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Original Articles

SDHC-related deficiency of SDH complex activity promotes growth and metastasis of hepatocellular carcinoma via ROS/NFκB signaling

Jibin Li[a,1], Ning Liang[b,e,1], Xiaoyu Long[a,1], Jing Zhao[a], Jin Yang[c], Xiaohong Du[a], Tao Yang[d], Peng Yuan[d], Xiaojun Huang[a], Jiansheng Zhang[d], Xianli He[b,**], Jinliang Xing[a,*]

[a] State Key Laboratory of Cancer Biology and Experimental Teaching Center of Basic Medicine, The Fourth Military Medical University, Xi'an, Shaanxi, 710032, China
[b] Department of General Surgery, Tangdu Hospital, The Fourth Military Medical University, Xi'an, 710038, China
[c] Institute of Preventive Genomic Medicine, School of Life Sciences, Northwest University, Xi'an, Shaanxi, 710000, China
[d] Department of Pain Treatment, Tangdu Hospital, The Fourth Military Medical University, Xi'an, Shaanxi, 710038, China
[e] Department of the Outpatient, PLA 32268 Troops, Dali 671000, China

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ABSTRACT

Succinate dehydrogenase is a heterotetrameric complex comprising four nuclear-encoded subunits, catalyzes the oxidation of succinate to fumarate in the tricarboxylic acid cycle. A subset of cancers have been found to be associated with mutations in the four SDH genes. However, the functional roles of the SDH complex in tumorigenesis remain largely unclear, especially in hepatocellular carcinoma (HCC). Here, we investigated the expression levels of the four SDH subunits and their clinical significance in HCC, followed by systematic exploration of the effects of SDH dysfunction on HCC cell survival and metastasis both *in vitro* and *in vivo,* as well as the underlying molecular mechanisms. Our results showed that the expression of the SDHA/B/C/D subunits was significantly downregulated in HCC, associated with poor patient prognosis, and contributed to SDH inactivation. Additionally, attenuated SDH activity following *SDHC* knockdown promoted HCC-cell growth and metastasis both *in vitro* and *in vivo* via elevated reactive oxygen species levels and subsequent activation of nuclear factor-κB signaling. These findings suggest a critical tumor-suppressive role for SDH and provide strong evidence supporting this enzyme as a potential drug target in the treatment of HCC.

1. Introduction

The tricarboxylic acid (TCA) cycle involves a series of enzyme-catalyzed chemical reactions that produce many biosynthetic precursors and promote aerobic respiration, thereby representing the common metabolic pathway for the production of glucose, fatty acids, and amino acids. Numerous studies report that mutation of genes involved in the TCA cycle, such as those encoding fumarate hydratase [1,2] and isocitrate dehydrogenase [3,4], causes abnormal accumulation of oncometabolites involved in cancer pathogenesis via epigenetic mechanisms.

Succinate dehydrogenase (SDH; also called mitochondrial respiratory chain complex II) is an enzyme that catalyzes the oxidation of succinate into fumarate in the TCA cycle and comprises four nuclear-encoded subunits (SDHA/B/C/D), among which SDHA and SDHB act as catalytic subunits, and SDHC and SDHD provide the binding site for ubiquinones (an element of the electron-transport chain). Cumulative evidence reveals a close relationship between SDH dysfunction and cancer [5],with somatic mutations in SDHB, SDHC and SDHD commonly observed in a number of malignancies, including paraganglioma, pheochromocytoma, gastrointestinal stromal tumors, and renal cell carcinoma [6–9]. Alteration of genes encoding members of the SDH

Abbreviations: CCCP, carbonyl cyanide m-chlorophenyl hydrazone; EdU, 5-ethynyl-2′-deoxyuridine; EMT, epithelial-mesenchymal transition; HCC, hepatocellular carcinoma; HE, hematoxylin-eosin; IHC, immunohistochemistry; NAC, N-acetyl cysteine; NF-κB, nuclear factor kappa-light-chain-enhancer of activated B cells; OS, overall survival; PCR, polymerase chain reaction; qPCR, quantitative PCR; qRT-PCR, quantitative reverse transcription PCR; RFS, relapse-free survival; ROS, reactive oxygen species; siRNA, small-interfering RNA; TUNEL, terminal deoxynucleotidyl transferase dUTP nick-end labeling; SDH, succinate dehydrogenase; SDHA, succinate dehydrogenase A; SDHB, succinate dehydrogenase B; SDHC, succinate dehydrogenase C; SDHD, succinate dehydrogenase D; TCA, tricarboxylic acid

* Corresponding author. State Key Laboratory of Cancer Biology and Experimental Teaching Center of Basic Medicine, the Fourth Military Medical University; 169 Changle West Road, Xi'an, 710032, China.
** Corresponding author. , Department of General Surgery, Tangdu Hospital, the Fourth Military Medical University, No. 1 Xinsi Road, Xi'an, 710038, China.
E-mail addresses: wanghe@fmmu.edu.cn (X. He), xingjl@fmmu.edu.cn (J. Xing).
[1] These authors contributed equally to this work.

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complex leads to impaired SDH function and subsequent cancer pathogenesis and progression [7]. However, the expressions of SDHA, SDHB, SDHC, and SDHD, their effects on SDH activity, and subsequent tumor progression remain poorly understood, especially in hepatocellular carcinoma (HCC).

SDH catalyzes the oxidation of succinate into fumarate in the TCA cycle and subsequent transfer of the extracted electrons to ubiquinone via the electron-transport chain, thereby leading to the production of reactive oxygen species (ROS) [10]. As a byproduct of electron transport, ROS are well-known promoters of tumorigenic signaling that contribute to the pathogenesis and progression of various human cancers [11–13]. Several previous studies report that mutations in SDH subunits are associated with increased production of ROS. Benjamin et al. reported that mutations in SDHC increase ROS production, which in turn induces metabolic stress, genomic instability, and promotes tumorigenesis [14]. Additionally, Ishii et al. reported that an SDHC mutation increases oxidative stress, thereby contributing to DNA damage in the nucleus, mutagenesis, and subsequent tumorigenesis [15]. These findings suggest ROS as a primary trigger of tumor progression resulting from mutation-mediated SDH dysfunction. However, the role of ROS in cancers under non-mutation conditions such as HCC are still largely unclear.

In this study, we investigated the expression of the four subunits of the SDH complex in HCC and analyzed the clinical implications, biological functions, and molecular mechanisms associated with the role of the complex in HCC progression.

2. Materials and methods

2.1. Ethics

This study was approved by the Ethics Committee of the Fourth Military Medical University (Shaanxi, China), and written informed consent was obtained from all participants.

2.2. Antibodies and reagents

The primary antibodies and their working concentration used in this study are listed in Table S2. A succinate detection kit was purchased from abcam (ab204718; Cambridge, UK), N-acetyl cysteine (NAC) and succinate (succinate acid) were purchased from Sigma-Aldrich (St. Louis, MO, USA). MK-2206 was purchased from Selleck Chemicals (Shanghai, China). IκBα siRNA (Serial number, s70549) was purchased from Thermo Fisher Scientific (Waltham, MA, USA). Commercial kits for SOD, CAT, and GPx activities were purchased from Nanjing Jiancheng Bioengineering Institute (Nanjing, China).

2.3. HCC cell culture and tumor tissues collection

Human HCC cell lines SNU-354, SNU-368, HLE, SNU-739, Huh-1, and HLF were obtained from the Japanese Collection of Research Bioresources (Osaka, Japan) and authenticated using short-tandem-repeat DNA tested by the FMMU Center for DNA Typing in 2017. Cells were routinely cultured in Dulbecco's modified Eagle's medium (Gibco, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (Hyclone Laboratories, San Angelo, TX, USA). Additionally, 224 human HCC tumor-tissue samples were collected, as described in the Supplementary Methods.

