 1, padj > 0.1). **d** Kaplan–Meier curves indicating the overall survival of NSCLC patients with high or low WNT5A expression (cutoff value = 368, Affy ID:213425_at), log-rank test. **e** Representative IHC staining and quantitative analysis showing the expression of WNT5A in lung tumors from NSCLC patients with or without BM. *P < 0.05, as determined by the t test. **f** qRT-PCR analysis of WNT5A-L expression in the plasma sample of BM− (n = 46) and BM + NSCLC patients (n = 48) **g** qRT-PCR analysis of WNT5A-L expression in the 35 plasma sample of BM + NSCLC patients with EGFR mutation (n = 25) or not (n = 10). *P < 0.05, as determined by the t test.

mL (Goldbio St. Loius, MO, USA). The presence of brain metastases was histopathologically confirmed.

Immunohistochemical (IHC) analysis

Immunohistochemical staining and quantitative evaluations were performed as previously described [22]. Antibodies used included Ki-67 (7309-1-AP, Proteintech) and CD31 (ab28364, Abcam). At least two pathologists blinded to the investigation assessed the degree of positivity. The IHC scoring system consisted of determining the percentage of positively stained tumor cells and estimating the intensity of staining. The percentage of Ki-67 positive cells was expressed as the ratio between the number of positive tumor

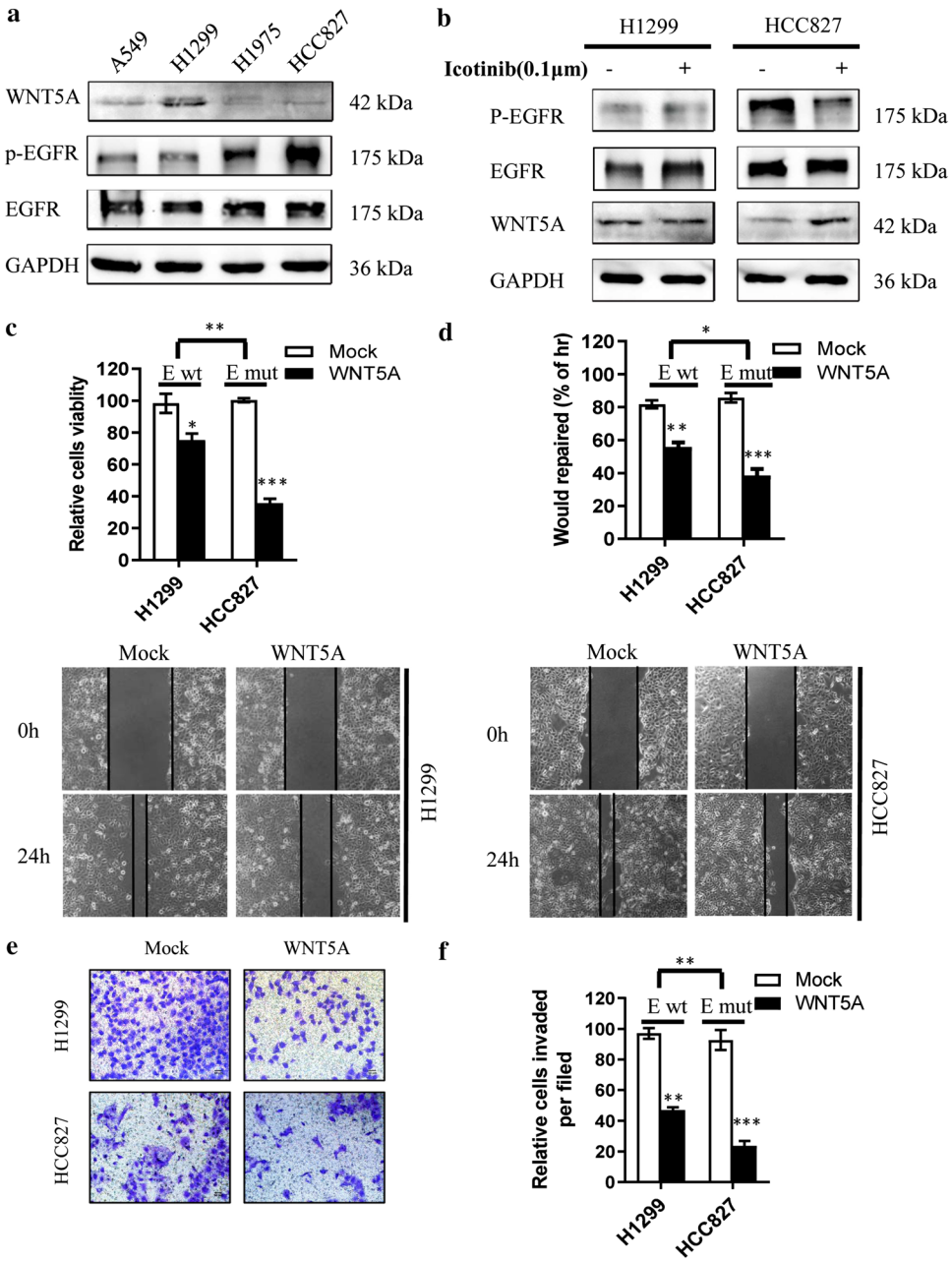


Fig. 2 WNT5A expression was downregulated in EGFR mutated NSCLC cell lines. **a** The expression of WNT5A in NSCLC cell lines, including EGFR-mutant cell (H1975, HCC827) and EGFR wt cell (A549, H1299) was detected by Western blotting. **b** cells were treated with icotinib (0.1 μM) for 48 h and proteins were evaluated for p-EGFR, total EGFR and WNT5A levels using Western blotting. **c** The proliferative ability of HCC827 and H1299 cells after transfection of WNT5A and vector (Mock) using CCK-8 assay. **d** Wound-healing assays were performed to assess NSCLC cells migration. Wound closure was determined 24 h after the scratch. **e**, **f** Representative images and quantification of transwell assays indicated the invasive capability of NSCLC cells stably transfected with WNT5A and mock. bar = 20 μm. Data are expressed as mean ± SEM, $^{*}P < 0.05$, $^{**}P < 0.01$, one-way ANOVA, unpaired two-sided *t* test for two groups

cells and the total number of tumor cells counted in the field. The mean number of micro-vessels in the five parenchymal areas detected by CD31 in tumor areas was regarded as the micro-vessel density (MVD).

