BTB and CNC homology 1 (Bach1) induces lung cancer stem cell phenotypes by inducing CD44 expression

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Research

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Abstract

Background

Growing evidence suggests that CSCs are responsible for cancer initiation in tumors. Bach1 has been identified to contribute to several tumor progression, including lung cancer. The role of Bach1 in CSCs remains poorly known. Therefore, the function of Bach1 on lung CSCs was focused currently.

Methods

The expression of Bach1, CD133, CD44, Sox2, Nanog and Oct4 mRNA was assessed using RT-qPCR. Protein expression of Bach1, CD133, CD44, Sox2, Nanog and Oct4 was analyzed by western blotting. EdU, colony formation, Flow cytometry analysis and transwell invasion assay were carried out to analyze cell proliferation, apoptosis and invasion. Tumor sphere formation assay was utilized to evaluate spheroid capacity. The relationship between Bach1 and CD44 was verified using ChIP-qPCR and dual-luciferase reporter assay. Ki-67 expression was determined by flow cytometry and IHC assay.

Results

The ratio of CD44 + CSCs from A549 and SPC-A1 cells were significantly enriched. Tumor growth of CD44 + CSCs was obviously suppressed in vivo. Bach1 expression was obviously increased in CD44 + CSCs. Then, via using the in vitro experiment, it was observed that CSC growth and invasion were greatly reduced by the down-regulation of Bach1. Loss of Bach1 was able to repress tumor-sphere formation and tumor-initiating CSC markers via acting as a transcription inducer of CD44. A repression of CSCs growth and metastasis of shRNA-Bach1 was confirmed using xenograft models. Furthermore, MAPK signaling pathway was selected and we proved the effects of Bach1 on lung CSCs were associated with the activation of the MAPK pathway. Inhibition of MAPK signaling remarkably restrained lung CSCs growth and CSCs properties.

Conclusion

In summary, our findings reveal that Bach1 positively regulated CD44 + lung cancer CSCs growth and we imply that Bach1 demonstrates great potential for the treatment of lung cancer metastasis and recurrence via regulating CD44 and MPAK signaling.

1. Introduction

Lung cancer is a most malignant tumor with a great rate of morbidity and mortality worldwide. Previous studies have reported that NSCLC can account for almost 85% of lung cancer cases [1]. The first reason for treatment failure and high mortality of NSCLC is due to its invasion and metastasis [2, 3]. Although it

has been confirmed that multiple tumor-related genes are involved in the modulation of NSCLC development, the detail molecular mechanism of NSCLC are still not clear.

Recently, it has been reported that there is a small group of cells within tumors and they can exhibit a significant role in cancer resistance. It is clear they participate in tumor progression and metastasis, which are called cancer stem cells [4]. It has been reported that leukemia stem cells are the first reported CSCs [5]. After that, cancer stem cells are widely recognized in many kinds of cancers, such as lung cancer, liver cancer and pancreatic cancer [6–8]. Lung CSCs are critical for lung cancer metastasis and drug resistance. For example, CD44 is functionally important to activate lung CSCs through regulating Wnt/β-catenin-FoxM1-Twist pathway [9]. Previously, we reported that EGCG can repress CSC-like properties through modulating miR-485 and CD44 in A549-cisplatin resistant cells [10]. NEAT1 induces the CSC-like traits of lung cancer cells via activating Wnt signaling [11]. Therefore, targeting lung CSCs can be provided as a useful lung cancer therapy.

Bach1 is a crucial transcriptional factor and it can play a significant role in oxidative stress, cell cycle, hematopoiesis, and immunity [12–14]. For example, in breast cancer, Bach1 functions as an inducer of metastatic genes including CXCR4 and MMP1 [15, 16]. The level of Bach1 indicates a higher risk of human cancers, such as colorectal cancer, glioblastoma and lung cancer [17–19]. Bach1 stabilization by antioxidants can induce lung cancer [20]. Nevertheless, the detailed function of Bach1 in lung cancer stem cells remains unclear.

Currently, we studied the role of Bach1 in lung cancer. Bach1 was highly expressed in lung CSCs. Knockdown of Bach1 repressed lung CSCs growth and metastasis. In addition, Bach1 interacted with CD44 and contributed to cell migration and invasion. For another, Bach1 activated the expression of genes related with MAPK signaling pathway. Therefore, our findings identified that Bach1 can serve as a significant transcriptional regulator of lung cancer development.

2. Materials And Methods

2.1 Cell culture

A549 and SPC-A1 cells were obtained from the American Type Culture Collection (Manassas, VA, USA). A549 and SPC-A1 cells were maintained at 37 °C in 5% CO2-humidified air in RPMI 1640 medium that contained 10% FBS (Sigma-Aldrich, St.Louis, MO, USA), 100 U/ml penicillin, and 100 mg/ml streptomycin/penicillin. CD44 + cells were isolated by CD44 microbead isolation kit (Miltenyi Bio-tec, Auburn, CA, USA).

2.2 5-Eyhynyl-2’-deoxyuridine (EdU) staining assay

Cell proliferation was evaluated with EdU staining, using a Cell-Light™ EdU Apollo®488 In Vitro Imaging Kit (RIBOBIO, Guangzhou, China). In brief, cells were grown into 96-well plate and cultured to normal

growth stage. EdU labeling was carried out using 100 µl reagent A. Cells were fixed by PFA and eluted using Triton X-100, and then Apollo and DAPI staining were conducted.

2.3 Cell transfection

A lentiviral vector of Bach1 and Bach1 shRNA were purchased from GenePharma (Shanghai, China). Cells were transduced by LV-Bach1 or LV-shBach1 at presence of 8 µg/mL polybrene (GenePharma, Shanghai, China) for 48 hours. Afterwards, a selection procedure using 10 µg/mL blasticidin for 72 hours was carried out. Surviving cells were collected and seeded into the six-well plates.