2.4. Quantitative reverse transcription PCR (qRT-PCR), western blot and immunohistochemistry

Total RNA extraction, cDNA synthesis, and PCR were performed as described in the Supplementary Methods. Primer sequences used in this study are provided in Table S1. HCC cell lines and tissues were processed for Western blot analysis, as previously described [16]. Quantification of IHC staining intensity was performed as described in the Supplementary Methods.

2.5. Measurement of ROS and activities of antioxidant enzymes

Intracellular ROS was measured by using the fluorescent probe 2′,7′-dichlorodihydrofluorescein diacetaten (S0033; Beyotime Biotechnology, Beijing, China), as previously described [16].

Activities of antioxidant enzymes of SOD, CAT and GPX were measured using commercial kits from Nanjing Jiancheng Bioengineering Institute (Nanjing, China). Activity detections were performed according to the manufacturer's instruction. One unit (U) of antioxidant enzyme activity was defined as the amount of enzyme consuming 1 μmol of substrate/min/mg total protein (U/mg prot).

2.6. Measurement of succinate concentration

Succinate concentrations were assessed colorimetrically using a commercially available succinate assay kit (ab204718, Abcam), with tissue and cell extractions performed according to manufacturer instructions. Samples were analyzed by loading onto a 96-well plate and measuring the absorbance at 450 nm.

2.7. Cell viability, proliferation, and apoptosis assays

Cell viability was determined by MTS assay (Promega, Madison, WI, USA) and colony formation assays, as described in the Supplementary Methods. To assess cell proliferation, a 5-ethynyl-2′-deoxyuridine (EdU)-incorporation assay kit (Ribobio, Guangdong, China) was used according to manufacturer instructions. Apoptosis was determined by flow cytometry using an Annexin V-FITC apoptosis detection kit (F-6012; Everbright USA, Inc., Redmond, WA, USA). Additionally, terminal deoxynucleotidyl transferase dUTP nick-end labeling(TUNEL) assays were performed to assess apoptosis in xenograft tissues, as described in the Supplementary Methods.

2.8. Cell migration and invasion

Transwell cell-migration and Matrigel invasion assays were performed to investigate the effect of suppressed SDH activity on cell migration/invasion, as described in the Supplementary Methods.

2.9. In vivo assays to evaluate tumor growth and metastasis

Male, athymic, 5-week-old Balb/c nude mice were used for *in vivo* tumor-growth and metastasis assays, as described in the Supplementary Methods. All animal studies were approved by the Institutional Animal Experiment Committee of the Fourth Military Medical University and carried out in accordance with the UK Animals (Scientific Procedures) Act, 1986.

2.10. Statistical analysis

SPSS version 17.0 software (SPS, Inc., Chicago, IL, USA) was used for all statistical analyses, with the detailed methods provided in the Supplementary Methods.

3. Results

3.1. SDHA/B/C/D expression is downregulated in HCC patients and associated with poor survival and disease progression

We first determined mRNA levels of the four subunits of the SDH complex in 30 paired HCC tissues. Quantitative PCR (qPCR) analyses showed that all four subunits were significantly downregulated in HCC tumor tissues relative to levels in peritumor tissues (Fig. 1A), with a

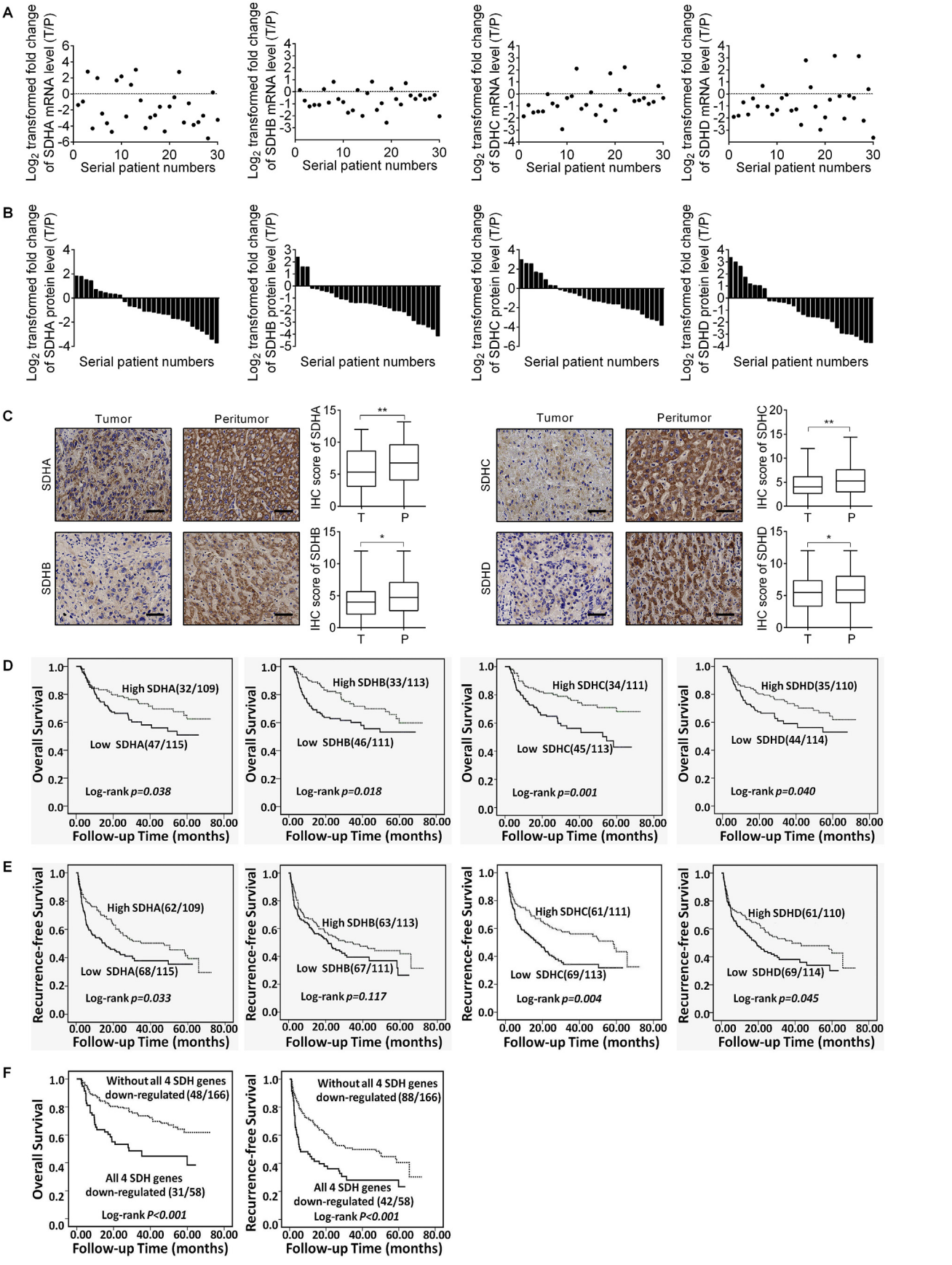

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Fig. 1. Subunits SDHA/B/C/D are frequently downregulated and associated with poor survival and disease progression in HCC patients. (A, B) qRT-PCR and Western blot analyses of subunit expression in 30 paired tissues from HCC patients. Relative expression ratio (tumor: peritumor) was $\log_2$-transformed. Patient serial number was rearranged according to expression level in Western blot results, and qRT–PCR data are displayed according to patient serial number.(C) Representative IHC-staining images (left) and IHC scores (right) for each subunit in 224 paired HCC tissues. $^*P < 0.05$; $^{**}P < 0.01$. Scale bar: 50 μm. (D, E and F) Kaplan–Meier analysis of OS and RFS in HCC patients according to subunit expression. Values for the number of deaths/recurrences/patients in each subgroup are presented.
P, peritumor; T, tumor.

similar pattern of protein translation observed by Western blot analysis (Fig. 1B and S1A). In addition, a significant positive correlation was found between subunit mRNA and protein levels in individual patients (Fig. S1B). Evaluation of the four SDH subunits by IHC staining in a large cohort of 224 paired HCC tissues revealed significant downregulation of all four subunits in HCC tumor tissues as compared with those in peritumor tissues (Fig. 1C), supporting the qPCR and Western blot results. Additionally, Kaplan–Meier analysis indicated that HCC patients exhibiting attenuated expression of the four subunits presented significantly poorer overall survival (OS) and recurrence-free survival (RFS) rates than those exhibiting elevated subunit expression(Fig. 1D and E). More importantly, patients with all four subunits down-regulated had significantly poorer overall OS and RFS than those without all four subunits down-regulated, which showed a better prediction of survival than each of the four SDH genes (Fig. 1F). These findings indicated that all four subunits were significantly downregulated and associated with poor prognosis in HCC patients.