Data mining of public datasets

The RNA sequencing datasets of NSCLC patients with or without BM had been deposited in Gene Expression Omnibus (GEO) and were accessible with the login number GSE126548. The differentially expressed genes identified by RNA Sequencing from GEO datasets GSE16476 were subjected to oncogenic signature enrichment analysis. Public GEO datasets GSE14340 were established using the Affymetrix Human Genome U133 Plus 2.0 Array platform (GPL570). Genes upregulated in MCF-7 cells (breast cancer) positive for ESR1 [GeneID = 2099] and engineered to express ligand-activable EGFR were accessed to find genes rich in EGPR signature. Differentially expressed genes between two groups were screened using unpaired Student's *t* test and correction multiple testing methods. The log-rank test and correction multiple testing analyses were applied to evaluate the survival significance of each gene in NSCLC patients.

Statistical analysis

Data from the GEO database (GSE126548) were processed as aforementioned. Results were reported as the mean ± SEM. The cutoff for gene expression was defined by average values. Comparisons between two groups were made using the unpaired *t* test. The differences between multiple groups were determined using the one-way ANOVA and the post hoc Tukey's HSD test. The statistical significance of overlap or the expression of correlation was determined using Fisher's exact test and Pearson's product-moment correlation analysis, respectively. The log-rank assay was employed to compare the difference in survival. $P < 0.05$ was considered to be statistically significant.

Results

WNT5A expression decreased in patients with brain metastasis of NSCLC

To unearth the genes crucial for BM of NSCLC, we performed RNA sequencing analysis to screen the differentially expressed genes in primary tissues of NSCLC patients with BM + and BM− (GSE126548). As shown in Fig. 1a, the EGFR signature was enriched according to the molecular signatures Database ($P < 0.05$) according to oncogenic signature enrichment analysis. Furthermore, the 182 genes rich in the EGPR signature were analyzed by accessing Gene Set EGFR_UP.V1_UP. As shown in Fig. 1b, c, the heatmap and volcano plot showed that WNT5A expression was significantly downregulated in the BM + group, suggesting that WNT5A expression has some potential relevance to BM in NSCLC.

Loss of WNT5A expression was associated with a poor clinical outcome in NSCLC

Analysis of the Kaplan–Meier plotter [23] revealed that the high expression of WNT5A in NSCLC patients was associated with a good outcome (Hazard ratio [HR] = 0.83, $P = 0.003$) (Fig. 1d), a HR below 1 suggests a smaller risk. A large number of studies have shown that the two isoforms of WNT5A, WNT5A-L and WNT5A-S possess intrinsically different activities and behave as tumor suppressor and oncogene, respectively [13, 24]. Owing to WNT5A expression was significantly downregulated in primary tissues of NSCLC patients with BM + from RNA-seq, suggesting that WNT5A may play an inhibitory role in brain metastasis of NSCLC, thus WNT5A-L was chose as an object in the next study. In addition, WNT5A expression was lower in lung tumor tissues of BM + NSCLC patients compared to the BM- group ($n = 5$), by employing IHC assay (Fig. 1e). Moreover, 94 plasma cases of NSCLC patients were evaluated by qRT-PCR, with WNT5A-L primers, and showed that WNT5A expression in BM + NSCLC patients ($n = 48$) was significantly lower compared with the BM- group ($n = 46$) ($P = 0.0111$) (Fig. 1f). Furthermore, we screened the BM + NSCLC that had been analyzed by NGS ($n = 35$). Notably, the expression of WNT5A in the EGFR-mutant group ($n = 25$) was significantly lower than that of the EGFR wild-type (wt) group ($n = 10$) (Fig. 1g) ($P = 0.0454$). We also found that EGFR mutation was more inclined to occur in BM, which was consistent with the previous reports (Supplementary Table 1). These data suggest that the loss of WNT5A might be involved in the development of BM in EGFR-mutant NSCLC.

WNT5A inhibits the growth and aggressiveness of EGFR-mutant NSCLC cells

To confirm the hypothesis that WNT5A was a key factor in EGFR-mutant BM in NSCLC in vitro, we explored the expression of WNT5A on EGFR-mutant or wt cell lines. Compared with EGFR wt cell lines (A549 and H1299), the expression of WNT5A was lower in EGFR-mutant cell lines (HCC827 and H1975), with an increase in the accompanying basal level of EGFR phosphorylation (p-EGFR) expression (Fig. 2a). In addition, we detected the mRNA levels of WNT5A-L in four cell lines, the results showed that the transcription levels of WNT5A-L were different in above cell lines, which were consistent with that of protein expression (Supplementary Fig. 1a). However, the expression of WNT5A-S was hardly detected (no data), indicated that it is mainly WNT5A-L that plays a major role, consistent with our previous hypothesis. Furthermore, icotinib (0.1 μM, 24 h), inhibit EGFR activation, resulted in the upregulation of WNT5A expression in HCC827, but on the contrary, no significant changes in the H1299 (Fig. 2b). These data suggest that the expression of WNT5A is dependent on EGFR-mutant-induced activation signaling in NSCLC.