2.4 Colony formation

Cells were grown at 500 cells in each well of 6-well plates and maintained in RPMI 1640 medium added with 10% FBS at 37 °C. Two weeks, after the cells were fixed. cells were stained using 0.1% crystal violet.

2.5 Cell invasion assay

Cell invasion was assessed using transwell chamber (8-µm pore size; Corning Co., Corning, USA) with Matrigel. At 48 h after transfection, cells were grown into the upper chamber with 10 µg/ml Matrigel. Medium containing 10% FBS was added into the lower chamber. After 48 h, cells that migrated across the membrane were fixed using methanol, stained by 0.1% crystal violet and counted under a microscope.

2.6 Flow cytometry assay

For CD44 + cell analyses, cells were incubated with fluorescence-conjugated monoclonal antibodies against human CD44-PE (BD Biosciences, Franklin Lakes, NJ, USA). We analyzed the samples on a FACS Aria III (BD Biosciences, Franklin Lakes, NJ, USA).

2.7 Western blot analysis

Total protein was isolated using RIPA lysis buffer. Extracted proteins were subject to 10% SDS-PAGE and transferred to PVDF membranes. Then, membranes were blocked by 5% milk and incubated with the primary antibodies at 4 °C for a whole night, followed by incubation with the corresponding horseradish peroxidase-conjugated secondary antibodies. The signals were detected by an enhanced chemiluminescence-detecting kit (Cell Signaling Technology, Danvers, MA, USA). Primary antibodies used were antibodies against Bach1, CD44, Sox2, Nanog, Oct4 and GAPDH, which were obtained from Abcam

2.8 qRT-PCR

Total RNA was isolated by Trizol reagent (Invitrogen). Reverse transcription was carried out with PrimeScript RT Reagent Kit (Takara, Dalian, China). qRT-PCR was conducted with SYBR Prime Script RT-PCR Kit (Takara, Dalian, China). The levels of mRNA were calculated using $2^{-\Delta\Delta Ct}$ method. The primers used were listed in Table 1.

Table 1
Primers used for real-time PCR.

Genes	Forward (5’-3’)	Reverse (5’-3’)
GAPDH	GCACCGTCAAGGCTGAGAAC	TGGTGAAGACGCCAGTGGA
CD44	GCATTGCAGTCAACAGTCGAAGA	CCTTGTTCACCAAATGCACCA
Sox2	GACAGTTACGCGCACATGAA	TAGGTCTG CGAGCTGGTCAT
Nanog	GTGATTTGTGGGCCTGA AGA	ACACAGCTGGGTGGAAGAGA
Oct4	GGTATTCAGCCAAACGA CCA	CACACTCGGACCACATCCTT
Bach1	GAACAGGGCTACTCGCAAAG	AAAGG GCAGTTGACGGAAC

2.9 CD44 promoter analysis

Briefly, cells were transfected with the Bach1-FLAG or control plasmid. 24 h later, cells were transfected with a β-galactosidase plasmid and the wild-type or mutated versions of the CD44 promoter plasmid or the pGL3-basic luciferase reporter plasmid. The luciferase activity was tested with a Luciferase Assay Kit (Promega, Madison, WI, USA). Then, β-Galactosidase activity was evaluated. Afterwards, the relative Luc activity was recorded as the ratio of Luc/β-gal activity.

2.10 Tumor sphere formation assay

Cells were maintained in serum-free DMEM/F12 medium with 20 ng/mL epidermal growth factor, 20 ng/mL basic fibroblast growth factor and B27. Afterwards, cells were plated into an ultra low-attachment 24-well plate at a density of 5000 cells in each well. Tumorsphere formation was photographed using a light microscope (Nikon, Japan).

2.11 Chromatin immunoprecipitation (ChIP) assay

Cells were grown into a 10 cm tissue culture dish. Next day, they were cross-linked with 1% formaldehyde for 10 min, followed by genomic DNA fragmentation. The chromatin fragments were immunoprecipitated with 5 μg of an antibody against Bach1 (R&D Systems, Minneapolis, MN, USA) or an antibody against CD44 (R&D Systems, Minneapolis, MN, USA). DNA extraction was carried using a Qiagen Purification kit. Subsequently, real-time PCR analysis was conducted with primers amplifying the promoters of Sox2, Nanog and Oct4.

2.12 Immunohistochemical (IHC) staining

Slides of tissue from xenografts were incubated with anti-Ki-67 rabbit polyclonal antibody at 4 °C overnight. Normal rabbit serum was employed as the negative control. Then, the incubation with a horseradish peroxidase-conjugated anti-rabbit secondary antibody (Santa Cruz, CA, USA) was followed at 37 °C for 1 h. Diaminobenzidine (DAB) substrate kit (Vector Laboratories, Burlingame, CA, USA) was used to evaluate the signals.

2.13 Xenograft experiments

Animal studies were approved by the Ethics Committee of Experimental Research at Zhongshan Hospital, Fudan University. Six-week-old nude mice (six mice per group, grouped as CD44+, CD44- LV-NC and LV-shBach1) were injected subcutaneously in the bilateral flank area with 5×10^6 cells in 200 μL PBS mixed with 100μL of Matrigel. Tumor volume was recorded every three days. ^{18}F-FDG PET/CT was carried out at 4 weeks after tumor cell injection to monitor the tumor growth. After PET/CT imaging, mice were sacrificed to harvest the tumor tissues for haematoxylin and eosin (HE) staining or IHC analysis. To carry out in vivo tumor metastasis assay, six nude mice per group were injected intraperitoneally with 5×10^6 cells in 200 μL of PBS. Four weeks later, lung tissues were observed.

2.14 Statistical analysis

Data were presented as the mean ± standard error of at least three independent assays. SPSS 20.0 and GraphPad Prism v 6.0 were utilized to do statistical analysis. Two group comparisons were conducted using a Student's t-test. one-way ANOVA was utilized to do multiple group comparisons. $P < 0.05$ was considered to be statistically significant.