3.2. Downregulated SDH-subunit expression results in decreased SDH activity and increased succinate and ROS levels in HCC cells

To explore the effect of subunit downregulation on the enzyme activity of SDH complex, the expression levels of four subunits in six HCC cell lines were determined by qPCR and Western blot analyses. HCC cells exhibiting relatively high expression of SDHA (SNU-354 cells), SDHB (Huh-1 cells), SDHC (HLF cells), or SDHD (SNU-368 cells) were respectively selected for the establishment of cell models for subunit knockdown/overexpression (Figs. S2A and S2B). As shown in Fig. 2A, SDH complex enzyme activity was significantly decreased following subunit knockdown in HCC cells, with the most substantial alteration in SDH complex activity observed following SDHC knockdown, suggesting that SDH activity was suppressed by subunit downregulation in HCC cells.

To investigate the biological effect of decreased SDH activity on HCC-cell progression, HCC-cell models exhibiting different SDH activities were established by *SDHC* knockdown or overexpression in HLE and HLF cells, respectively (Figs. S2C and S2D). We observed a significant accumulation of the SDH substrate succinate in SDHC-knockdown HLF cells, whereas succinate was significantly reduced in SDHC-overexpressing HLE cells (Fig. 2B). Additionally, measurement of succinate levels in 30 paired HCC tumor tissues and peritumor tissues revealed a significantly higher average succinate level in HCC tumor tissues relative to that in peritumor tissues (Fig. 2C).

ROS plays an important role in cancer initiation and progression [17]. Given that mutations in genes encoding the SDH subunits can result in increased levels of ROS, which function as second messenger that regulate multiple oncogenic signaling pathways during cancer progression, we measured ROS levels by flow cytometry following *SDHC* knockdown or overexpression. As shown in Fig. 2D, SDHC-knockdown HLF cells displayed significantly elevated ROS levels, whereas SDHC-overexpressing HLE cells displayed significantly reduced ROS levels. These results demonstrated that SDH-subunit downregulation decreased SDH enzyme activity and elevated ROS and succinate levels. In addition, the effect of SDHC deficiency on the antioxidant activity were further determined through measuring the activities of three major antioxidant enzymes of SOD, CAT and GPX in HCC cells. As shown in Fig. 2E, SDHC knockdown did not notably affect the activity of SOD, while increased the activities of CAT and GPX. In contrast, the activities of CAT and GPX were suppressed when SDHC was over-expressed, indicating that elevated ROS level induced by decreased SDH enzyme activity is accompanied by a elevation of antioxidant capability, perhaps for detoxifying the elevated ROS level. Collectively, these results demonstrate that downregulation of the SDH subunits leads to the decrease of SDH enzyme activity and elevation of ROS and succinate levels.

3.3. Deficient SDH activity promotes HCC-cell growth

We then determined the effect of decreased SDH activity on HCC-cell growth. MTS and colony formation assays showed that *SDHC*-knockdown HLF cells grew faster than control cells, whereas *SDHC*-overexpressing HLE cells displayed a slower growth rate than control cells (Fig. 3A–B). Because increased cell growth can be caused by both uncontrolled proliferation and apoptosis resistance, we evaluated the effect of decreased SDH activity on HCC-cell proliferation, finding significantly increased EdU incorporation in *SDHC*-knockdown HLF cells as compared with that observed in control cells, whereas the opposite effect was observed in *SDHC*-overexpressing HLE cells (Fig. 3C). Evaluation of the potential functional role of the SDH complex in HCC-cell apoptosis indicated that *SDHC* knockdown significantly inhibited carbonyl cyanide m-chlorophenyl hydrazone (CCCP)-induced apoptosis in HLF cells, whereas we observed a higher percentage of apoptotic cells in *SDHC*-overexpressing HLE cells (Fig. 3D). These results were subsequently confirmed by the significant inhibition of both cytochrome *c* release and lower levels of cleaved caspase-9 and -3 in *SDHC*-knockdown HLF cells following CCCP treatment, whereas these activities were upregulated in *SDHC*-overexpressing HLE cells(Fig. 3E–F).

We then established a subcutaneous xenograft model in nude mice using HCC cells exhibiting stable *SDHC* knockdown or overexpression (Fig. S3). As shown in Fig. 4A–B, mice injected with *SDHC*-knockdown HLF cells displayed increased tumor growth as compared with mice injected with control cells, whereas the growth of xenografts developed from *SDHC*-overexpressing HLE cells was lower than that in controls. Additionally, xenografts developed from *SDHC*-knockdown HLF cells exhibited increased Ki-67-positivestaining and decreased TUNEL-positive staining, whereas those established with *SDHC*-overexpressing HLE cells displayed significantly decreased Ki-67-positive staining and increased TUNEL-positive staining (Fig. 4C–D). These findings suggested that suppressed SDH activity promoted HCC growth by accelerating cell proliferation and inhibiting apoptosis.

3.4. Deficient SDH activity promotes HCC-cell migration and invasion

We then investigated the effect of decreased SDH activity on HCC metastasis. Scratch-wound-healing assays showed that *SDHC* knockdown significantly increased the migratory ability of HLF cells, whereas *SDHC*-overexpressing HLE cells displayed significantly inhibited migratory capability relative to control cells (Fig. 5A). Additionally, Matrigel invasion assays indicated enhanced invasion capacity by *SDHC*-knockdown HLF cells as compared with that observed in control cells, whereas *SDHC*-overexpressing HLE cells showed decreased invasion capacity (Fig. 5B). Epithelial–mesenchymal transition (EMT) is an important contributing factor in tumor metastasis and associated with reduced cell–cell contact and increased cell motility. To investigate the