To explore the role of WNT5A in EGFR-mutant NSCLC, the H1299 and HCC827 cells stable transfection of WNT5A overexpression and knockdown lentivirus were constructed (Supplementary Fig. 1b–d). Remarkably, WNT5A overexpression transfected EGFR-mutant cell lines exhibited more apparent inhibition ability of cell proliferation per the CCK-8 assay (Fig. 2c). Also, wound-healing assay and matrigel invasion assays revealed that WNT5A more significantly inhibited the migration and invasiveness of EGFR-mutant cells in comparison with EGFR wt cells (Fig. 2d–f). Conversely, knockdown of WNT5A in cells resulted in increased of cell viability, migration and invasiveness, more apparent in EGFR-mutant cell lines compared with EGFR wt cells

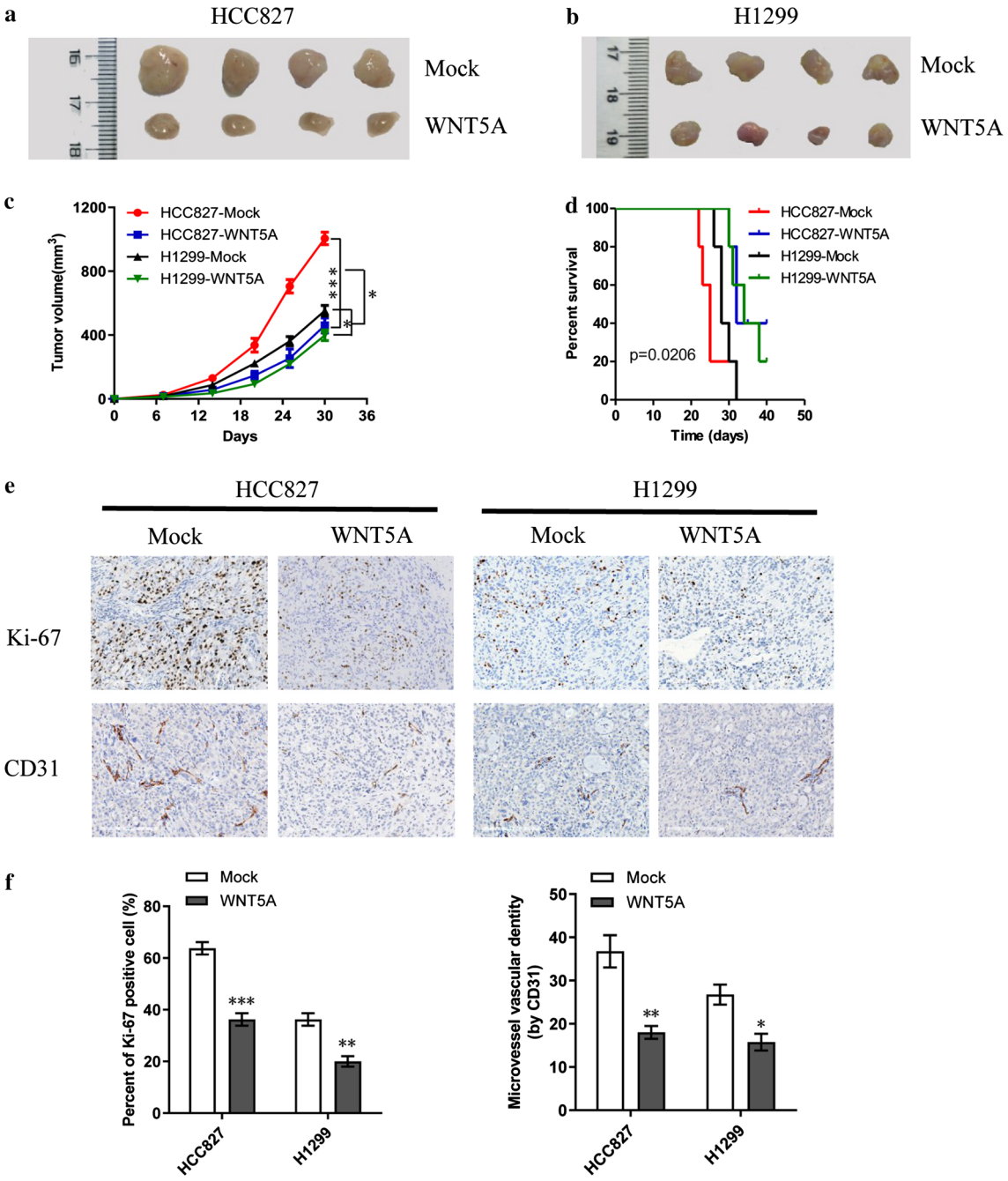
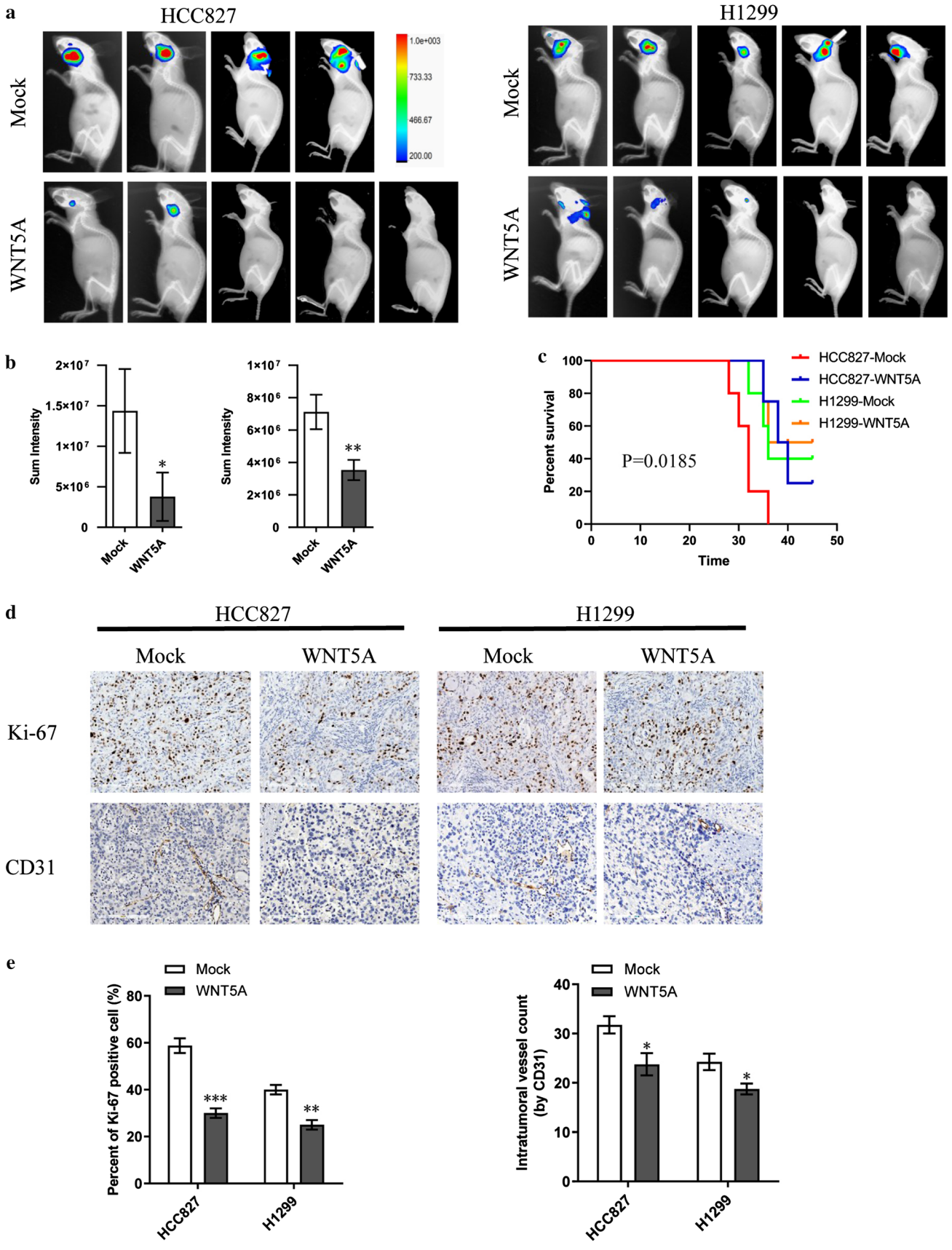