3. Results

3.1 Lung CSCs were successfully enriched from A549 and SPC-A1 cells.

Recently, CD44 is identified as a tumor-initiating CSC marker in various cancers. We isolated CD44 + lung cancer cells using flow cytometric analysis as shown in (Fig. 1A and 1B). In Fig. 1C and 1D, we found that Bach1, CD44, Sox2, Nanog and Oct4 protein expression was significantly increased in CD44 + A549 and SPC-A1 CSCs as evaluated using western blotting analysis. These indicated that Bach1 played a key role in CSC progression. Then, we confirmed the tumor growth of lung CSCs in xenografts. Mice were injected with CD44 + A549 and CD44- cells respectively. In Fig. 1E, tumors were peeled form the two different groups. As displayed in Fig. 1F, tumor volume was decreased in CD44- mice group compared to the CD44 + group. Then, in In Fig. 1G and 1H, Ki-67 positive cell ratio was obviously increased in CD44 + group. For another, in Fig. 1I and 1J, Bach1 and CD44 expression was greatly elevated in CD44 + mice group.

3.2 Knockdown of Bach1 inhibited lung CSCs proliferation, enhanced cell apoptosis and repressed cell invasion.

Moreover, to study the potential role of Bach1 against CSCs growth, CD44 + cells were transfected with LV-shBach1 for 48 hours. After 48 hours of post transfection, EdU assay was performed and cell proliferation was repressed by LV-shBach1 as shown in Fig. 2A. In Fig. 2B, colony formation assay was conducted and we found cell colony formation capacity was reduced by loss of Bach1. In addition, cell

apoptosis was triggered by LV-shBach1 as manifested in Fig. 2C. Transwell invasion assay evidenced that test cell invasion ability was suppressed by lack of Bach1 (Fig. 2D).

3.3 Knockdown of Bach1 restrained lung CSCs properties.

Next, we proved that CD44, Sox2, Nanog and Oct4 mRNA and protein expression in lung CSCs was greatly inhibited by LV-shBach1 (Fig. 3A and 3B). In Fig. 3C, tumor spheroid assay indicated that cell sphere formation was remarkably reduced by loss of Bach1. In Fig. 3D, ChIP-qPCR was carried out and it revealed that Bach1 and CD44 co-occupied the promoters of CSC-related genes using anti-Bach1 or anti-CD44 antibodies. Furthermore, overexpression of Bach1 significantly increased the luciferase activity of the CD44 promoter (Fig. 3E).

3.4 Lack of Bach1 repressed the tumor growth and tumor metastasis of lung CSCs.

Then, mice were injected with CD44 + A549 cells transfected with or without Bach1 respectively. PET-CT was employed and we found that tumor growth of lung CSCs in xenografts was significantly reduced by LV-shBach1 (Fig. 4A). In Fig. 4B and 4C, tumor volume and tumor weight were both repressed by loss of Bach1 in vivo. IHC staining of Ki-67 was demonstrated in Fig. 4D. In Fig. 4E, loss of Bach1 inhibited the metastasis of lung CSCs in xenografts evaluated using HE assay.

3.5 Relation of MAPK signaling with lung CSCs.

Moreover, microarray analysis was carried out to explore the underlying molecular mechanism of Bach1. A549 CSCs transfected with LV-NC ore LV-Bach1 were analyzed using RNA sequences analysis. Volcano plot of the data showing altered gene expression in A549 CSCs transfected with or without LV-shBach1 in Fig. 5A. KEGG pathway analysis indicated the potential association between Bach1 and MAPK signaling pathway as shown in Fig. 5B and 5C. Then, in Fig. 5D, top 20 genes involving MAPK signaling pathway was exhibited. We proved that p-p-38 and p-AKT protein expression were significantly depressed by Fig. 5E and 5F.

3.6 Inhibitors of MAPK signaling repressed CD44 + CSCs characteristics.

LY2228820 has been commonly used as MAPK inhibitors. Colony formation assay implied that A549 CSCs cell colony formation capacity was induced by overexpressing Bach1 while LY2228820 repressed this process in Fig. 6A. Consistently, cell invasion ability was also reduced by MAPK inhibitors in Fig. 6B. Additionally, tumor spheroid assay evaluated cell sphere formation ability and we found that LY2228820 restrained cell sphere formation as shown in Fig. 6C. In Fig. 6D, Bach1, CD44, p-p-38 protein expression was greatly reduced by LY2228820 A549 CSCs.

4. Discussion

Lung cancer patients with metastasis demonstrates low survival rates [21]. Mounting evidence has indicated CSCs are mainly responsible for cancer aggressiveness, drug resistance, and tumor relapse. The studies of genes involved in lung CSCs is critical to understand their therapeutic potential. In our present study, the effect of Bach1 in the progression of lung CSCs was studied. To our knowledge, we first reported the potential mechanism of Bach1 in lung CSCs. We demonstrated that Bach1 was significantly increased in lung CSCs. Loss of Bach1 obviously reduced growth, invasion and CSC-like properties in lung CSCs in vivo and in vitro via activating CD44 expression. In addition, we found that knockdown of Bach1 inactivated MAPK signaling pathway.

Accumulating evidence has reported that Bach1 contributes to tumor metastasis [15]. For example, knockdown of Bach1 gene restrains invasion of breast cancer cells via targeting the expression level of matrix metalloproteinase-9 and CXCR4 receptor [22]. Bach1 silencing significantly restrains the migration of colon cancer cells via inhibiting metastasis-related genes [23]. It has been shown that Bach1 can form a complex with MAFG and the DNA methyltransferase DNMT3B, which results in the repression of tumor suppressor genes [24, 25]. In our present study, we observed that Bach1 was highly expressed in lung CSCs. The up-regulation of Bach1 in lung CSCs may be associated with the increases of CSC-associated genes. However, the specific mechanisms of the elevation of Bach1 in lung CSCs have yet to be investigated.

