Research Square

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HINT3 suppresses AKT/mTOR signaling activity during breast cancer tumorigenesis through transcriptionally activation of PTEN

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Abstract

Background

Histidine triad nucleotide-binding (HINT) protein belongs to the histidine triad proteins family. Recent studies have shown that HINT1 and HINT2 play pivotal roles in cancer growth. However, the function of HINT3 in cancers, including breast cancer (BRCA) remains to be determined. In the present study, we investigated the role of HINT3 in BRCA.

Methods

The clinical relevance of HINT3 in BRCA was analyzed using the datasets from The Cancer Genome Atlas. HINT3 was over-expressed and knocked down using lentivirus system. qRT-PCR and Western blot assays were performed to detect mRNA and protein expression. CCK-8 and colony formation assays were used to assess cell proliferation. Migration was analyzed using Transwell assay. Luciferase reporter activity assay was performed using pGL3.Basic/TK system. Xenogrfted tumorigenesis was performed to evaluate the effect of HINT3 on tumor development.

Results

HINT3 was down-regulated in BRCA tissues based on TCGA analysis and our qPCR analysis. TCGA database also showed that HINT3 transcript was much lower in BRCA tissues with higher stage. *In vitro*, HINT3 knockdown promoted the cell proliferation, colony growth and EDU cooperation in MCF7 and MDA-MB-231 cells. Oppositely, HINT3 overexpression suppressed the DNA synthesis and proliferation in both cells. *In vivo*, HINT3 ectopic expression blunted the xenografted tumorigenesis of MDA-MB-231 cells. Furthermore, HINT3 silencing or overexpression enhanced and inhibited the migration capacity of MCF7 and MDA-MB-231 cells, respectively. Lastly, HINT3 upregulated PTEN at transcription level, which resulted in inactivation of AKT/mTOR signaling *in vitro* and *in vivo*.

Conclusions

Taken together, HINT3 inhibits PTEN/AKT/mTOR signaling pathway and suppresses the proliferation, growth, migration and tumor development of BRCA cells.

Background

Breast cancer (BRCA) is the most commonly diagnosed malignancy among women worldwide [1]. Approximately 2.1 million BRCA patients were newly diagnosed and more than 0.62 million of the patients died in 2018 [2]. Based on the absence or presence of human epidermal growth factor 2 (HER2), estrogen receptors (ER) or progesterone receptors (PR), BRCA was divided into three subtypes, including

ER+/HER2-, HER + and ER-/PR-/HER2- [3]. Even though most of the patients are diagnosed at non-metastatic and curable stage, the remaining advanced BRCA women are faced with irreparable predicament with no effective therapy. Thus, identifying novel molecular biomarkers or essential contributors for BRCA may help develop effective therapies against this malignancy.

The HIT protein superfamily is classified into at least three subgroups, containing HINT, FHIT and GalT [4]. Increasing evidences have demonstrated that the HINT subfamily functions as tumor suppressors in various cancers. Depletion of HINT1 in mice promotes the development of N-nitrosomethylbenzylamine-induced squamous tumors in forestomach and 7,12-dimethylbenz[a]anthracene-induced breast cancer [5, 6]. Long term observation shows that HINT1 knockout mice exhibit higher incidence of spontaneous tumors than widetype mice [6]. Reduced expression of HINT1 is found in gastric cancer (GC) and hepatocellular carcinoma (HCC) [7, 8]. Similar to HINT1, HINT2 is also identified as a tumor suppressor in cancers. HINT2 is down-regulated in HCC and colorectal cancer [9, 10]. Reduction of HINT2 promotes the proliferation, migration and xenografted tumorigenesis of cancer cells [10, 11]. Nevertheless, the significance of HINT3 in BRCA remains unclear.

In this study, we explored the clinical relevance of HINT3 and its potential role in BRCA. We used cell lines and nude mice model to investigate the function of HINT3 in BRCA growth. We found that HINT3 was down-regulated in BRCA tissues. Down-regulation of HINT3 promoted the proliferation, growth and migration of BRCA cells. *In vivo*, xenografted tumor formation was blunted by HINT3 overexpression. Molecular experiments showed that PTEN/AKT/mTOR signaling pathway was suppressed by HINT3. Our study demonstrated HINT3 as a tumor suppressor in BRCA.

Methods

TCGA database analysis

The clinical information of each patients and the transcriptome expression datasets were downloaded from The Cancer Genome Atlas (http://cancergenome.nih.gov). 1. 1104 cancer and 113 normal tissues were used for analyzing the HINT3 expression in BRCA tissues and normal tissues. 2. 114 normal, 183 stage I, 615 stage II, 247 stage III and 20 stage IV BRCA tissues were used for analyzing the correlation between HINT3 expression and BRCA stage. 3. 114 normal, 516 N0, 362 N1, 120 N2 and 77 N3 BRCA tissues were used for analyzing the relationship between HINT3 expression and nodal metastasis status.