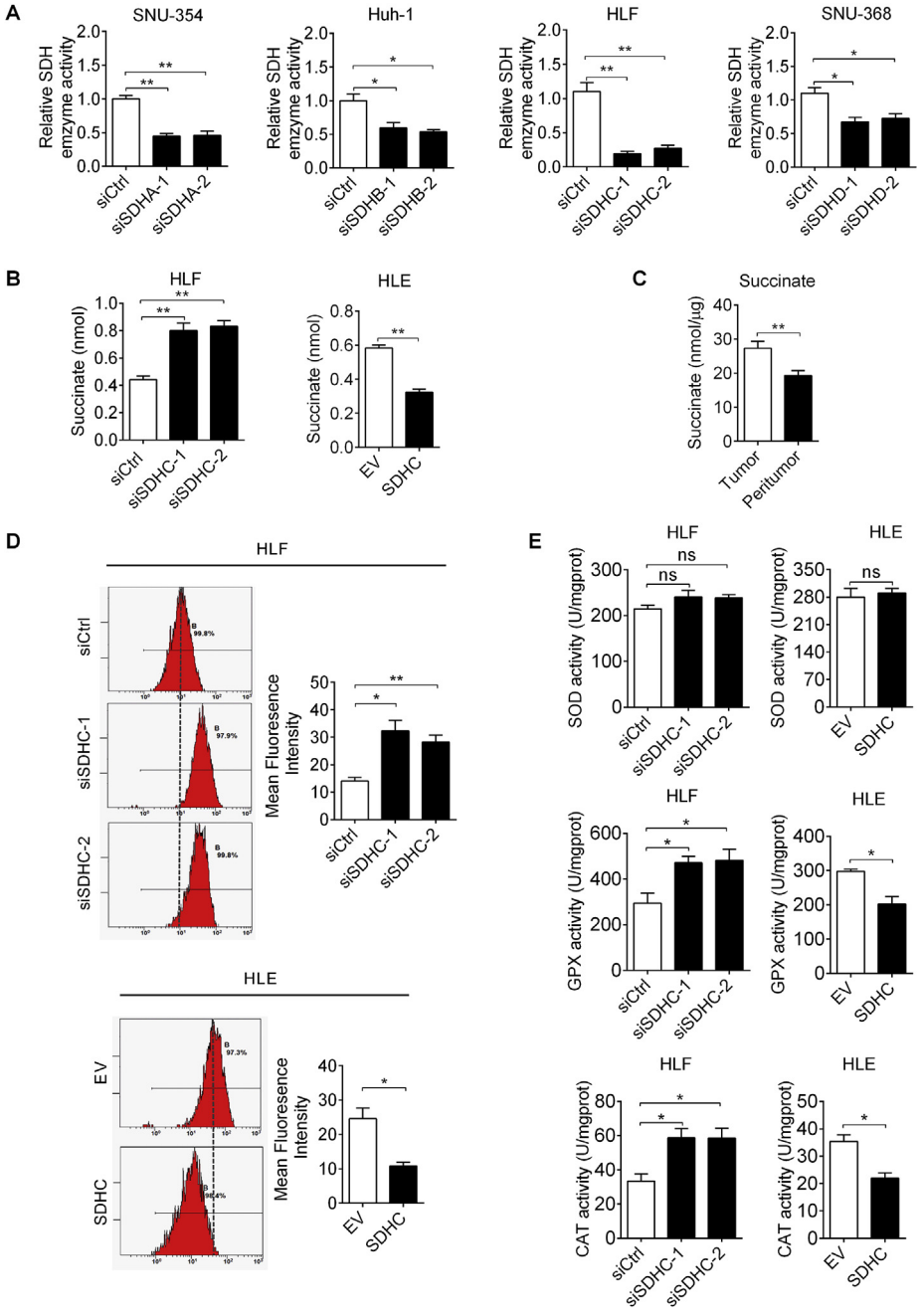

Fig. 2. SDH-subunit downregulation decreases SDH activity and increases succinate and ROS levels in HCC cells. (**A**) SDH activity in SNU-354, Huh-1, HLF, and SNU-368 cells following the indicated treatment. (**B**) Intracellular succinate levels measured in HLF and HLE cells treated as indicated. (**C**) Succinate levels measured in 30 paired tissues from HCC patients. (**D**) Intracellular ROS levels analyzed by flow cytometry in HLF and HLE cells treated as indicated. (E) Activities evaluation for three major antioxidant enzymes of SOD, CAT and GPX in HCC cells.
EV, empty vector; P, peritumor; siCtrl, control siRNA; siSDHA/B/C/D, siRNAs targeting each subunit; SDHC, expression vector encoding SDHC; siSDHC-1/2, siRNAs against *SDHC*;T, tumor; ns, not significant.

involvement of EMT in regulating HCC-cell invasion and migration following suppression of SDH activity, we performed qRT-PCR and Western blot analyses, with the results indicating that *SDHC* knockdown significantly increased the expression of the mesenchymal markers vimentin, and N-cadherin and decreased the expression of the epithelial markers E-cadherin and zonula occludens-1, whereas the opposite effect was observed following *SDHC* overexpression (Fig. 5C–D).

To explore the role of decreased SDH activity in tumor metastasis *in vivo*, HLF and HLE cells exhibiting stable *SDHC* knockdown and overexpression, respectively, were transplanted into the livers of nude mice to asses intrahepatic metastasis. Interestingly, the number and size of the metastatic nodules in the liver were dramatically increased in the *SDHC*-knockdown group as compared with controls, with the opposite effect observed in the *SDHC*-overexpressing group (Fig. 5E). These findings indicated that deficient SDH activity significantly enhanced HCC-cell migration and invasion both *in vitro* and *in vivo*.

3.5. Deficient SDH activity activates ROS/nuclear factor (NF)-κB signaling in HCC cells

Given that suppressed SDH activity significantly increased ROS levels in HCC cells (Fig. 2D) and its reported role in regulating oncogenic signal-transduction pathways, such as those associated with NF-κB [18,19], we evaluated whether NF-κB signaling is increased following attenuation of SDH activity by *SDHC* knockdown. Western blot analysis indicated that *SDHC* knockdown significantly increased levels of phosphorylated p65 (Ser536), suggesting increased NF-κB activity (Fig. 6A). Additionally, immunofluorescence staining showed significantly higher levels of p65 nuclear localization in *SDHC*-knockdown HLF cells relative to controls (Fig. 6B), which was further confirmed by immunoblot results (Fig. 6C). Moreover, examination of phosphorylated p65 (Ser536) levels in 224 HCC tissues by IHC staining indicated a significant negative correlation between *SDHC* and phosphorylated p65 (Ser536) levels (Fig. 6D). Furthermore, nuclear localization of p65