Emerging roles of CD44 have been demonstrated in CSCs and it might be a promising biomarker [26]. Mounting evidence has shown that CD44 are crucial CSC markers and critical in regulating cancer self-renewal, tumor initiation, and metastasis. Therefore, CD44 is widely used to enrich CSCs through fluorescence-activated cell sorting of dissociated single cells [27]. It has been reported that lung cancer cells expressing CD44 are enriched for CSCs properties [28]. In our present study, single cells were isolated from tumor cell cultures of A549 and SPC-A1 cells. Then, we found that Bach1 expression was increased in lung CSCs and it was positively correlated with CSC-related gene expression in lung CSCs. In addition, we proved that loss of Bach1 greatly reduced the expression of the CSC-related genes Sox2, Nanog and Oct4. The relationship between CD44 and Bach1 remains unknown. Currently, a positive correlation between CD44 and Bach1 was observed in lung CSCs. In addition, loss of Bach1 was able to repress CD44 expression. Subsequently, we confirmed that CD44 is a binding partner of Bach1 in lung CSCs. More studies are expected to explore the correlation between CD44 and Bach1 in lung CSCs.

MAPK signaling pathway is a significant signaling cascade which can serves for cell survival and differentiation [29–31]. It has revealed that activation of MAPK signaling pathway plays a positive role in tumor metastasis in numerous human malignancies, including lung cancer [32]. LncRNA TUC338 can promote invasion of lung cancer through MAPK pathway [33]. In addition, lncRNA SNHG12 triggers multidrug resistance by activating the MAPK/Slug pathway in lung cancer [34]. In our study, we identified that silence of Bach1 significantly repressed MAPK pathway. Therefore, our study investigated the function of MAPK signaling pathway in Bach1 in promoting lung CSCs growth and CSC-like properties.

We proved that MAPK inhibitors reversed the CSCs growth and CSC-like characteristics induced by overexpression of Bach1.

In conclusion, we revealed that Bach1 induced CSC self-renewal, which is relevant to the growth of tumors and the recurrence of lung cancer through activation of the CD44 and MAPK signaling. These data might provide Bach1 as a potential target of lung CSCs.

Abbreviations

Bach1: BTB domain and CNC homolog 1; **EdU:** 5-ethynyl-2'-deoxyuridine4', 6-diamidino-2-phenylindole; CSCs: Cancer Stem cells; **IHC:** Immunohistochemistry; **MAPK:** mitogen-activated protein kinase; **CHIP:** Chromatin immunoprecipitation assay kit; **qRT-PCR:** Quantitative Real-time Polymerase Chain Reaction; **CXCR4:** C-X-C chemokine receptor type 4; **NEAT1:** Nuclear Enriched Abundant Transcript 1; **NSCLC:** Non Small Cell Lung Cancer

Declarations

Acknowledgements

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Ethics approval

All animal procedures in this study were approved by the Experimental Animal Ethics Committee of Zhongshan Hospital, Fudan University and carried out in accordance with the guidelines for the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Consent for publication

Not applicable.

Data Availability Statement

The data that support the findings in this study are available from the corresponding author upon reasonable request.

Disclosures

No conflicts of interest.

Author contributions

P.J., and S.H. conceived and designed research; P.J, and L.X. conducted experiments; Z, L., L.X., W.H, H.W., and P.J. analyzed data; P.J. and S.H. interpreted data of experiments; P.J. prepared figures; P.J. and S.L. drafted manuscript; Z, L., L.X., W.H, H.W., and P.J. edited and revised manuscript; S.L. approved final version of manuscript.