Cell culture

Human breast cancer cell lines MCF7 and MDA-MB-231 were purchased from ATCC. These cells were cultured in (Hyclone) medium, which was supplied with 10% fetal bovine serum (Gbico) and 1% penicillin and streptomycin solution (Hyclone). The cell culture was maintained at 37°C with 5% CO_2.

Lentivirus-mediated HINT3 knockdown and overexpression

The lentivirus vector was used to knock down HINT3 in MCF7 and MDA-MB-231 cells. This system composes three vectors: pGCSIL-GFP (inserted with the targeted shRNA), pHelper1.0 (gag/pol) and Helper2.0 (VSVG). The sequences of the shRNA were as follow: negative control, 5’-TTCTCCGAACGTGTCACGT-3’; HINT3#1, 5’-GCGAGAATGAGGACCTAATTT-3’ HINT3#2, 5’-GAGTCAATTCCTATTGGTTTA-3’. After cloning the shRNA into the pGCSIL-GFP vectors, the vectors were co-transfected with pHelper1.0 and Helper2.0 into 293FT cells using Lipofectamine 2000 (Invitrogen). Viral supernatants were harvested, filtered and centrifuged for subsequent infection. Knockdown efficiency was detected by qRT-PCR and Western blot assays.

Western blot

The protein from shCtrl, shHINT3#1 and shHINT3#2, Ctrl and HINT3 overexpressed MCF7 and MDA-MB-231 cells were lysed using lysis buffer (Beyotime). BCA protein assay kit (Beyotime) was performed to detect the protein amount. The proteins were loaded on SDS-PAGE gels for separation and transferring onto the PVDF membranes. Subsequently, the membranes were blocked by 5% non-fat milk for 60 minutes at room temperature and incubated with indicated primary antibodies at 4°C overnight. Antibody against HINT3 was from Abcam. Antibodies against β-actin and the secondary antibodies were from Proteintech.

qRT-PCR

shCtrl, shHINT3#1 and shHINT3#2, Ctrl and HINT3 overexpressed MCF7 and MDA-MB-231 cells were lysed using Trizol (Invitrogen). Total RNA was extracted using Ultrapure RNA Kit from CWBIO, according to the manufacturer’s protocols. After reversely transcribing the RNA to cDNA, qPCR reaction was conducted on the Biorad machine with SYBR master mixture. The sequences of qPCR primers are as follows: HINT3 forward, 5’-CTGGTTGAGAACATGGTAACT-3’, and reverse, 5’-TGATCAGCTGTGATAAACCAAT-3’; and GAPDH forward, 5’-TGACTTCAACAGCGACACCCA-3’, and reverse, 5’- CACCCTGTTGCTGTAGCCAAA-3. GAPDH is the internal control.

CCK assay

Equal number of shCtrl, shHINT3#1, shHINT3#2, Ctrl and HINT3 overexpressed MCF7 and MDA-MB-231 cells were seed into 96-well plates, which contained 200ul culture medium. 1, 2, 3 and 4 days later, 20ul of CCK regent was added into each well and incubated at 37°C for 3 hours. The optical density (OD) was detected at 450 nm on the micro-plate reader.

Colony formation

Equal number of shCtrl, shHINT3#1, shHINT3#2, Ctrl and HINT3 overexpressed MCF7 and MDA-MB-231 cells were seed into the 6-well plates. After cultured for 10 days, the colonies were fixed by methanol and stained by Giemsa solution. The images of the colonies were taken with the camera and the numbers of the colonies were counted using Photoshop CS5.

Transwell assay

8.0-μm filter migration chambers (BD Biosciences, USA) was applied to detect the cell migration of shCtrl, shHINT3#1 and shHINT3#2 MCF7 and MDA-MB-231 cells. In brief, equal number of the cells were seeded onto the upper surface of the chambers. After incubating at 37°C for 24 hours, the cells on the upper surface were removed by cotton tips. The cells on the lower surface were fixed by methanol and stained by crystal violet. The stained cells were photographed using the microscope.

Dual luciferase activity reporter assay

The promoter sequence of PTEN was inserted into pGL3.basic reporter vectors. The coding sequence of HINT3 was inserted into pCDNA3.1 vectors. MCF7 cells were transfected with pGL3.basic, TK and siRNAs or pCDNA3.1 vectors. Luciferase activity was measured using Dual luciferase reporter kit (Promega), following manufacturer's protocols.