Fig. 3 WNT5A inhibited the tumorigenesis in vivo. **a**, **b** HCC827 and H1299 cells stably transfected with Mock and WNT5A were injected subcutaneously into nude mice ($n=4$). Forty days after the injection, mice were photographed and killed. Tumor growth curves were plotted. **c** Compare differences in body weight data between the four groups ($P<0.05$). **d** Kaplan–Meier curves of nude mice treated with subcutaneous injection of HCC827 and H1299 cells stably transfected with Mock or WNT5A, ($n=5$ for each group). log-rank test for survival comparison. **e**, **f** Representative IHC staining and quantitative analysis of Ki-67 and CD31 in xenograft tumors. Scale bars: 200 μm. The data are expressed as the mean ± SEM, $*P<0.05$, $**P<0.01$, $***P<0.001$, as determined by unpaired two-sided *t* test

(Supplementary Fig. 2a–e). These results suggest that WNT5A suppresses cell growth and aggressiveness, specifically in EGFR-mutant NSCLC cells in vitro.

WNT5A suppressed tumorigenesis and brain metastasis in vivo

To further elucidate the anti-tumor effect of WNT5A in EGFR-mutant NSCLC cells in vivo, the xenograft model


a
HCC827
H1299
Mock
WNT5A
1.0e+003
733.33
466.67
200.00
b
Sum Intensity
2×10^7
1.5×10^7
1×10^7
5×10^6
0
1×10^7
8×10^6
6×10^6
4×10^6
2×10^6
*
**
Mock
WNT5A
c
Percent survival
100
80
60
40
20
0
P=0.0185
0
10
20
30
40
50
Time
HCC827-Mock
HCC827-WNT5A
H1299-Mock
H1299-WNT5A
d
HCC827
H1299
Mock
WNT5A
Ki-67
CD31
e
Mock
WNT5A
Percent of Ki-67 positive cell (%)
80
60
40
20
0
***
**
HCC827
H1299
Intratumoral vessel count (by CD31)
40
30
20
10
0
*
*

◂**Fig. 4** WNT5A inhibited the brain metastasis in vivo. **a**, **b** Representative bioluminescent images in brain metastatic mice. $P=0.0325$ in HCC827 group, P = 0.01 in H1299 group. HCC827 and H1299 cells stably transfected with Mock and WNT5A were injected into the intracarotid artery of nude mice ($n=5$). One mice died in HCC827 group during observed period. $*P<0.05$, $**P<0.01$, as determined by unpaired two-sided t test. **c** Kaplan–Meier curves of nude mice treated with tail vein injection of HCC827 and H1299 cells stably transfected with mock or WNT5A ($n=5$ for each group). Log-rank test for survival comparison. **d**, **e** Representative IHC staining and quantitative analysis of Ki-67 and CD31 in brain xenograft tumors**.** Scale bars: 200 μm. Data are representative of twice independent experiments and expressed as the mean ± SEM, $*P<0.05$, $**P<0.01$, $***P<0.001$, as determined by unpaired two-sided t test

and BM model of mice were established by implanting stably-transfected HCC827 and H1299 cells with WNT5A overexpression. The overexpression of WNT5A significantly inhibited tumor growth and tumor size in HCC827 group. In contrast, the inhibitory effect in the H1299 group was slight (Fig. 3a–c and Supplementary Fig. 2f). In addition, a higher survival probability was noted in nude mice treated with HCC827 cells stably overexpressing WNT5A, but not evident in the H1299 group ($P<0.05$) (Fig. 3d). Moreover, IHC staining showed that the expression of Ki-67 and CD31 diminished significantly in cells overexpressing WNT5A ($P<0.05$) (Fig. 3e–f).

Consistently, in the BM model of mice, the overexpression of WNT5A not only significantly inhibited the growth of BM but also reduced the occurrence of BM in the EGFR-mutant group ($P<0.05$) (Fig. 4a, b and Supplementary Fig. 2g). A greater survival probability of mice was noted by stably overexpressing WNT5A in the HCC827 group, compared with the H1299 group (Fig. 4c). Additionally, IHC staining indicated that the expression of Ki-67 and CD31 diminished significantly with WNT5A overexpressed, especially in the HCC827 group ($P<0.05$) (Fig. 4d–e). All these findings show that WNT5A decreased the growth and aggressiveness of EGFR-mutant cell lines and is a potential therapeutic target in BM of EGFR-mutant NSCLC cells in vivo.