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Figures

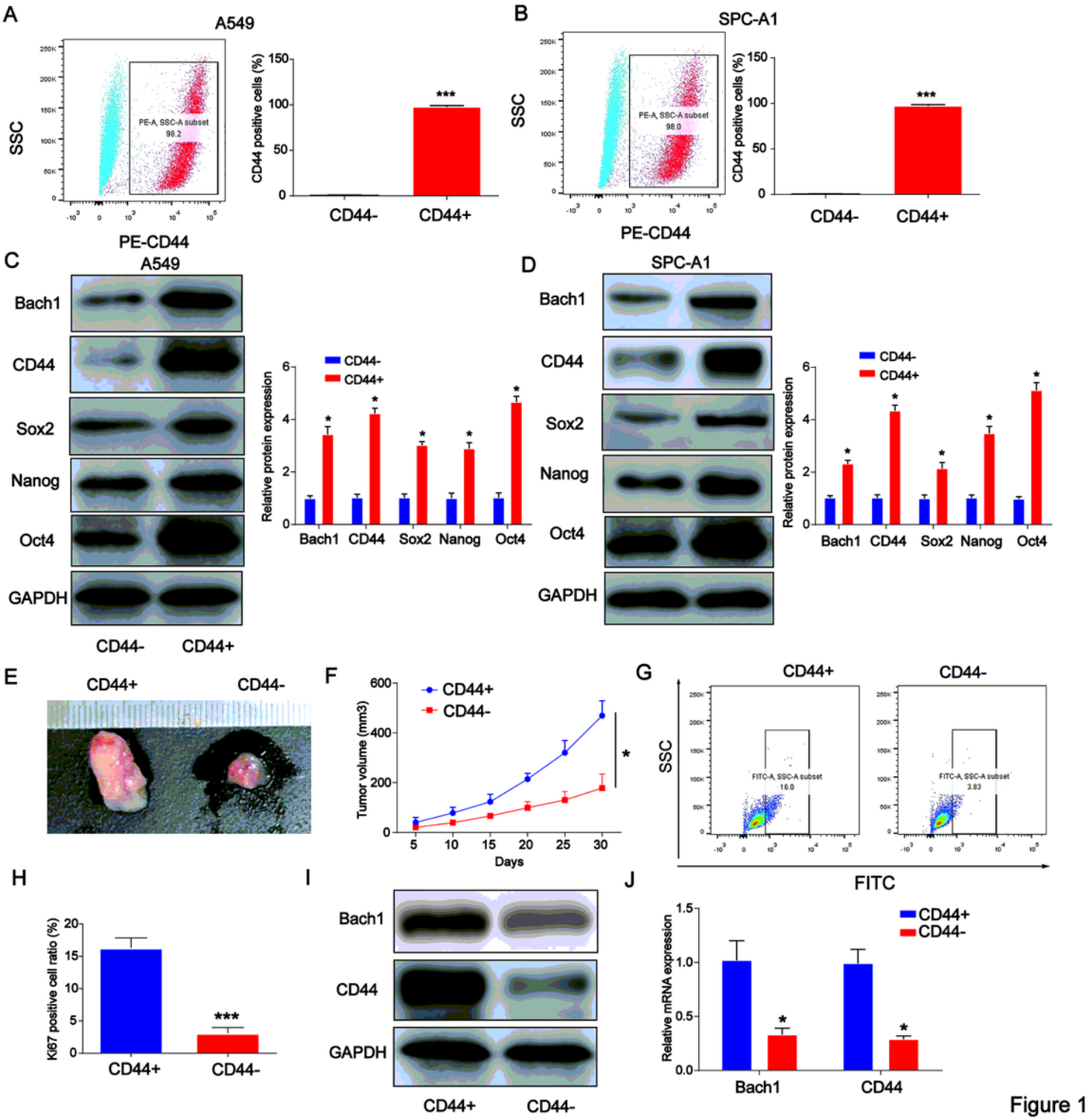

Figure 1

Figure 1

Lung CSCs were successfully enriched from A549 and SPC-A1 cells. (A and B) Flow cytometry analysis demonstrates the percentage of tumorigenic CD44+ A549 and SPC-A1 cells. (C and D) CD44+ A549 and SPC-A1 CSCs were analyzed by anti-Bach1, anti-CD44, anti-Sox2, anti-Nanog and anti-Oct4 using western blotting analysis. (E) The tumor growth of lung CSCs in xenografts. Tumors from mice injected with CD44+ A549 and CD44- cells are shown. (F) Tumor volume was shown in the two different groups. (G

and H) Ki-67 positive cell ratios. (I) Bach1 protein expression was evaluated using IHC western blotting analysis. (J) Bach1 and CD44 mRNA expression was assessed using qRT-PCR analysis in the tumor tissues. *P < 0.05, ***P < 0.001.

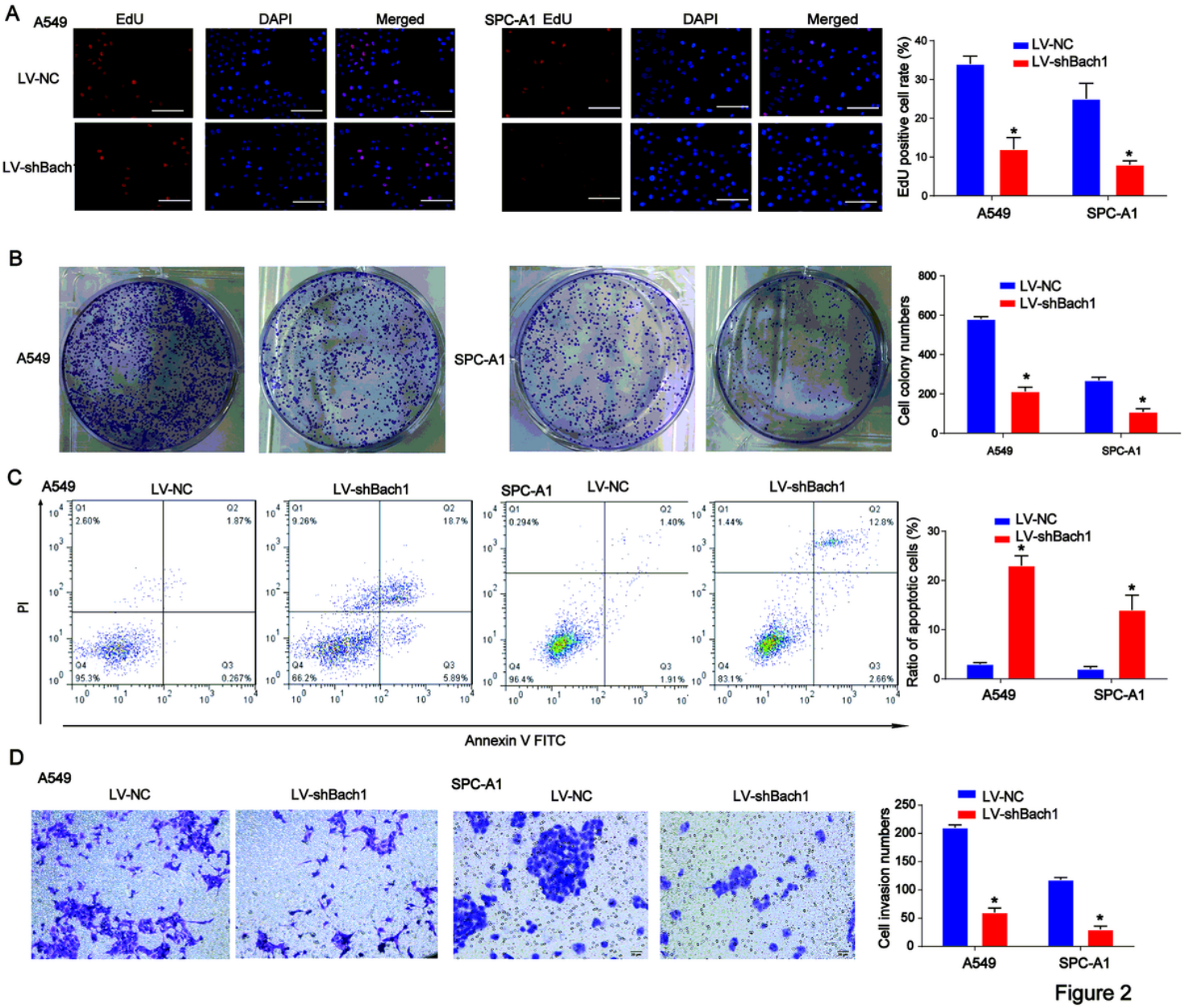

Figure 2

Role of Bach1 on lung CSCs proliferation, apoptosis and invasion. (A) CD44+ cells were cultured at 70% confluency and transfected with LV-shBach1 for 48 hours. After 48 hours of post transfection, EdU assay was carried out to test cell proliferation. (B) Colony formation assay was performed to assess cell colony formation capacity. (C) Flow cytometry assay was utilized to detect cell apoptosis. (D) Transwell invasion assay was used to test cell invasion ability. *P < 0.05.