In vivo xenograft assay

Balb/c nude mice (14-16 g per mice) were purchased from Animal Center of Beilun District People's Hospital and were fed with standard diet and water in a specific pathogen-free room. The temperature was maintained at 25°C and the room light was kept with 12-h light/12-h dark cycles. Ten mice were randomly divided into two groups (n=5 in each group). A total of $3*10^7$ MDA-MB-231 cells were subcutaneously implanted into the right armpit of 5-week old female balb/c nude mice. The volume of the xenogrfted tumors was calculated using the following formula: $v=ab^2/2$ (a, the long diameter, b, the short diameter). The mice were sacrificed by peritoneal injecting with 0.4 mg/g chloral hydrate per mice at 45 days after implantation. Animal experiments were approved by the Ethics Committee of and were performed according to the animal guideline of Beilun District People's Hospital.

Statistical analysis

The statistical analysis was conducted using GraphPad prism. The data were shown as mean ± standard error of mean (SEM). Students't tests, one-way or two-way analysis of variance (ANOVA) were used to determine the statistical significance. Statistical difference was considered significant when $P<0.05$.

Results

The expression of HINT3 is reduced in BRCA tissues and is inversely correlated with the tumor stage.

TCGA database has a large amount of information on the transcript abundance of various genes in different cancer types. To evaluate the clinical relevance of HINT3 in breast cancer, we analyzed the mRNA expression of HINT3 from TCGA. The analysis showed that HINT3 was down-regulated in BRCA tissues as compared with the normal tissues (Figure 1A). We collected the normal and BRCA tissues and subjected them to qPCR detection of HINT3 mRNA level. The results showed that the mRNA level of HINT3 was obviously decreased in BRCA tissues (Figure 1B and 1C). Since breast cancer is a malignancy

with faster disease progression and a high potential of metastasis, we analyzed HINT3 expression in BRCA tissues of different stage and metastasis status. HINT3 was much lower in the cancer tissues of higher stage or metastasis status (Figure 1C and 1D). Collectively, HINT3 may participate in breast cancer development.

HINT3 inhibits the proliferation and colony formation of BRCA cells.

To explore the role of HINT3 in BRCA cells, we firstly used lentivirus-mediated overexpression of HINT3 in BRCA cells. Western blot results showed that HINT3 was overexpressed in both cells (Figure 2A). Then, we examined the effect of HINT3 on BRCA cell proliferation using CCK and colony formation assays. HINT3 overexpression inhibited the cell proliferation of MCF7 and MDA-MB-231 cells (Figure 2B). Up-regulation of HINT3 also suppressed the colony formation of both cells (Figure 2C). Next, knockdown efficiency of HINT3 was assessed by Western blot in these cells. The results showed that the protein abundance of HINT3 was significantly reduced in shHINT3-1 and shHINT3-2 MCF7 and MDA-MB-231 cells (Figure 2D). HINT3 reduction obviously accelerated the proliferation of MCF7 and MDA-MB-231 cells (Figure 2E). Consistently, down-regulation of HINT3 enhanced the colony formation capacity in MCF7 and MDA-MB-231 cells (Figure 2F and 2G). Taken together, HINT3 functions as a tumor suppressor protein in breast cancer.

HINT3 reduces the EDU cooperation in BRCA cells.

Proliferative cells require increased DNA synthesis. EDU cooperation is an assay to analyze the DNA synthesis. We then detected the DNA synthesis using EDU staining in BRCA cells. Compared with the EDU staining density in shCtrl MCF7 and MDA-MB-231 cells, the positive signal of EDU was dramatically increased in shHINT3#1 and shHINT3#2 cells (Figure 3A). In contrast, the EDU cooperation was suppressed by HINT3 overexpression in MCF7 and MDA-MB-231 cells (Figure 3B). Collectively, HINT3 represses the DNA synthesis in BRCA cells.

HINT3 suppresses the migration of MCF7 and MDA-MB-231 cells

Breast cancer is a malignancy with high potential of metastasis. We herein explored the role of HINT3 in BRCA metastasis using transwell assay. The results showed that HINT3 knockdown increased the migration of MCF7 cells, while HINT3 ectopic expression suppressed the migration capacity (Figure 4A and 4B). Similar results were found in HINT3 silenced and overexpressed MDA-MB-231 cells (Figure 4C and 4D).