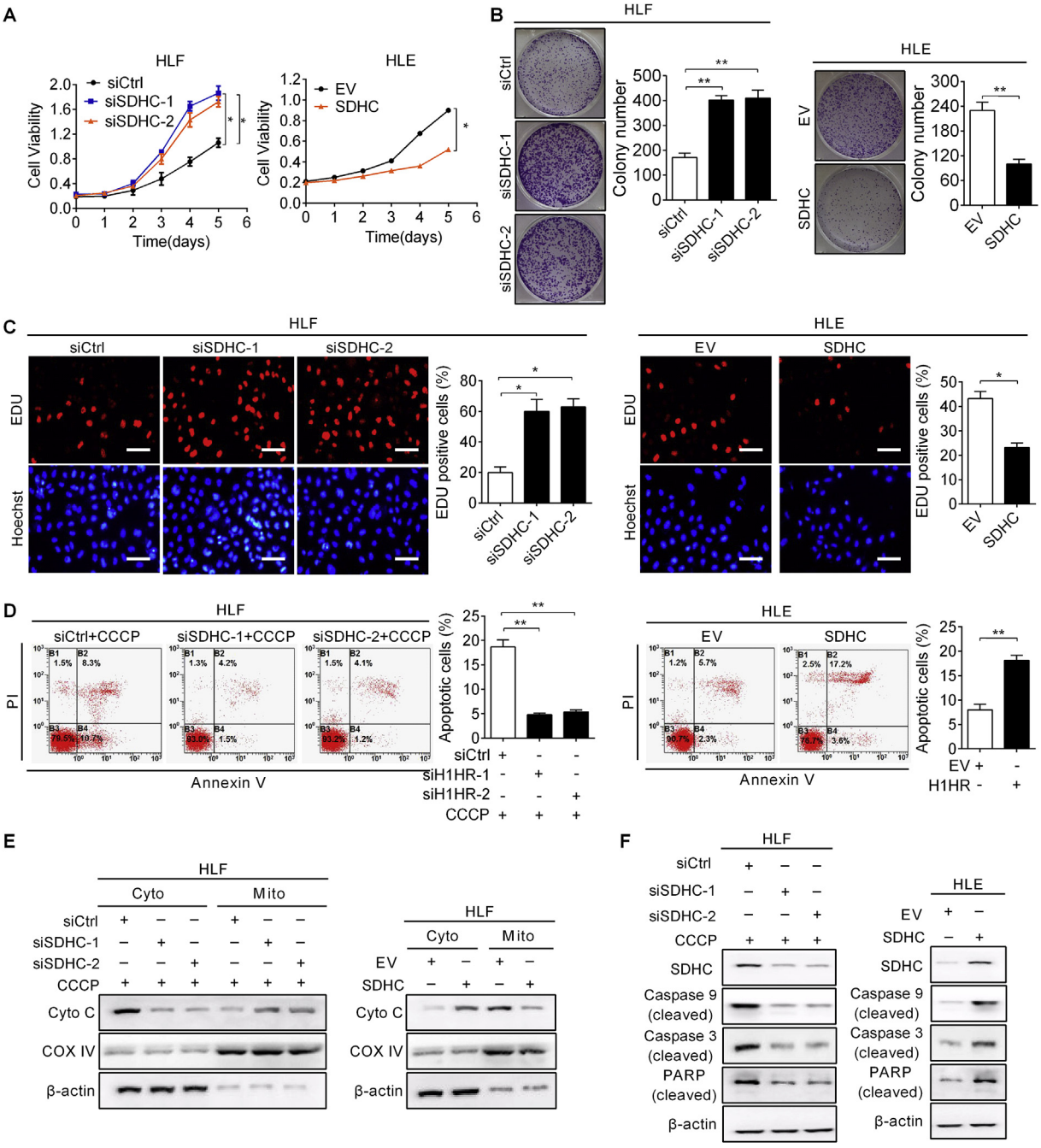

Fig. 3. Deficient SDH activity promotes HCC-cell growth *in vitro*. (**A**) MTS assay was performed using both HLF and HLE cells treated as indicated. (**B**) Colony formation assay was performed using HLF and HLE cells treated as indicated. (**C**) Cell proliferation was evaluated by EdU-incorporation assay in HLF and HLE cells treated as indicated. Scale bar: 20 μm. (**D**) Apoptosis was examined by flow cytometry using Annexin V (an indicator of apoptosis) and propidium iodide staining in HLF and HLE cells treated as indicated. HLF cells were also treated with CCCP (150 μM) for 4 h prior to evaluating apoptosis. **(E)** Protein levels of cytochrome C in the cytoplasm and mitochondria of HLF and HLE cells were detected by Western blot. β-actin and COX4 were used as loading controls for cytoplasm and mitochondria, respectively. **(F)** Protein levels of SDHC, cleaved caspase-9, cleaved caspase-3, and cleaved PARP in HLF and HLE cells treated as indicated.
COX4, cyclooxygenase 4; Cyto, cytoplasm; EV, empty vector; Mito, mitochondria; PARP, poly ADP ribose polymerase; SDHC, expression vector encoding SDHC; siSDHC-1/2, siRNAs against *SDHC*.

(Fig. 6E–F) was significantly reversed by NAC (an ROS scavenger) or H_2O_2 treatment, suggesting that deficient SDH activity might activate NF-κB signaling through increased ROS production. These results indicated that deficient SDH activity activated ROS/NF-κB signaling in HCC cells.

3.6. ROS/NF-κB signaling mediated by SDH activity regulates HCC-cell growth and metastasis

Given the crucial role of NF-κB signaling in cancer development and progression, we hypothesized that suppressed SDH activity promotes HCC-cell growth and metastasis through activation of NF-κB signaling. To test this hypothesis, we treated HCC cells with BAY11-7082 or a specific small-interfering (siRNA) targeting IκBα in order to inhibit or

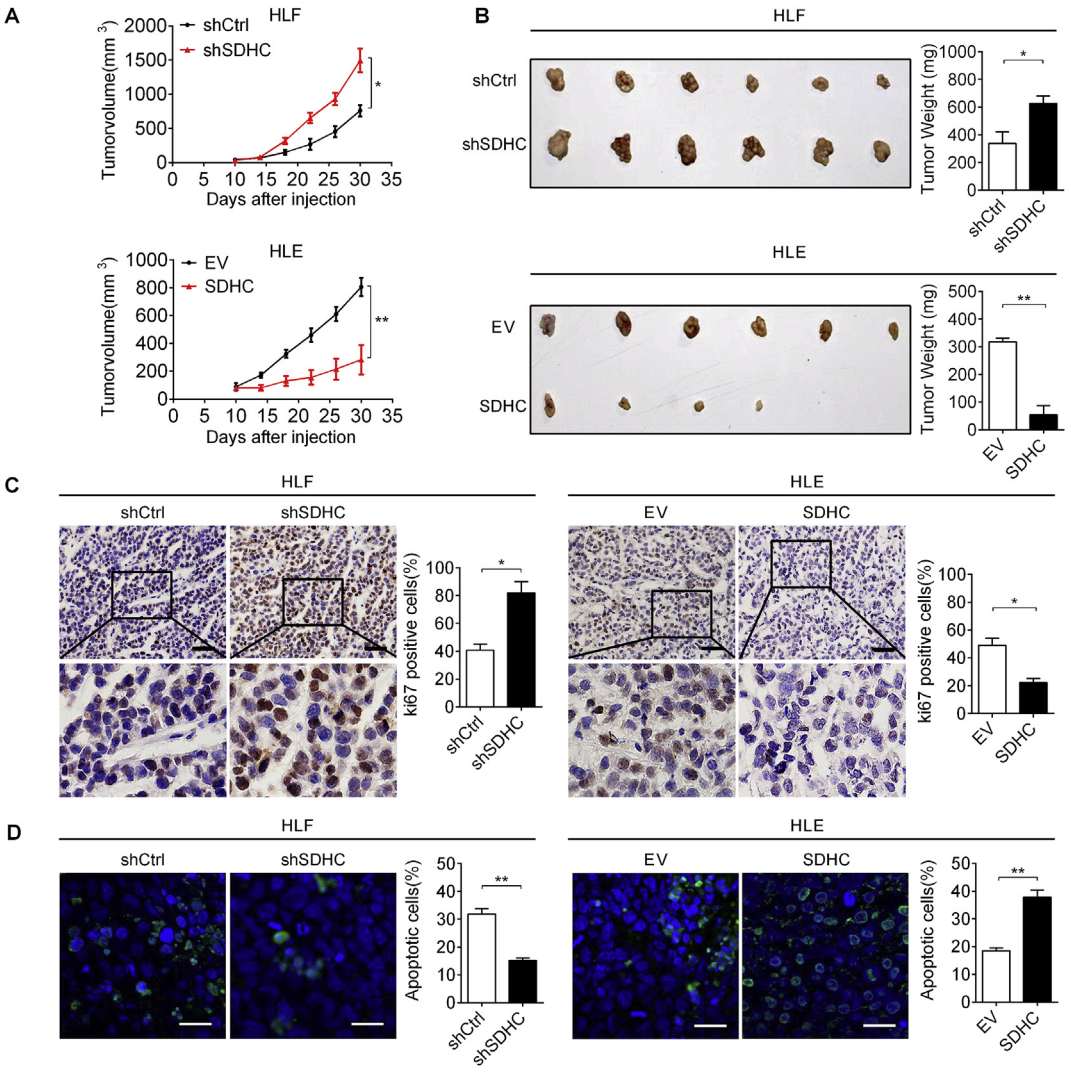

Fig. 4. Deficient SDH activity promotes HCC-cell growth *in vivo*.(**A**) Subcutaneous tumor-growth curves for HCC cells stably transfected with short-hairpin RNA targeting *SDHC* for *SDHC* knockdown or an *SDHC*-expression. (**B**) Dissected tumors from sacrificed mice (left panel) and tumor weight (right panel). (**C**) Ki-67 staining of tumor tissues from subcutaneous xenografts established as indicated. Scale bar, 50 μm. (**D**) TUNEL staining of tumor tissues from subcutaneous xenografts established as indicated. Scale bar, 50 μm*P < 0.05; **P < 0.01.

activate NF-κB signaling, respectively. Notably, inhibition of NF-κB by Bay11-7082 decreased SDHC-knockdown HLF-cell proliferation, migration, and invasion (Fig. 7A–D), whereas these activities in SDHC-overexpressing HLE cells were significantly increased following siRNA knockdown of IκBα (Figs. S4A–S4D).