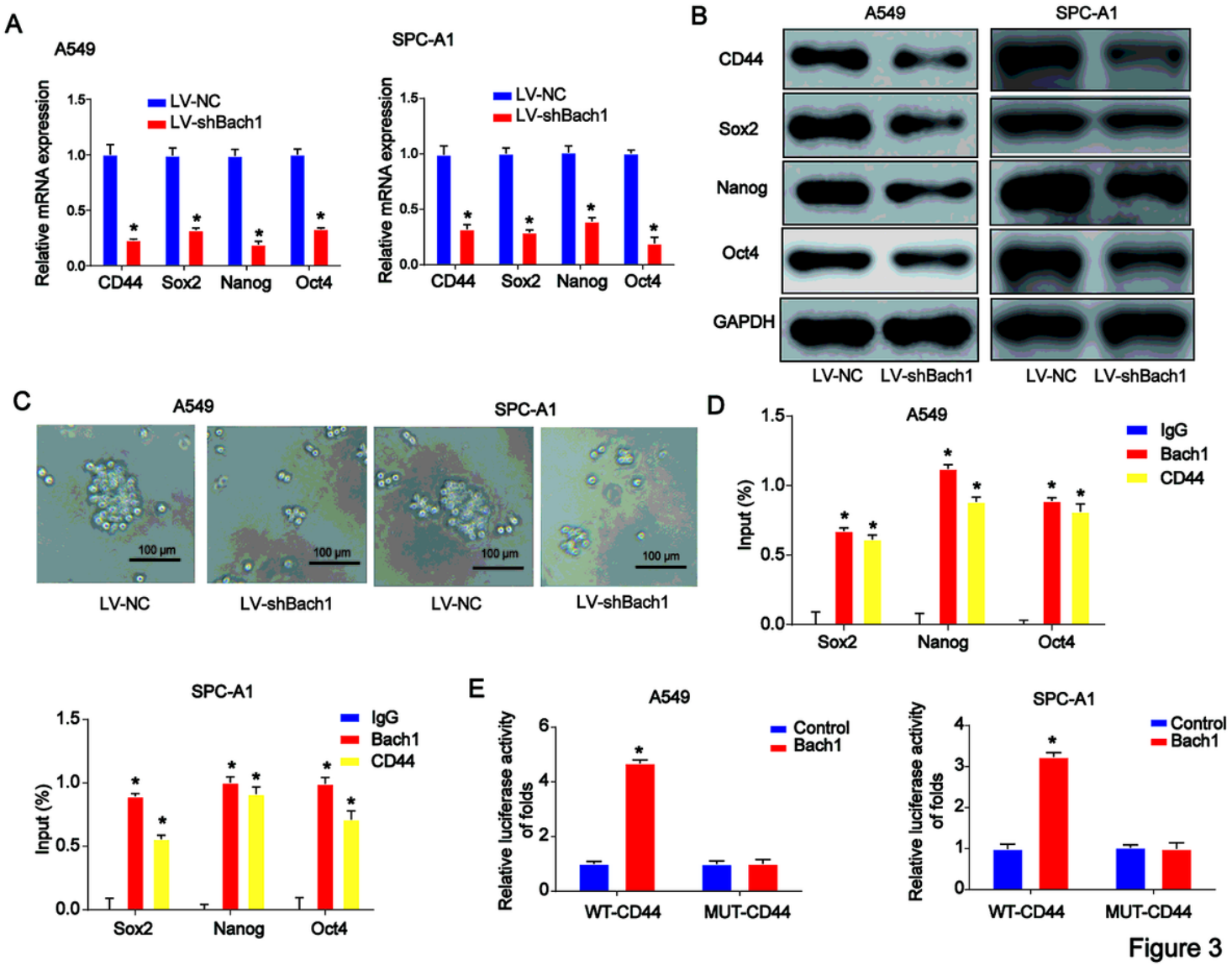

Figure 3

Role of Bach1 on lung CSCs properties. (A) CD44, Sox2, Nanog and Oct4 mRNA expression in lung CSCs. (B) CD44, Sox2, Nanog and Oct4 protein expression in lung CSCs. (C) Tumor spheroid assay was carried out to evaluate cell sphere formation ability. (D) ChIP-qPCR was conducted with anti-Bach1 or anti-CD44 antibodies. (E) Analysis of CD44 promoter activity. Cells were transfected with the Bach1-flag for 24 h. *P < 0.05.