HINT3 suppresses PTEN/AKT/mTOR signaling in BRCA cells

Next, we explored the molecular changes in MCF-7 cells with up-regulation and down-regulation of HINT3. We found that HINT3 knockdown and overexpression led to down-regulation and up-regulation of PTEN at the mRNA level, respectively (Figure 5A and 5B). HINT3 is a nuclear protein which may participate in regulating the transcription activity of downstream genes. We then performed luciferase activity reporter assay to check whether HINT3 regulates the transcription activity of PTEN. The results showed that

HINT3 promoted the transcription activity of PTEN in MCF7 cells (Figure 5B). Western blot results showed that PTEN protein expression was reduced by HINT3 silencing and stimulated by HINT3 ectopic expression (Figure 5C). Consistently, the phosphorylation but not the protein expression of AKT and S6 was enhanced and suppressed by HINT3 knockdown and overexpression, respectively in MCF7 cells (Figure 5C). Taken together, PTEN/AKT signaling may participate in the tumor suppressive role of HINT3 in BRCA.

HINT3 inhibits AKT activity and xenografted tumor growth

To explore the function of HINT3 *in vivo*, MDA-MB-231 cells infected with Ctrl or HINT3 overexpressed lentivirus were subcutaneously implanted into the right armpit of 4-week old female nude mice. The mice were sacrificed by the day of 45. The results showed that HINT3 overexpression inhibited the tumorigenesis and progression of MDA-MB-231 cells (Figure 6A-6C). We also found that HINT3 overexpression repressed AKT activity in the tumors (Figure 6D). Collectively, HINT3 blocked AKT activity and the tumor growth of BRCA cells in nude mice.

Discussion

Unlike HINT1 and HINT2, the role of HINT3 is not known in cancer development. Here in this study, we found that HINT3 was a tumor suppressor in BRCA. *In vitro*, HINT3 knockdown promoted the proliferation, colony growth, EDU cooperation and migration of BRCA cells. By contrast, opposite results were observed in HINT3 overexpressed BRCA cells. *In vivo*, xenografted tumorigenesis of MDA-MB-231 cells was repressed by HINT3 ectopic expression.

Approximately ten percent of the BRCA patients are associated with inherited genetic variants. Among them, mutations of BRCA1 and BRCA2, which are involved in DNA repair, are important events during BRCA development [12–15]. Besides, the most commonly mutated genes or amplified genes, such as TP53, PIK3CA, MYC, PTEN, CCND1, ERBB2, FGFR1 and GATA3, also play important roles in BRCA progress [16]. These triggers give the probability of drugs to treat BRCA patients. The representative drug is poly (ADPribose) polymerase (PARP) inhibitor to treat the BRCA patients with BRCA mutations [17]. However, the effectiveness is unsatisfactory and the usage is limited. It is necessary to identify novel drug targets to cure this malignancy. In this study, we revealed HINT3 as a tumor suppressor in BRCA. HINT3 was down-regulated in BRCA tissues. HINT3 expression was much lower in BRCA tissues of higher stage or higher metastasis status. These results suggested that HINT3 was inversely correlated with the disease progression of BRCA. Functional experiments showed that HINT3 ectopic expression repressed the proliferation and migration capacity in MCF7 and MDA-MB-231 cells. The DNA synthesis, which is the hallmark of cell cycle progression, was also suppressed by HINT3. In vivo, HINT3 overexpressed MDA-MB-231 cells developed xenografted tumors slower than Ctrl MDA-MB-231 cells. These findings indicated that HINT3 exhibited tumor suppressive function in BRCA cells.

Phosphatase and tensin homolog (PTEN) is a well-known tumor suppressor [18]. Inactivation of PTEN leads to enhanced phosphorylation and activity of AKT. Activation of AKT contributes to cancer

development through regulating distinct downstream targets, including GSK3β, FOXO, p21 and caspase 9 [19]. Absence or mutations of PTEN are commonly observed in BRCA [20–22]. However, the upstream regulator of PTEN is less known in BRCA. Here, we found that HINT3 negatively regulated the mRNA and protein expression of PTEN. HINT3 knockdown and overexpression up-regulated and down-regulated the phosphorylation level of AKT. These results suggested that HINT3 suppressed the development of BRCA at least partly through inactivating PTEN/AKT signaling pathway.

Conclusion

In summary, we provided for the first time that HINT3 was down-regulated in BRCA tissues and its mRNA level was inversely correlated with the disease stage. Furthermore, reduction of HINT3 contributed to the development of BRCA via activating PTEN/AKT signaling pathway. Our study added a new tumor suppressor and signaling cascade in BRCA.

Abbreviations

HINT, Histidine triad nucleotide-binding; BRCA, breast cancer; HER2, epidermal growth factor 2; ER, estrogen receptors; PR, progesterone receptors; GC, gastric cancer; HCC, hepatocellular carcinoma; SEM, standard error of mean; PARP, poly (ADPribose) polymerase; PTEN, phosphatase and tensin homolog.

Declarations

Funding

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Author contribution

Q.J., Y.L., B.L. and J.L. designed the study. Q.J., Y.L., B.L., H.L., D.C., Y.G. and Y.W. conducted the experiments and analyzed the results. Q.J. and J.L. wrote and revised the manuscript. All authors read and approved the final manuscript.