E2F1 regulated the repression of WNT5A in EGFR-mutant NSCLC

To investigate the underlying mechanism of WNT5A loss following EGFR mutation signaling in NSCLC, we analyzed publicly available datasets UCSC Genome Browser and Genomatix to filter any transcriptional factors (TFs) (Supplementary Table 4) that may regulate WNT5A expression. Based on the overlapping analyses of these genes, five potential TFs, namely E2F1, AP-1 transcription factor subunit (AP-1), NF-κB, paired box 2 (PAX2), and POU class 2 homeobox 1 (POU2F1) were identified (Fig. 5a). According to a p-value and HR value, E2F1 was the only TF considered, the high expression of E2F1 was associated with poor outcomes in NSCLC (HR = 1.46, $P=5E{-}09$), and correlating negatively with the expression of WNT5A ($R=-0.28$, $P<0.01$) (Fig. 5b, c). The other 4 TFs were AP-1 (HR = 1.13, $P=2.6E{-}05$), NF-κB (HR = 0.91, $P=0.15$), PAX2 (HR = 0.75, $P=0.056$), and POU2F1 (HR = 0.79, $P=9.3E{-}05$) (Supplementary Fig. 3a, b). Accordingly, these data suggest that E2F1 has the potential ability to suppress WNT5A expression at the transcriptional level in NSCLC.

To explore the impact of E2F1 on the expression of WNT5A in EGFR-mutant NSCLC, the overexpression and knockdown vector of E2F1 were transfected into HCC827 and H1975 cells, respectively (Supplementary Fig. 3c, d). Notably, the mRNA levels of WNT5A decreased significantly in NSCLC cells overexpressing E2F1, whereas, knocking down E2F1 produced the opposite effect (Fig. 5d), indicating that E2F1 regulated the repression of WNT5A in EGFR-mutant NSCLC cells.

Subsequently, to verify E2F1 acts as a transcriptional regulator of WNT5A, we analyzed the potential binding motif of the WNT5A promoter region by mining public databases UCSC Genome Browser and JASPAR, and unearthed two putative E2F1-binding sites with the consensus sequence of GGCGCCAAA (Fig. 5e and Supplementary Fig. 3e). Considering the WNT5A-L isoform exerts anti-tumor effects, site1 was chosen as the potential binding site. ChIP assays were performed with E2F1 antibodies and revealed that endogenous E2F1 recruitment to the promoter of WNT5A, and the binding ability was mainly reflected in the HCC827 group compared with the H1299 group (Fig. 5f and Supplementary Fig. 4a). In addition, the rescue experiments indicated that the ectopic expression of E2F1 abolished the change in the promoter enrichment of WNT5A induced by treatment with icotinib in EGFR-mutant NSCLC cells (Fig. 5g, h).

Furthermore, HEK293T cells transfected with E2F1 were subjected to luciferase reporter assays, revealed that the ectopic expression of E2F1 suppressed the transcriptional activation of WNT5A, whereas sh-E2F1 facilitated such an ability (Supplementary Fig. 4b). The ectopic expression of E2F1 also prevented increased transcriptional activation of WNT5A induced by icotinib in HCC827 and H1975 cells (Fig. 5i). Additionally, the overexpression of E2F1 suppressed the transcriptional and protein levels of WNT5A in EGFR-mutant cells and reversed the increased WNT5A expression induced by icotinib (Fig. 6a, b). Overall, findings suggest that E2F1 binds to the promoter of WNT5A directly and suppresses the expression of WNT5A at transcriptional and protein levels in EGFR-mutant cells.

The E2F1/WNT5A axis downregulates β-catenin activity mediated by the ERK1/2 pathway in EGFR-mutant cells

WNT5A has been reported to inhibit canonical Wnt/β-catenin signaling [14], to determine whether β-catenin signaling is a potential pathway for WNT5A mediation of BM in NSCLC, the expression and activity of β-catenin were measured in EGFR-mutant cells. Indeed, both overexpression and knockdown of WNT5A did not inhibit the levels of β-catenin efficiently but reduced the mRNA levels of its downstream target genes, including CCDN1 (cyclin D1), CD44 (CD44 molecule), and ZEB1 (zinc finger E-box-binding homeobox 1) significantly (Fig. 6c, d and Supplementary Fig. 4c, d). Furthermore, the TOP/FOP flash assay showed that ectopic expression of WNT5A dramatically attenuated β-catenin activity in EGFR-mutant cells significantly (Fig. 6e). Conversely, knockdown of WNT5A increased the activity of β-catenin (Supplementary Fig. 5a).

WNT5A has been implicated in antagonizing canonical WNT/β-catenin signaling via binding with the receptor ROR2 in human disease [14]. To explore the potential mechanism by which WNT5A inhibits β-catenin activity, ROR2 was silenced by siRNA in EGFR-mutant cells, and the results showed that ROR2 is important in regulating the WNT5A-β-catenin pathway in NSCLC. The knockdown efficiency of ROR2 was confirmed by qRT-PCR and western blot (Supplementary Fig. 5b, c). In addition, knockdown of ROR2 reversed the downregulation of β-catenin activity and the downstream genes expression by ectopic expression of WNT5A (Supplementary Fig. 5d, h), indicating that ROR2 was the vital receptor that induced the WNT5A antagonizing β-catenin activity in EGFR-mutant NSCLC.

ERK1/2 and PI3K/AKT singling are classic pathways that mediate EGFR signals [25]. Furthermore, U0126 (a MEK inhibitor) inactivated ERK1/2 singling and suppressed the E2F1-mediated repression of WNT5A in EGFR-mutant cells (Fig. 6f). However, LY294002 (a PI3K inhibitor) inhibited the AKT pathway but no significant effect on the expression of the E2F1/WNT5A axis (Supplementary Fig. 6a). These data indicated EGFR mutation occurs via ERK1/2 signaling, instead of the PI3K/AKT pathway, to enhance E2F1 binding to the WNT5A promoter and repress its activity, thereby increasing β-catenin activity and the levels of downstream genes (Fig. 6g-h and Supplementary Fig. 6b, c). Collectively, the data suggest that E2F1 downregulates WNT5A expression at transcriptional levels and relieves the inhibition of β-catenin, which in turn upregulating β-catenin-induced reporter gene expression and promoting EGFR-mutant-induced BM in NSCLC, and these processes mainly depend on the ERK1/2 pathway (Fig. 6i).