To further evaluate the potential role of NF-κB in *SDHC*-mediated HCC-cell growth and metastasis *in vivo*, we treated xenografted and metastatic mice with Bay11-7082 three times weekly for 4 weeks by intraperitoneal injection of 10 mg/kg, a dose previously shown to inhibit NF-κB *in vivo* [20,21]. The results showed that Bay11-7082 treatment significantly inhibited tumor growth and metastasis regulated by *SDHC* knockdown (Fig. 7E–G). These results indicated that NF-κB activation is involved in HCC tumor growth and metastasis promoted by deficient SDH activity, suggesting this pathway as a potential therapeutic target for the treatment of HCC subsets exhibiting suppressed SDH activity.

Except NF-κB, activation of several other oncogenic signaling pathways, including Erk1/2, Hif-1α and AKT signaling pathways, have also been reported to be involved in ROS-mediated tumor progression. We thus determined the effect of suppressed SDH on the activities of Erk1/2, Hif-1α and AKT in HCC cells. As shown in Fig. S5A, SDHC knockdown did not notably affect the activation of Erk1/2 and Hif-1α, while activated AKT in HLF cells. In contrast, SDHC over-expression suppressed AKT in HLE cells. AKT activity was inhibited by treatment with MK2206 in HLF cells. Additionally, we found that the proliferation, migration, and invasion capabilities of HLF cells were significantly decreased in HLF cells with either normal or down-regulated SDHC expression upon treatment with MK2206, suggesting that AKT activation is involved in the oncogenic functions of SDH in HCC (Figs. S5B–S5E). Previously, we and many others have demonstrated that NFkB is a major downstream target of AKT in different types of cancer cells [16,22–25]. Considering that SDHC knockdown activated both AKT and NFkB, we hypothesized that AKT may be in the upstream of SDHC knockdown activated NF-κB. As shown in Figs. S5F–S5G, AKT inhibition by MK2206 significantly attenuated NF-κB activation when SDHC was knockeddown, while NF-κB inhibition by BAY11-7082 has no effect on the activity of AKT, indicating that the ROS/AKT/NFkB signaling axis is involved in the oncogenic functions of SDH in HCC.

Elevated levels of succinate, the SDH substrate, has been linked to tumorigenesis [26]. therefore, we determined whether the tumor-suppressive role of SDHC in HCC is also associated with increased succinate levels. Administration of succinate slightly increased the proliferation, migration, and invasion capabilities of HLF cells exhibiting either normal or increased *SDHC* expression (Figs. S6A–S6D), suggesting that

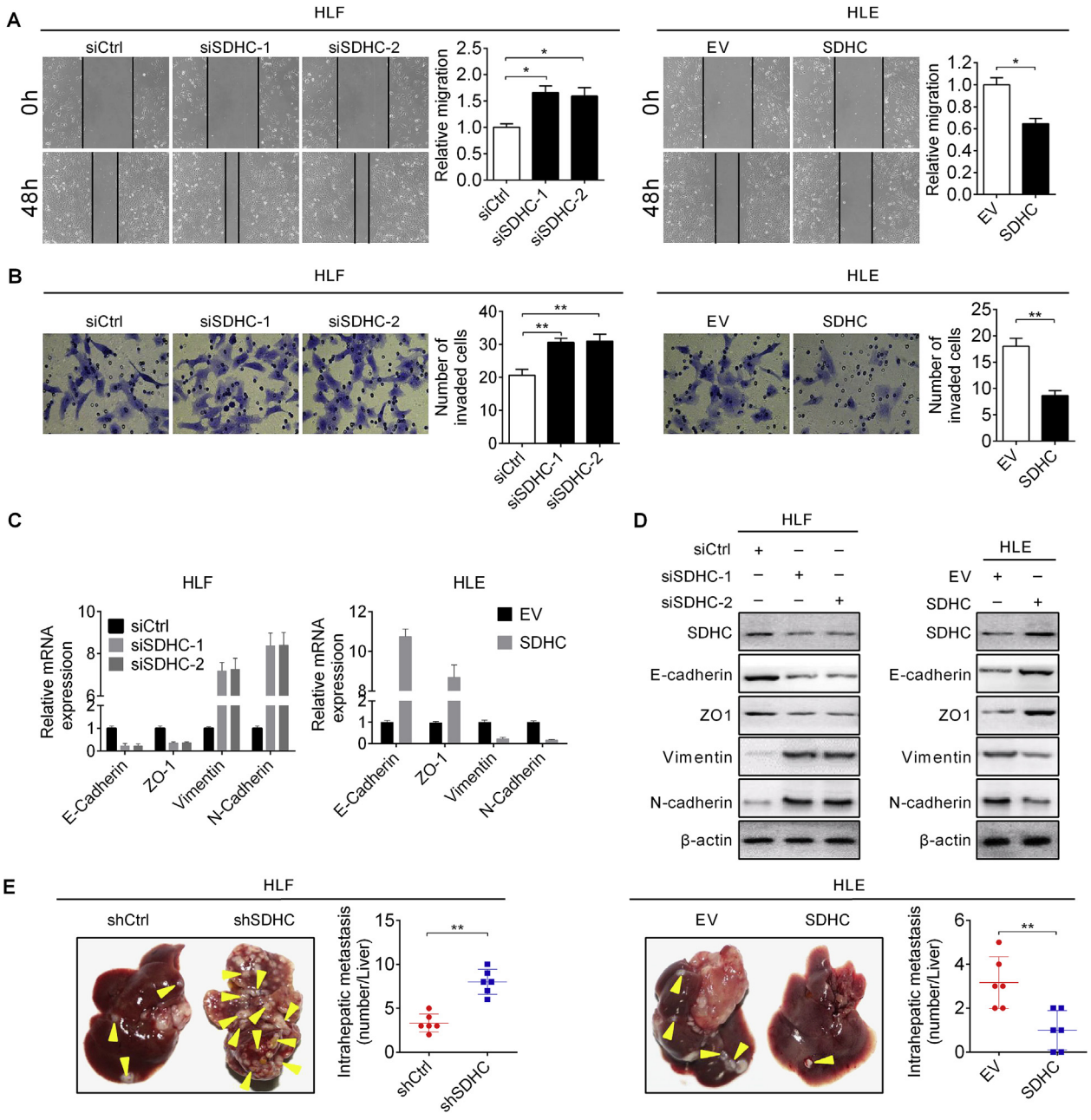

Fig. 5. Deficient SDH activity promotes HCC-cell migration and invasion. (**A**) Scratch-wound-healing assay to determine cell-migration ability of HLF and HLE cells treated as indicated. (**B**). Transwell assay to determine cell-invasion ability of HLF and HLE cells treated as indicated. (**C**) qPCR analysis of mRNA levels of EMT markers in HLF and HLE cells treated as indicated. (**D**) Western blot analysis of protein levels of EMT markers in HLF and HLE cells treated as indicated. (**E**) Incidence of intrahepatic metastasis and the number of metastatic foci observed in nude mouse models. Arrows indicates metastatic foci. *P < 0.05; **P < 0.01.

increased succinate might also be involved in the oncogenic functions of SDH in HCC.

4. Discussion

Recently, several studies reported mutations in the four subunits of the SDH enzyme complex, which represents an important component of the TCA cycle, contributing to tumor formation and progression. In addition, downregulated expression of *SDHA* and *SDHB* has also been reported in thyroid cancer and HCC [27,28]. Moreover, Tseng et al. have revealed a correlation between decreased SDHB expression and HCC patient OS [29]. However, the clinical significance of the other three subunits of SDH in HCC remains unclear. The present study demonstrated significant downregulation of all four subunits in HCC cells and tissue samples, as well as their association with poor OS and RFS in HCC patients. Furthermore, patients with all four subunits down-regulated had significantly poorer overall OS and RFS than those without all four subunits down-regulated, which shows a better prediction of survival than each of the four subunits. These results agreed with previous findings for nasopharyngeal carcinoma, where patients exhibiting attenuated *SDHB* expression presented a significantly shorter OS rates compared with those exhibiting elevated *SDHB* expression [30]. By contrast, a previous study of renal-cell carcinoma showed that patients with higher levels of *SDHB* expression displayed a worse OS rate relative to that observed in patients exhibiting attenuated *SDHB* expression [31]. These different observations might suggest the tumor-specific nature of SDH-subunit expression.