Availability of data and materials

All data generated during this research are available.

Ethics approval and consent to participate

The study was approved by the Ethics Committee of Beilun District People's Hospital. Written informed consents were collected from all patients.

Consent for publication

All of the authors agreed to publish our article in your journal.

Competing interests

These authors declare no conflicts of interest.

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Figures

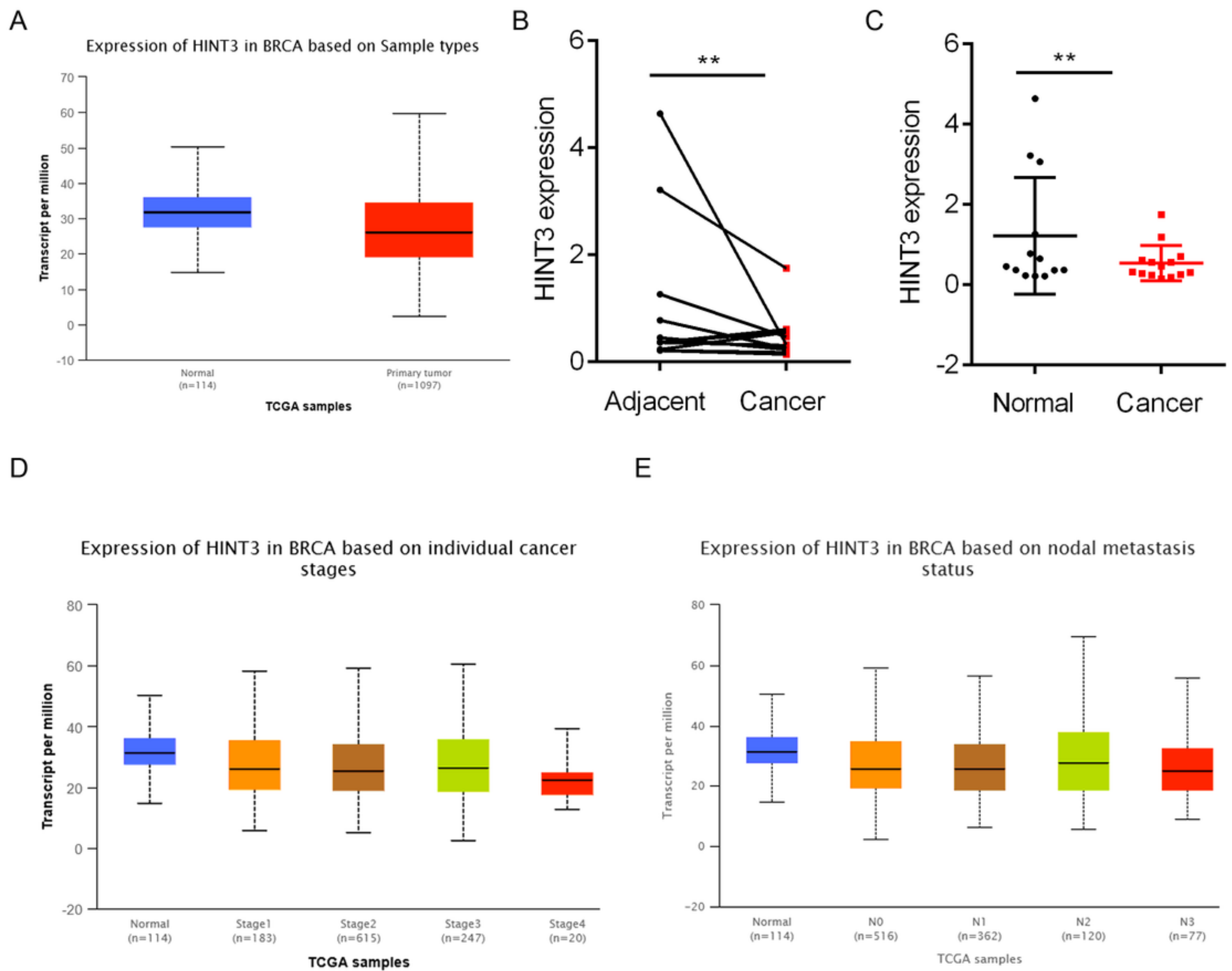

Figure 1

HINT3 is down-regulated in BRCA tissues. (A) The transcript of HINT3 was analyzed in BRCA tissues (n=1097) and normal tissues (n=114) from the TCGA database. p<0.001. (B) HINT3 mRNA expression was determined by qRT-PCR assay in BRCA tissues and paired-normal tissues. (C) HINT3 mRNA expression was determined by qRT-PCR assay in BRCA tissues and normal tissues. (D) The expression of HINT3 was analyzed in BRCA tissues of different individual stages from the TCGA database. *p<0.05, **p<0.01, ***p<0.001. (E) The expression of HINT3 was analyzed in BRCA tissues of different metastasis status from the TCGA database. **p<0.01, ***p<0.001.