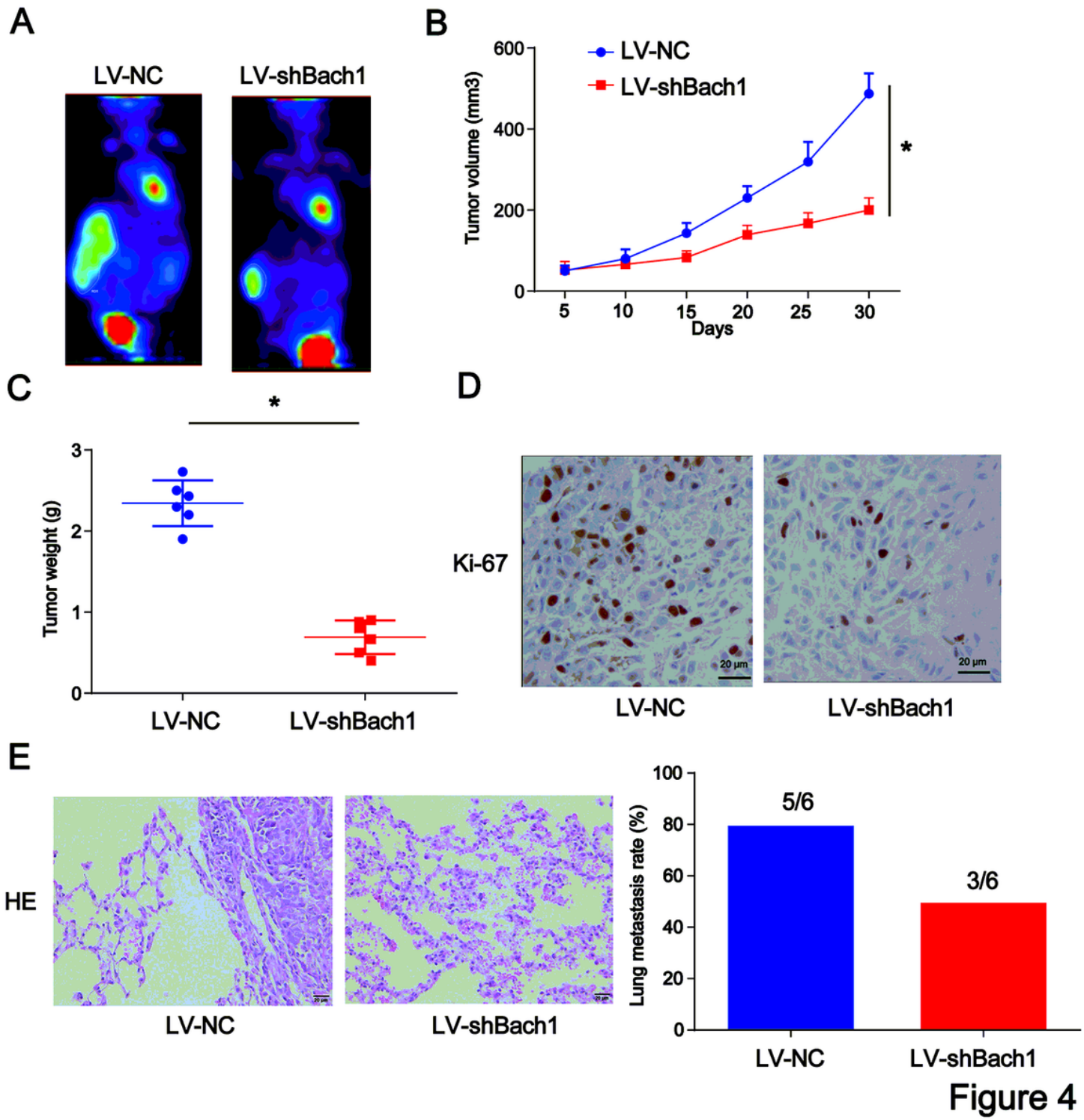

Figure 4

Role of Bach1 on the tumor growth and tumor metastasis of lung CSCs. (A) PET-CT was utilized to observe tumor growth of lung CSCs in xenografts. (B) Tumor volume in a time dependent manner. (C) Tumor weight. (D) IHC staining of Ki-67. (E) Loss of Bach1 inhibited the metastasis of lung CSCs in xenografts evaluated using HE assay. Metastasized lung tissues from mice were shown using HE staining. *P < 0.05.