Discussion

BM is one of the major causes of death in advanced NSCLC and is associated with short survival and reduced quality of life [26, 27]. The EGFR mutation status is a predictive factor response to EGFR-TKIs, which could significantly prolong the progression-free survival (PFS) and objective response rate (ORR) of NSCLC patients with BM [27, 28]. Accumulating evidence shows that EGFR-mutant NSCLC patients are more prone to develop BM [29–31]. However, the underlying mechanisms of how EGFR mutation contributes to BM in NSCLC remain largely unknown. In this study, we demonstrate that the E2F1/WNT5A axis is a novel mechanism involved in the occurrence of BM in EGFR-mutant NSCLC. Our initial evidence reveals that upregulation of WNT5A is associated with less BM in EGFR-mutant NSCLC and better outcome of patients. Our findings indicate that the WNT5A is negatively regulated by E2F1 at transcription levels and the process was dependent on the ERK1/2 pathway. The activation of ERK1/2-E2F1-WNT5A pathway contributes to the occurrence of BM in EGFR-mutant NSCLC. These outcomes represent a promising step in the diagnosis and treatment of EGFR-mutant NSCLC.

WNT5A belongs to Wnt family, exerting a critical role in the regulatory processes of an organism and performing anti- or pro-tumorigenic roles in cancer progression [32–34]. Indeed, the role of aberrant expression of WNT5A in human cancers is still controversial, which might be associated with the expression of different isoforms of WNT5A, namely WNT5A-L and WNT5A-S, and behaves as tumor suppressor and oncogene, respectively [13, 35]. The object of our study is WNT5A-L, of which expression decreased in the BM group remarkably and exerted tumor-suppressive functions, inhibiting the growth, aggressiveness and brain metastasis of EGFR-mutant NSCLC cells, via influencing the activity of β-catenin and downregulating the downstream gene expression, including CCND1, CD44 and ZEB1. A lot of studies have shown that WNT5A plays opposing roles in different types of cancer dependent on the cancer context, such as the existence of different receptors [14, 16, 24, 36]. For example, Frizzled and ROR2 receptors, and recently evidences showed that WNT5A mediating its inhibitory role through the activation of ROR2 [14]. ROR2 has been reported suppressed β-catenin signaling, leading to the inhibit tumor progression [37]. We further confirmed that WNT5A inhibited the β-catenin activity and its downstream genes' expression in EGFR-mutant NSCLC via ROR2 receptor by knockdown ROR2 receptor. Of course, there may also be other factors which mediate the anti-tumor effect of WNT5A, such as promoter methylation modification or others [38], which will be further investigation in our next research.