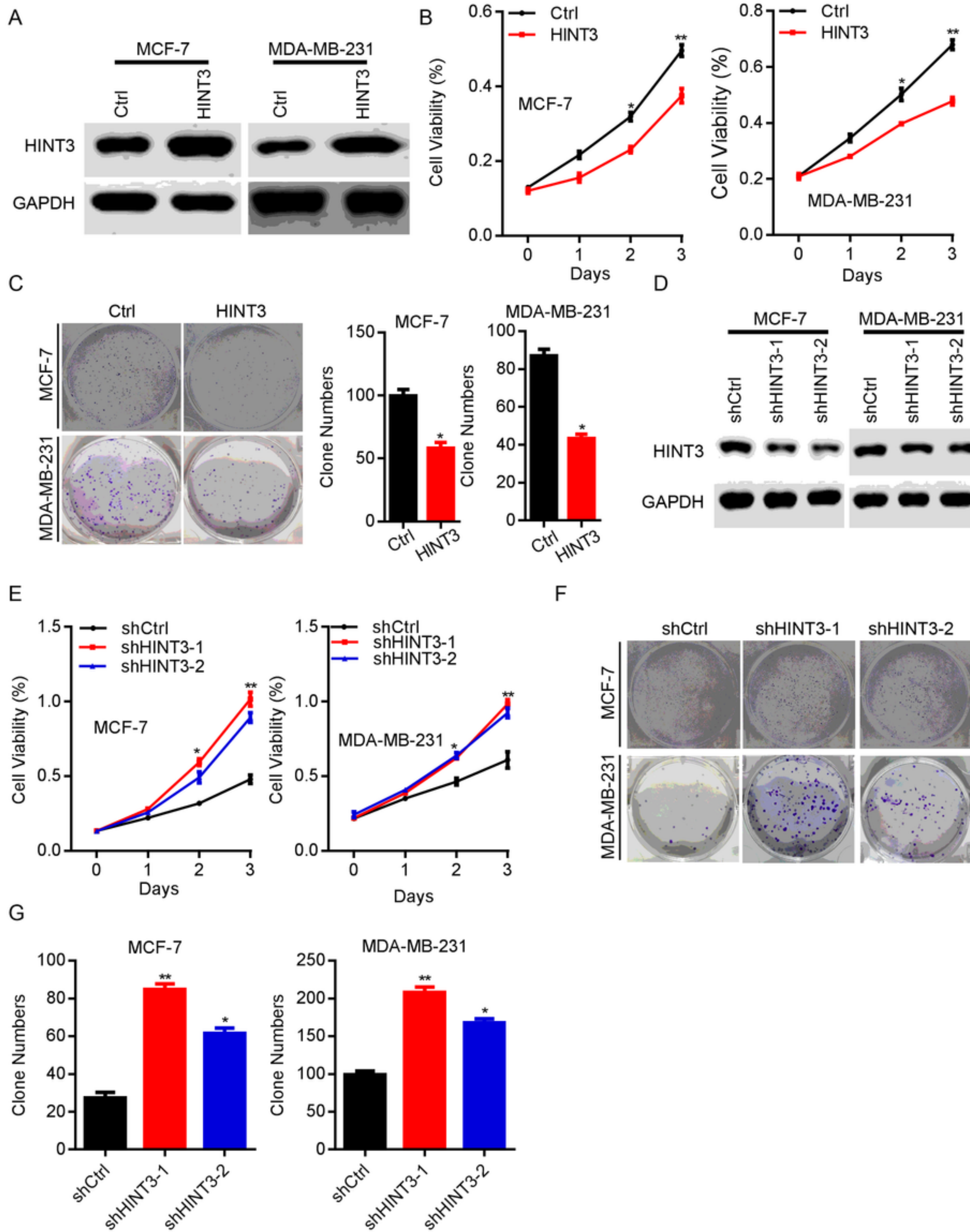

Figure 2

HINT3 supresses the proliferation of BRCA cells. (A) Western blot of HINT3 in Ctrl and HINT3 overexpressed MCF7 and MDA-MB-231 cells. GAPDH is the internal control. (B) Cell proliferation was determined by CCK assay in cells as indicated. *p<0.05, **p<0.01. (C) Ctrl and HINT3 overexpressed MCF7 and MDA-MB-231 cells were subjected to colony formation assay. Left, colony images. Right, quantification results. *p<0.05. (D) Western blot of HINT3 in shCtrl, shHINT3-1 and shHINT3-2 MCF7 and

MDA-MB-231 cells. GAPDH is the internal control. (E) Cell proliferation was determined by CCK assay in cells as indicated. *p<0.05, **p<0.01. (F and G) shCtrl, shHINT3-1 and shHINT3-2 MCF7 cells were subjected to colony formation assay. F, colony images. G, quantification results. *p<0.05, **p<0.01.