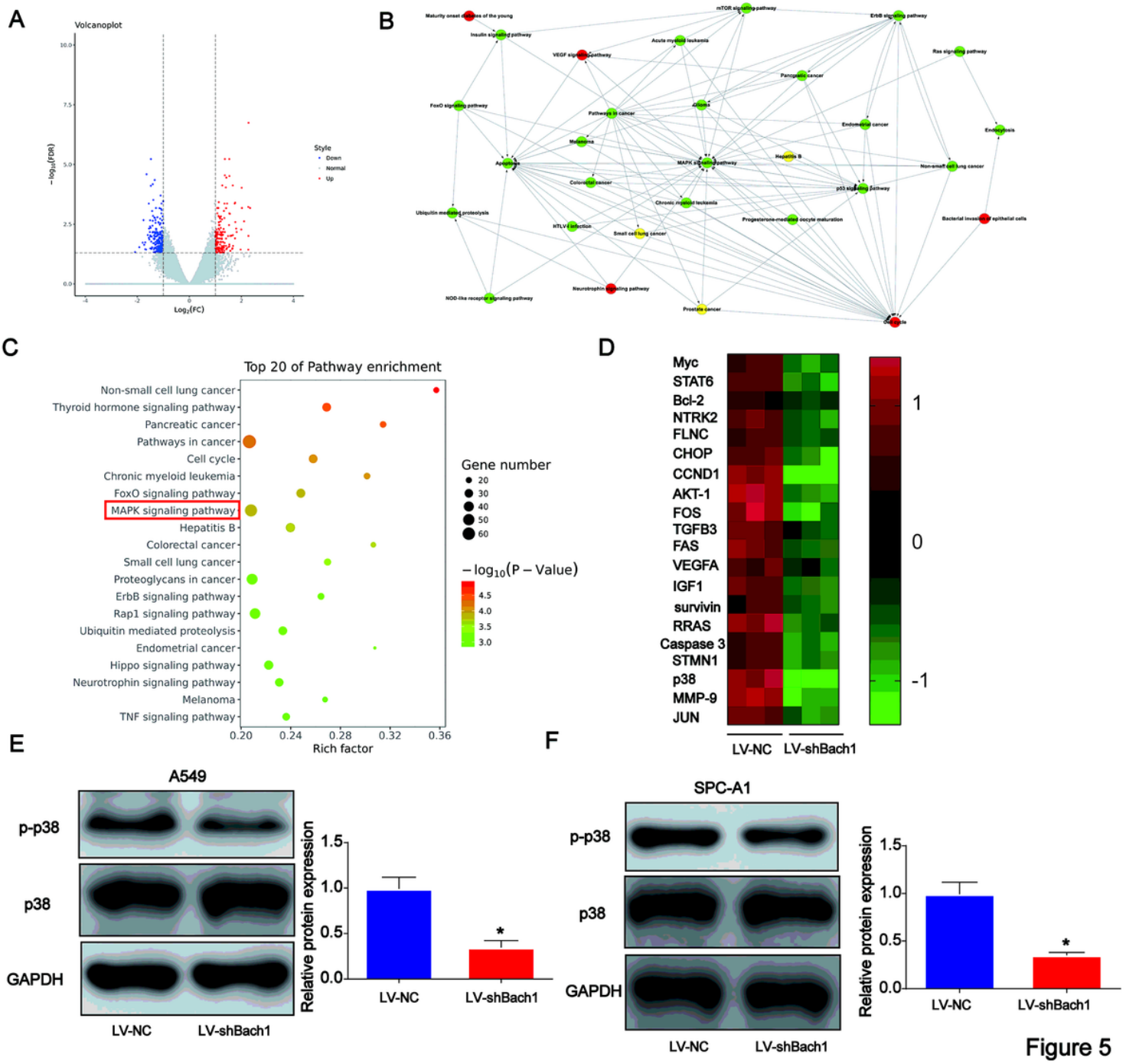

Figure 5

Figure 5

Relation of MAPK signaling with lung CSCs. Microarray analysis was applied to screen the mRNAs which can be regulated by Bach1. (A) Volcano plot of the data showing altered gene expression in A549 CSCs transfected with or without LV-shBach1. (B and C) KEGG pathway analysis revealed the signaling pathways potentially involved in Bach1-mediated functions. (D) Top 20 genes involving MAPK signaling pathway. (E and F) p-38 and AKT protein expression.

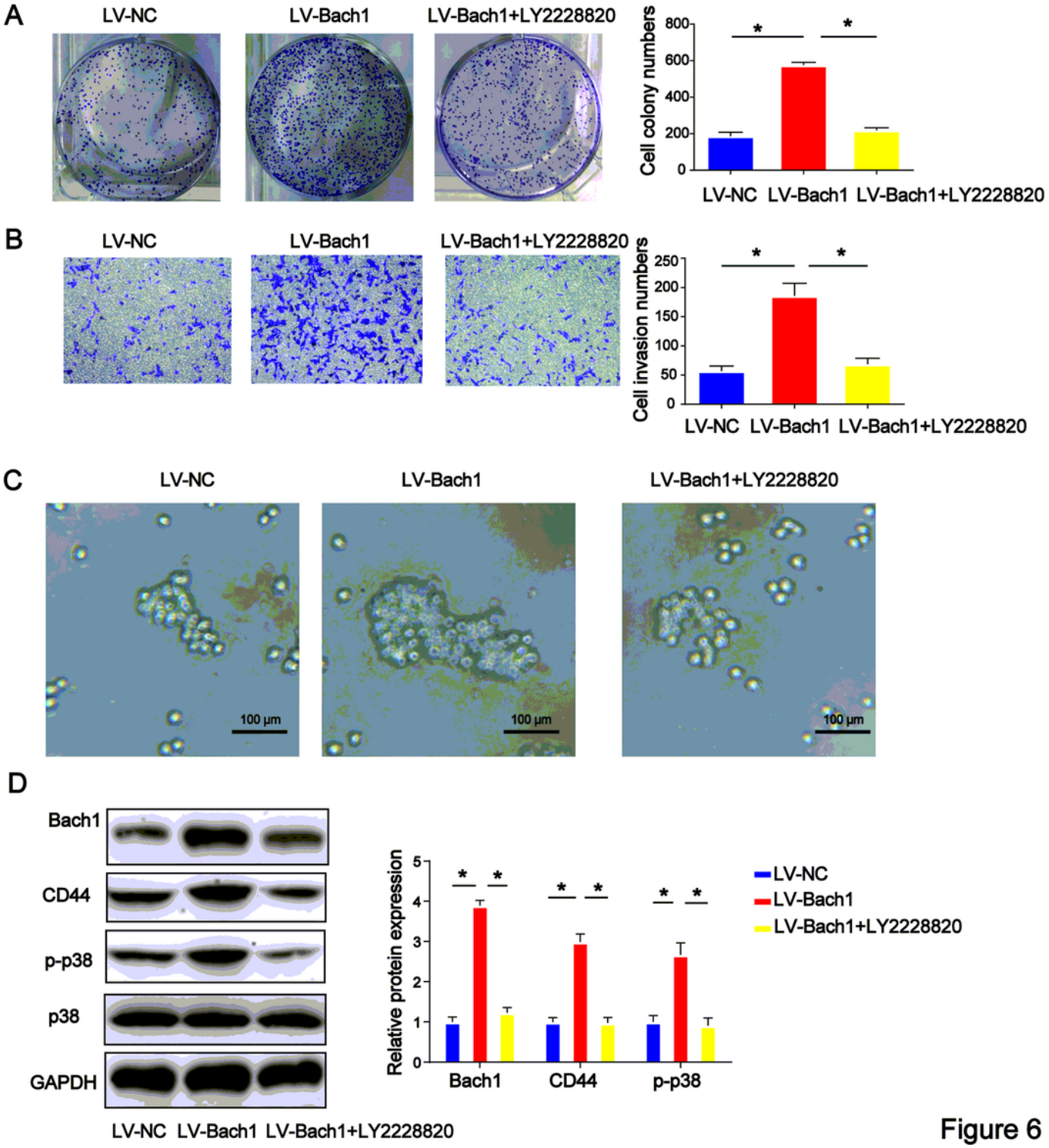

Figure 6

Inhibitors of MAPK signaling repressed CD44+CSCs characteristics. (A) Colony formation capacity. (B) Transwell invasion assay was carried out to test cell invasion ability. (C) Tumor spheroid assay was used to evaluate cell sphere formation ability. (D) Bach1, CD44, p-p-38 protein expression in A549 CSCs. *P < 0.05.