Mutations in each of the four SDH subunits disrupt formation of the

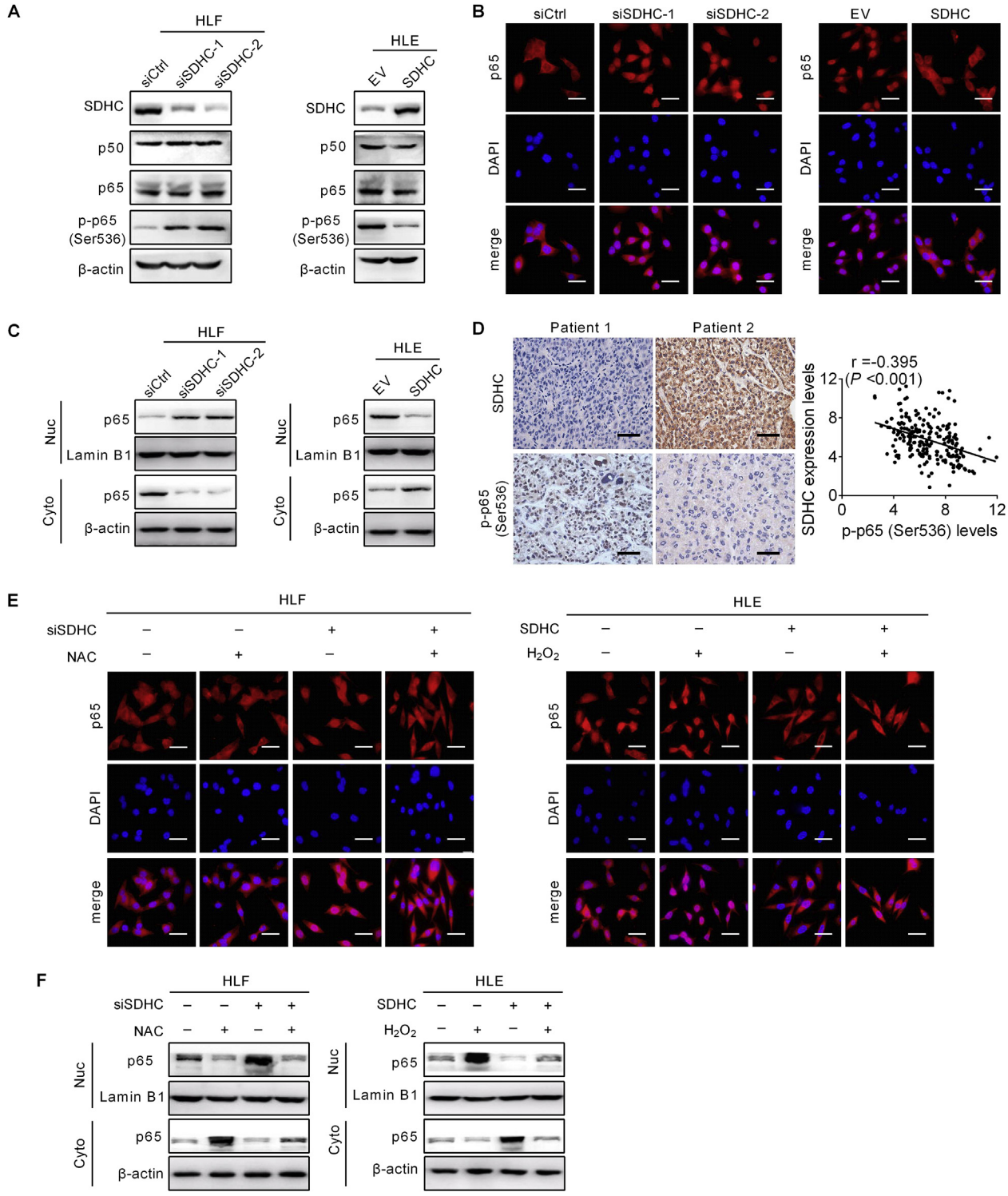

Fig. 6. Deficient SDH activity activates ROS/NF-κBsignaling in HCC cells.(**A**) Western blot analysis in HCC cells treated as indicated. (**B**). NF-κB nuclear translocation assessed by immunofluorescence staining for p65 (red) in HLF and HLE cells treated as indicated. Nuclei were counterstained with DAPI (blue). Scale bars: 50 μm. (**C**) Western blot analyses for nuclear and cytosolic protein levels of p65 in HCC cells with treatment as indicated. (**D**) Left panel: representative immunohistochemical staining images of SDHC and phosphorylated p65 (Ser536) in 224 tumor tissues of HCC (Scale bars, 50 μm). Right panel: the relationship between the expression of SDHC and phosphorylated p65 (Ser536) was analyzed based on IHC staining. (**E**) NF-κB nuclear translocation was assessed by immunofluorescence staining for p65 (red) in HLF and HLE cells treatments as indicated (100 mM H_2O_2 or 20 mM NAC for 12 h). Nuclei were counterstained with 4′,6-diamidino-2-phenylindole. Scale bars: 50 μm. (**F**) Western blot analyses of nuclear and cytosolic levels of p65 in HLF and HLE cells treated as indicated. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article).