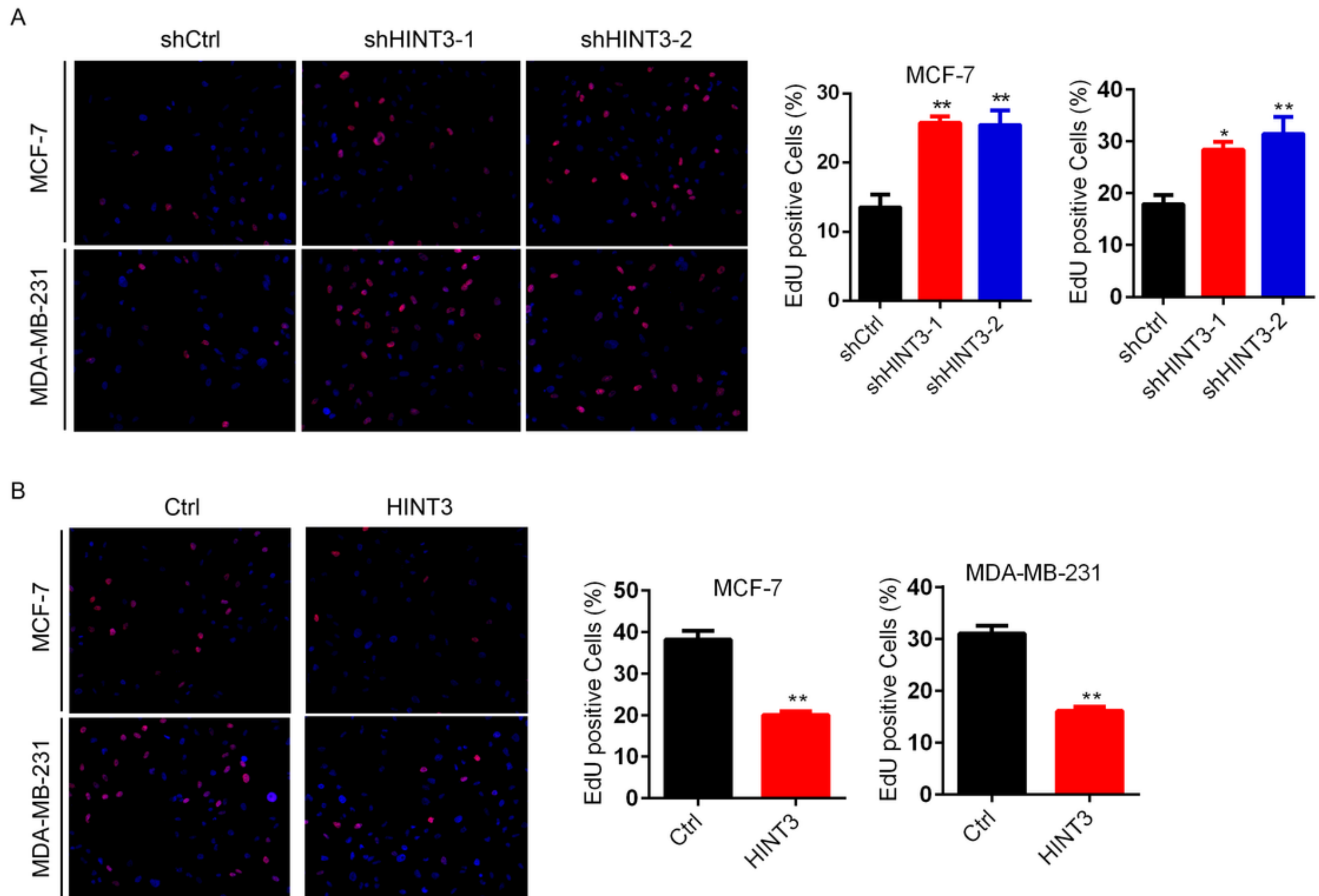

Figure 3

HINT3 inhibits the EDU cooperation of BRCA cells. (A) EDU staining was performed in shCtrl, shHINT3-1 and shHINT3-2 MCF7 and MDA-MB-231 cells. Left, staining images. Right, quantification results. **p<0.01. **p<0.01. (B) EDU staining was performed in Ctrl and HINT3 overexpressed MCF-7 and MDA-MB-231 cells. Left, staining images. Right, quantification results. **p<0.01.