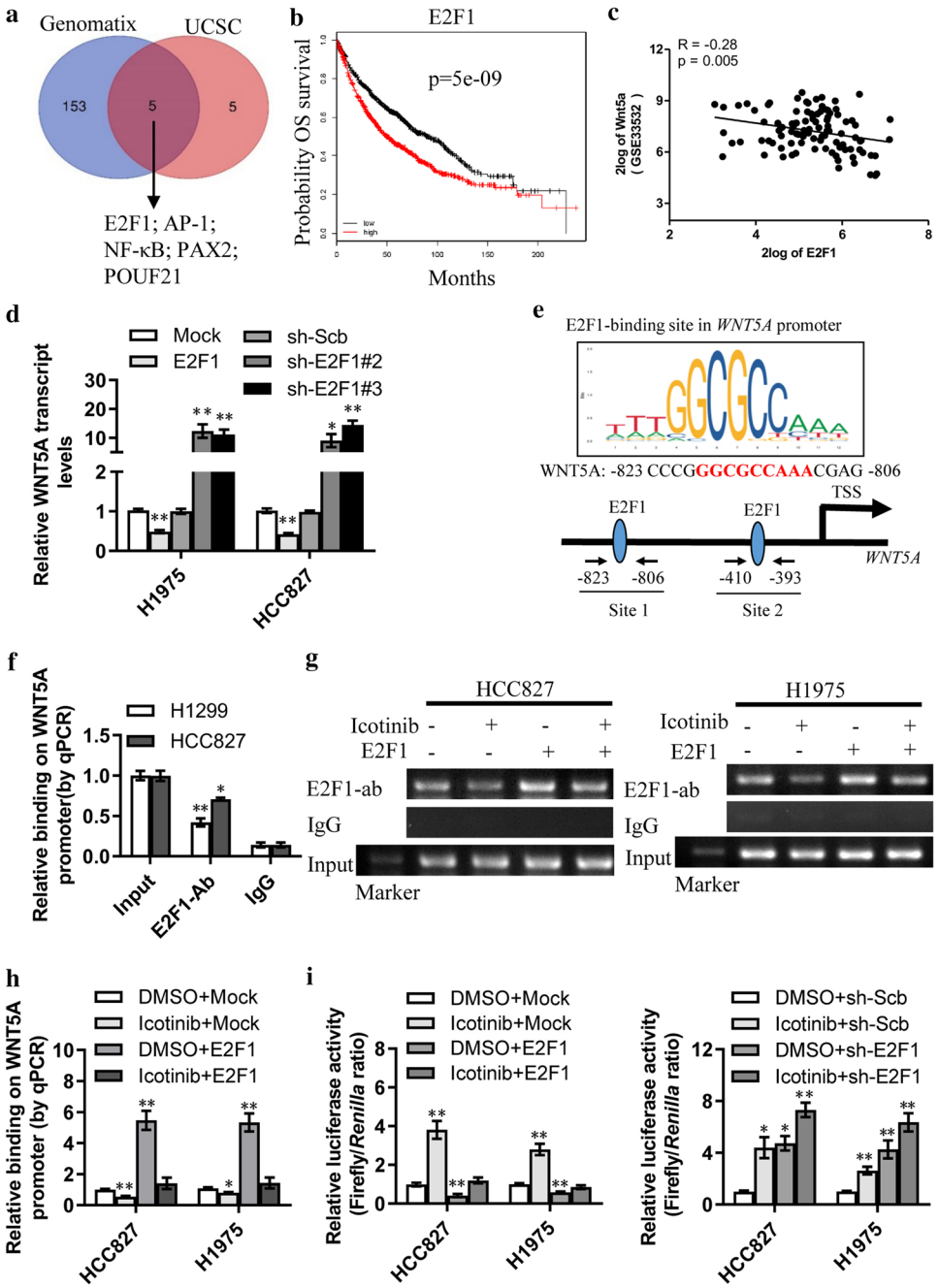
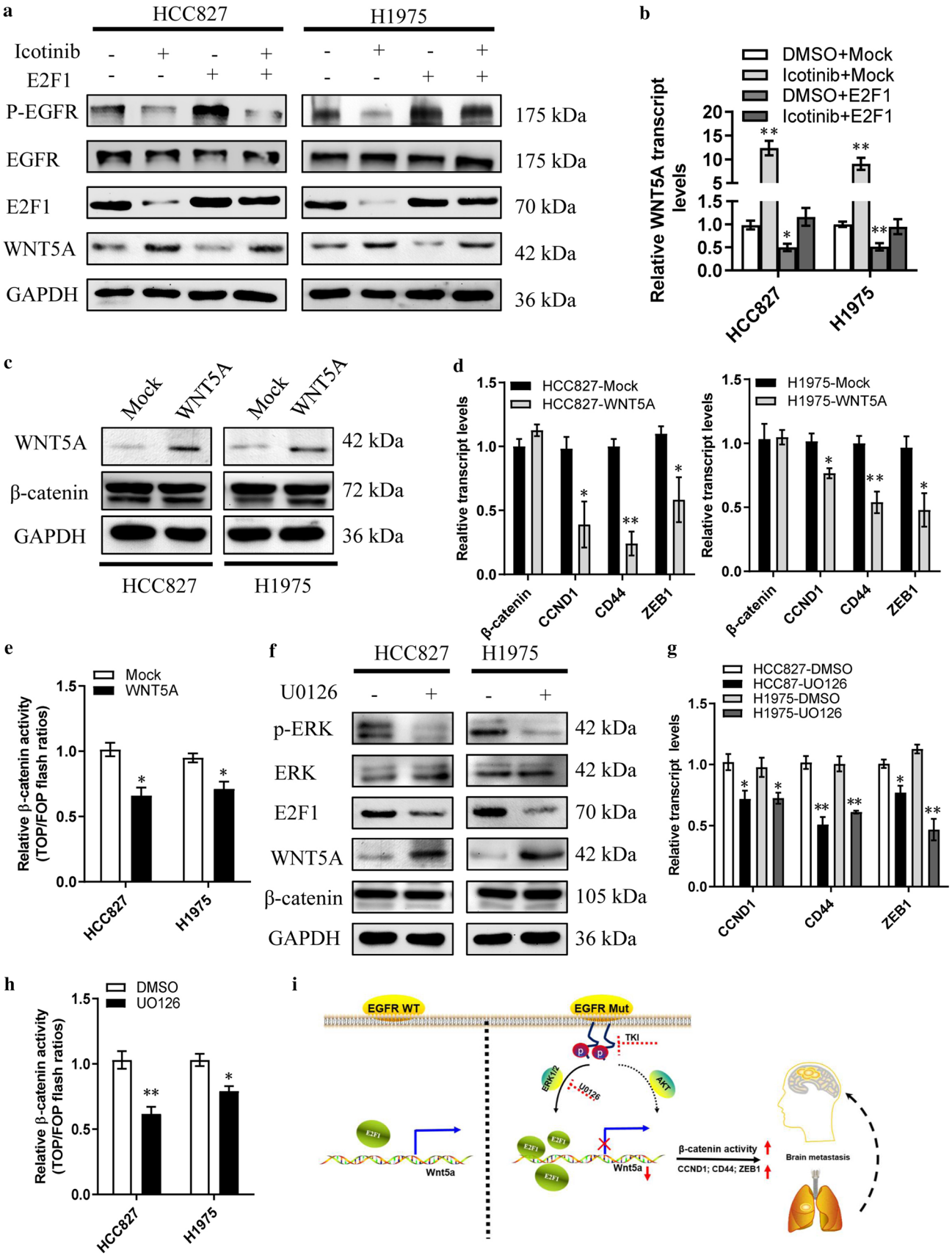


Fig. 5 E2F1 was identified as transcription factor bind to the WNT5A promoter. **a** Venn diagram revealing the identification of transcription factors using public datasets of UCSC and Genomatix database. **b** Kaplan–Meier curves indicating the overall survival of NSCLC patients with high or low E2F1 expression. Log-rank test. **c** The correlation of E2F1 and WNT5A expression from GSE33532 database. Pearson's product-moment correlation analysis. **d** The expression of WNT5A in H1975 and HCC827 cell by transfected E2F1 overexpression or knockdown vector and determined by qRT-PCR. **e** JASPAR analysis the core motif and detail sequence of E2F1 binding on the WNT5A promoter. **f** ChIP-qPCR analysis of E2F1 binding on the WNT5A promoter in H1299 and HCC827 cell by use of E2F1 antibody or rabbit immunoglobulin G as control. **g**, **h** ChIP and qPCR assay showed the enrichment level of E2F1 on WNT5A promoter with treatment of icotinib (0.1 μM) and overexpression of E2F1 in EGFR-mutant cell. **i** WNT5A luciferase reporter was transfected in H1975 and HCC827 cell with or without icotinib (0.1 μM) and overexpression or knockdown of E2F1. Data shown were the average of three independent experiments. $^{*}P<0.05$, $^{**}P<0.01$. Student's *t* test analyzed the difference. Differences among multiple groups were determined by one-way ANOVA with post hoc Tukey's HSD test

Multiple studies have highlighted the critical role of transcription regulation in the progression of cancers [39, 40]. E2F1 was reported mediating transcriptional activation or inhibition in a variety of tumors, such as downregulating the expression of the tumor-suppressor gene ARHI in breast cancer cells via complexing with histone deacetylase (HDAC) and enhancing the metastasis of tumor cells by upregulating vascular endothelial growth factor [41–43]. E2F1 also promotes the invasion of NSCLC and is associated with poor prognosis in NSCLC [44]. Moreover, E2F1 is required for ligand-independent EGFR activation and promotes gut regeneration via EGFR/MAPK signaling [45]. In our study, we showed that E2F1 decreased the WNT5A activity by binding to its promoter, resulting in the transcriptional repression of WNT5A. It has been indicated that WNT5A promoter being epigenetically activated during transformation in Glioblastoma [46]. Whether epigenetic modification also involved in the regulation of WNT5A promoter by E2F1 warrants further investigation.