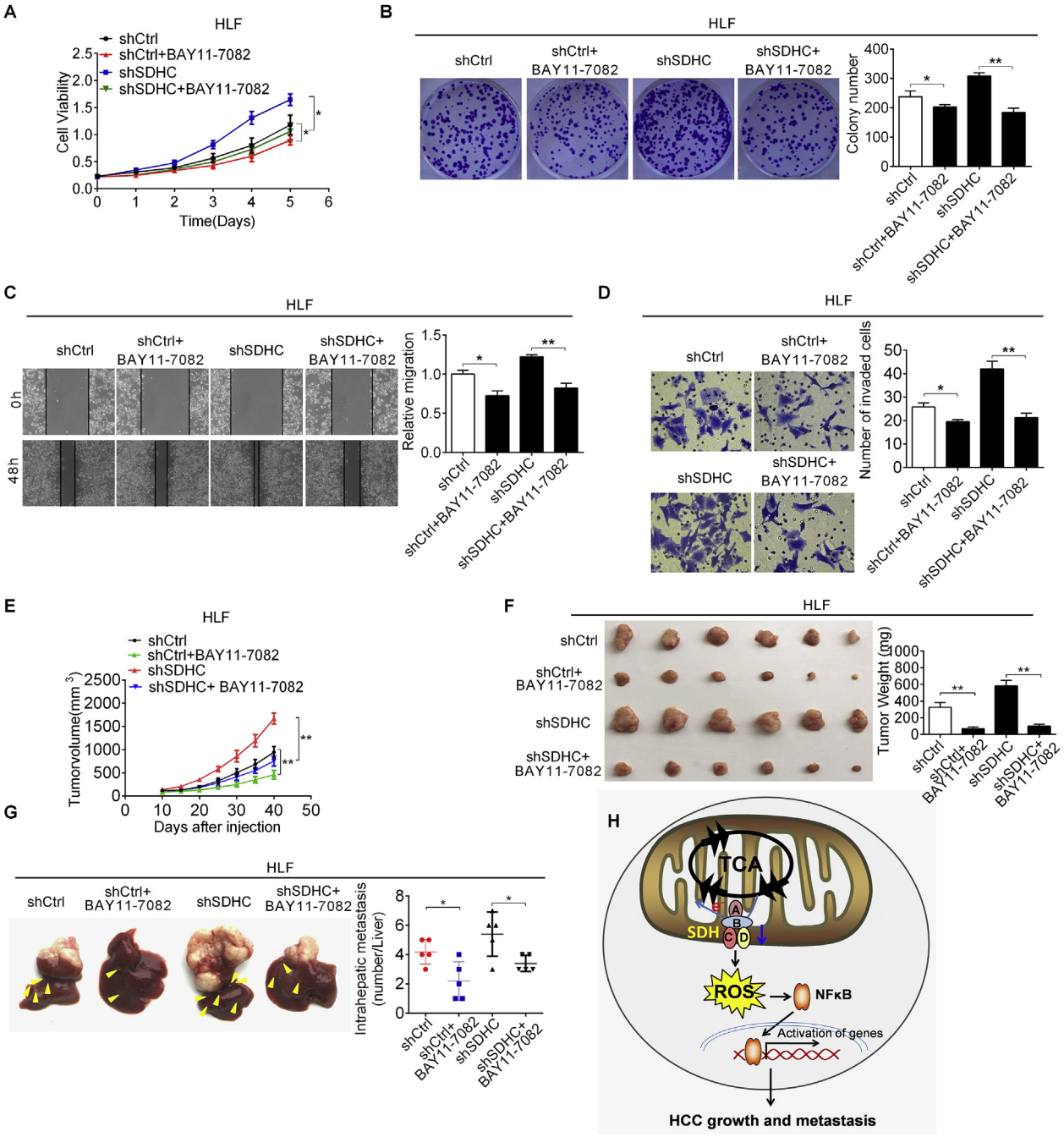

Fig. 7. ROS/NF-κB signaling mediated by SDH activity affects HCC-cell growth and metastasis. (**A, B**) MTS and colony formation assays were performed to assess HCC-cell growth following transient transfection of siRNA targeting *SDHC*, followed by treatment with the NF-κB inhibitor Bay11-7082 (12.5 mM) for 12 h. (**C, D**) Scratch-wound-healing and Matrigel invasion assays were performed to assess HCC-cell migration following treatment with the NF-κB inhibitor Bay11-7082 (12.5 mM) for 12 h. (E) Tumor-bearing mice injected with SDH-control (shCtrl) or SDHC-knockdown (shSDHC) HCC cells were treated with 10 mg/kg BAY11-7082 three times weekly by intraperitoneal injection. Tumor-growth curves are shown. (F) Dissected tumors (left) and their weights (right). (G) Incidence of intrahepatic metastasis and the number of metastatic foci in nude mouse models. Arrows indicate metastatic foci. (H) Schematic depicting the regulation of HCC growth and metastasis by deficient SDH activity in HCC. $*P < 0.05$; $**P < 0.01$.

enzyme complex and subsequently decrease enzymatic activity [7]. The results of the present study consistently indicated that downregulated expression of the four subunits suppressed SDH activity. Due to the central role of SDH in energy metabolism, previous studies focused on the role of SDH in regulating reprogrammed metabolism in cancer, with Pollard et al. demonstrating that compared with sporadic tumors, SDH-deficient paragangliomas display increased glycolysis [32]. Additionally, several previous studies reported that loss of SDH activity altered the metabolism of non-essential amino acids [33]. However, SDH mutations have been found only in several particular types of malignancies, especially several hereditary cancers [5–7,34]. Our cBioPortal-based analysis indicated that the mutation rates of SDHA, SDHB, SDHC and SDHD in HCC were only 8%, 0%, 0% and 0%, respectively (data not shown). The expressions and biological functions of the four subunits of SDH in cancers under non-mutation conditions such as HCC are still largely unclear. In the present study, we found that all of the four subunits of SDH were downregulated in HCC cells, leading to lower SDH enzymatic activity and thus increased epithelial-

mesenchymal transition, which is consistent with a previous study in ovarian cancer, showing that knockdown of SDHB resulted in an epithelial-to-mesenchymal transition (EMT) [35]. It is generally considered that high ROS level causes DNA damage, cell apoptosis or transformation, whereas moderate ROS increase stimulates proliferation and metastasis of cancer cells. Previous studies have indicated that SDH-related mutations lead to genomic instability and subsequent malignant transformation due to the overwhelming oxidative stress [14–16]. Benjamin et al. showed that SDHC mutations result in increased ROS production, which induces metabolic stress and genomic instability and promotes tumorigenesis [14]. Additionally, Ishii et al. reported that SDHC mutation increases oxidative stress, thereby contributing to nuclear DNA damage, mutagenesis, and, ultimately, tumorigenesis [15]. However, our results showed a moderate increase of ROS level in SDHC downregulated HCC cell, suggesting that our findings in HCC under non-mutation conditions may be different to those in SDH mutation-caused tumors. MicroRNA plays an important epigenetic role in the gene-expression regulation network. Several frequently overexpressed miRNAs in HCC, such as miR-210 and miR-31, have been demonstrated to be specifically target of SDHD and SDHA respectively [36,37], suggesting that elevated oncogenic miRNAs may be involved in the downregulation of the four subnunits of SDH in HCC.

Mechanistically, we found that activation of ROS/NF-κB pathway was involved in the regulation of HCC-cell growth and metastasis promoted by deficient SDH activity. However, Tseng et al. showed that silencing of SDHB contributed to malignancy of HCC by inducing the Warburg effect [29]. These contradictory results suggest that the SDHB and SDHC subunits promote HCC progression through different mechanisms, which might be associated with the distinct functional properties conferred by each subunits to SDH activity. In addition to NF-κB, our results also suggested that activation of AKT signaling could also be involved in ROS-mediated tumor progression, which still needs further clarification. Moreover, Our results showed that altered levels of the SDH substrate succinate increased the proliferation, migration, and invasion capabilities of HLF cells exhibiting either normal or upregulated levels of *SDHC* expression. Therefore, we cannot eliminate the possibility that these factors might be also involved in SDH-related oncogenic functions in HCC, with these functions requiring further clarification.

Succinate has been well established as one of the most common oncometabolites, whose abnormal accumulation promotes malignant transformation [38]. As intermediates in the tricarboxylic acid (TCA) cycle, succinate accumulation could result in reprogrammed mitochondria oxidative phosphorylation, HIF-1 activation and ROS production [38–40], all of which are indispensable metabolic elements in oncogenesis and tumor progression [38]. In addition, succinate could also lead to the activation of oncogenic signaling of extracellular regulated kinase (ERK) 1/2 and signal transducer and activator of transcription 3 (STAT3) through binding to succinate receptor 1 (SUCNR1), an orphan molecule that belongs to the G protein-coupled receptor [41]. Our results in showed that administration of succinate increased the proliferation, migration and invasion capabilities of HLF cells with either normal or increased SDHC, indicating that increased succinate could also be involved in the oncogenic functions of SDH in HCC.

In summary, our findings demonstrated that expression of the four SDH subunits (SDHA/B/C/D)was frequently downregulated in HCC tissues and associated with poor prognosis via the resulting inactivation of SDH. Moreover, deficient SDH activity played a critical role in regulating HCC-cell growth and metastasis primarily through elevated ROS levels and subsequent activation of NF-κB signaling. These findings collectively suggest a critical tumor-suppressive role for SDH in HCC and support this enzyme as a potential drug target for the treatment of this malignancy.

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Author contributions

J.L., N.L. and X.L. performed most experiments, analyzed data; J.L, wrote the manuscript; X.G. and X.H. performed prognosis and public microarray data analysis; J.Z., J.Y. and T.Y. participated in the *in vitro* study; X.D., P.Y. participated in the *in vitro and vivo* study. J.Z. collected HCC tissue samples and clinical data; J.X. and X.H. designed the overall study, supervised the experiments and revised the paper.

Conflicts of interest

The authors declare that they have no competing interests.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.canlet.2019.07.001.

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