The PI3K/AKT and ERK1/2 signaling pathways are constitutively activated in NSCLC tissues with EGFR mutation compared to EGFR wt [47, 48]. The aberrant


a
HCC827
H1975
Icotinib
E2F1
P-EGFR
EGFR
E2F1
WNT5A
GAPDH
175 kDa
175 kDa
70 kDa
42 kDa
36 kDa
b
DMSO+Mock
Icotinib+Mock
DMSO+E2F1
Icotinib+E2F1
Relative WNT5A transcript levels
HCC827
H1975
c
Mock
WNT5A
WNT5A
β-catenin
GAPDH
42 kDa
72 kDa
36 kDa
HCC827
H1975
d
HCC827-Mock
HCC827-WNT5A
H1975-Mock
H1975-WNT5A
Realtive transcript levels
β-catenin
CCND1
CD44
ZEB1
e
Mock
WNT5A
Relative β-catenin activity (TOP/FOP flash ratios)
HCC827
H1975
f
HCC827
H1975
U0126
p-ERK
ERK
E2F1
WNT5A
β-catenin
GAPDH
42 kDa
42 kDa
70 kDa
42 kDa
105 kDa
36 kDa
g
HCC827-DMSO
HCC87-UO126
H1975-DMSO
H1975-UO126
Relative transcript levels
CCND1
CD44
ZEB1
h
DMSO
UO126
Relative β-catenin activity (TOP/FOP flash ratios)
HCC827
H1975
i
EGFR WT
EGFR Mut
TKI
ERK1/2
U0126
AKT
E2F1
Wnt5a
β-catenin activity
CCND1; CD44; ZEB1
Brain metastasis

◂**Fig. 6** WNT5A regulated β-catenin activity and downstream genes expression dependent of ERK1/2 pathway in EGFR mutated cells. **a**, **b** The expression of indicated molecules was determined in H1975 and HCC827 cell with or without icotinib (0.1 μM) and E2F1 expression by qRT-PCR and Western blotting. **c** The expression of β-catenin in H1975 and HCC827 cell by Western blotting with or without transfected with WNT5A. **d** The expression of β-catenin and downstream genes CCDN1, CD44 and ZEB1 in H1975 and HCC827 cell by qRT-PCR with or without transfected with WNT5A. $*P<0.05$, $**P<0.01$, determined by unpaired two-sided *t* test. **e** TOP/FOP flash assay indicating the β-catenin activity in H1975 and HCC827 cells transfected with WNT5A or vector (Mock). $*P<0.05$, as determined by unpaired two-sided *t* test. **f** The expression of indicated molecules was determined in H1975 and HCC827 cell with U0126 (10.0 μM) by Western blotting. **g** The expression of target genes was determined in H1975 and HCC827 cell with U0126 (10.0 μM) by qRT-PCR. $*P<0.05$, $**P<0.01$, determined by unpaired two-sided *t* test. **h** TOP/FOP flash assay indicating the β-catenin activity in H1975 and HCC827 cells transfected with WNT5A and in those treated with solvent control (DMSO) or U0126 (10.0 μM) for 24 h. Data shown were the average of three independent experiments with similar results. $*P<0.05$, $**P<0.01$, determined by unpaired two-sided *t* test. The data are presented as the mean ± SEM. **i** model depicting the role of EGFR, ERK1/2, E2F1 and WNT5A in EGFR-mutant lung cancer cells. E2F1-mediated WNT5A expression at transcriptional levels dependent on ERK1/2 pathway in EGFR mutated cells

activation of the complex intracellular signaling pathways has been causally linked to cancers, inflammation, and angiogenesis [25]. In the current study, we identified that E2F1-mediated suppression of WNT5A in EGFR-mutant NSCLC was mainly regulated by the ERK1/2, rather than the PI3K/AKT pathway. There is possibility that other signaling pathways are also involved, and the full mechanisms of our outcomes remain to be elucidated in a future study. Previous studies have shown that the activation of EGFR signaling inactivated Rb, leading to the release of E2F1 from its sequestration to perform transcriptional regulatory functions [49], which may be the potential mechanism of E2F1 plays a role in EGFR-mutant NSCLC, worth exploring further, our evidence indicates that the E2F1-WNT5A axis in EGFR-mutant NSCLC, at least in part, mediated by ERK1/2 pathway.

Conclusion

In summary, our study shows that WNT5A (WNT5A-L isoform) inhibited EGFR-mutant cell proliferation, metastasis, and tumorigenesis both in vitro and in vivo in NSCLC. The suppression of WNT5A was regulated by E2F1 and the E2F1/WNT5A axis was mediated in part via the ERK1/2 pathway. These findings have significant implications for understanding of EGFR-mutant NSCLC are more likely to have BM, and offer a new possible therapeutic strategy for NSCLC.

Author contributions XRD conceived the study. HHL, LP, and JJW performed the experiments. HHL, FT, MR and RGZ analyzed the data. XRD and HHL wrote the manuscript. All authors read and approved the final manuscript.

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Compliance with ethical standards

Conflict of interest The authors declare no conflicts of interest.

Ethics approval and consent to participate This study was approved by the Institutional Review Board of Huazhong University of Science and Technology. Written informed consent was obtained from all legal guardians of the patients. All animal experiments were conducted in agreement with the Guide for the Care and Use of Laboratory Animals and were approved by the Committee on Animal Handling of Huazhong University of Science and Technology.

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