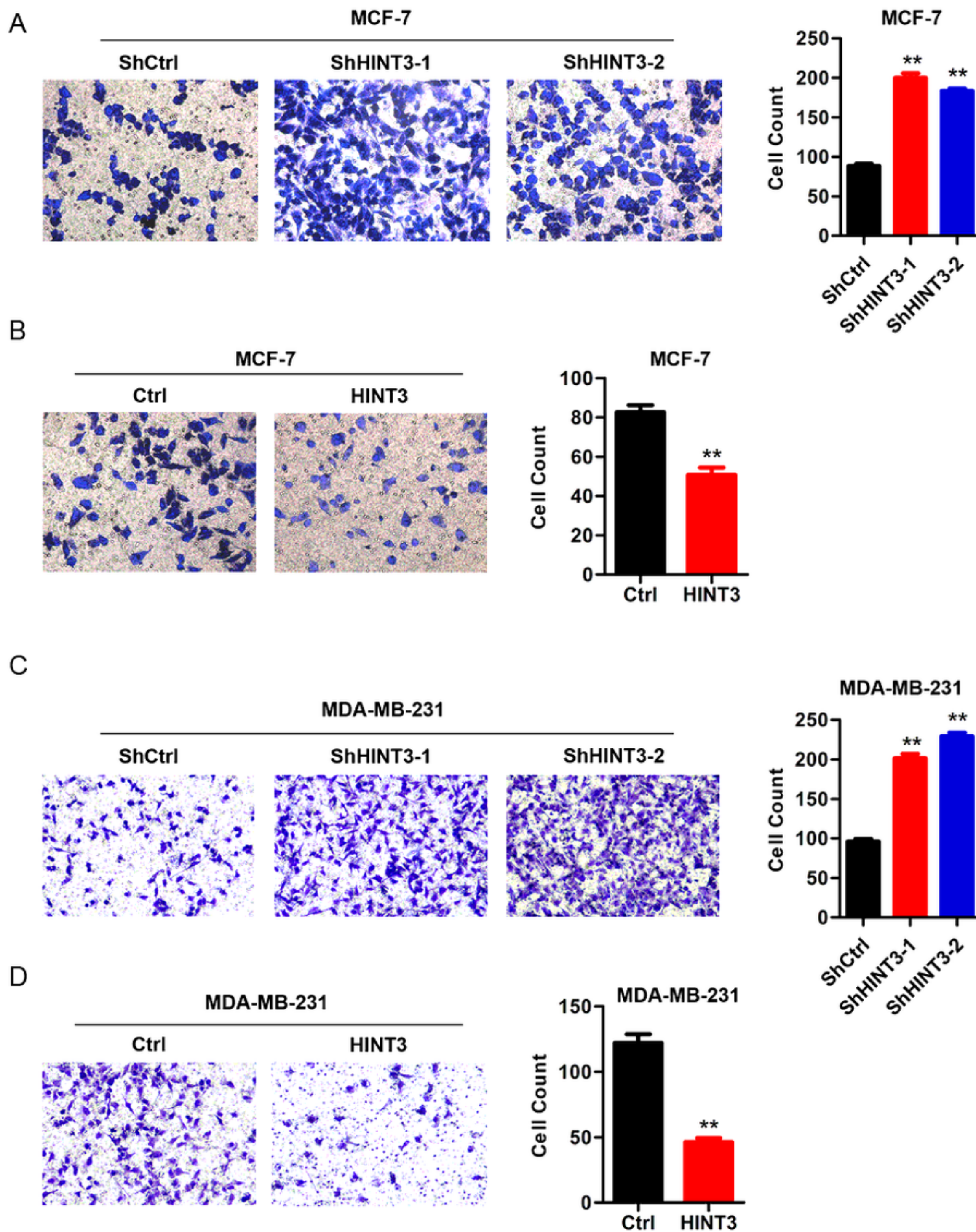

Figure 4

HINT3 suppresses the migration of BRCA cells. (A) Transwell analysis of migration was performed in shCtrl, shHINT3-1 and shHINT3-2 MCF7 cells. Left, representative images. Right, quantification results. **p<0.01. (B) Transwell analysis of migration was performed in Ctrl and HINT3 overexpressed MCF7 cells. Left, representative images. Right, quantification results. **p<0.01. (C) Transwell analysis of migration was performed in shCtrl, shHINT3-1 and shHINT3-2 MDA-MB-231 cells. Left, representative

images. Right, quantification results. **p<0.01. (D) Transwell analysis of migration was performed in Ctrl and HINT3 overexpressed MDA-MB-231 cells. Left, representative images. Right, quantification results. **p<0.01.

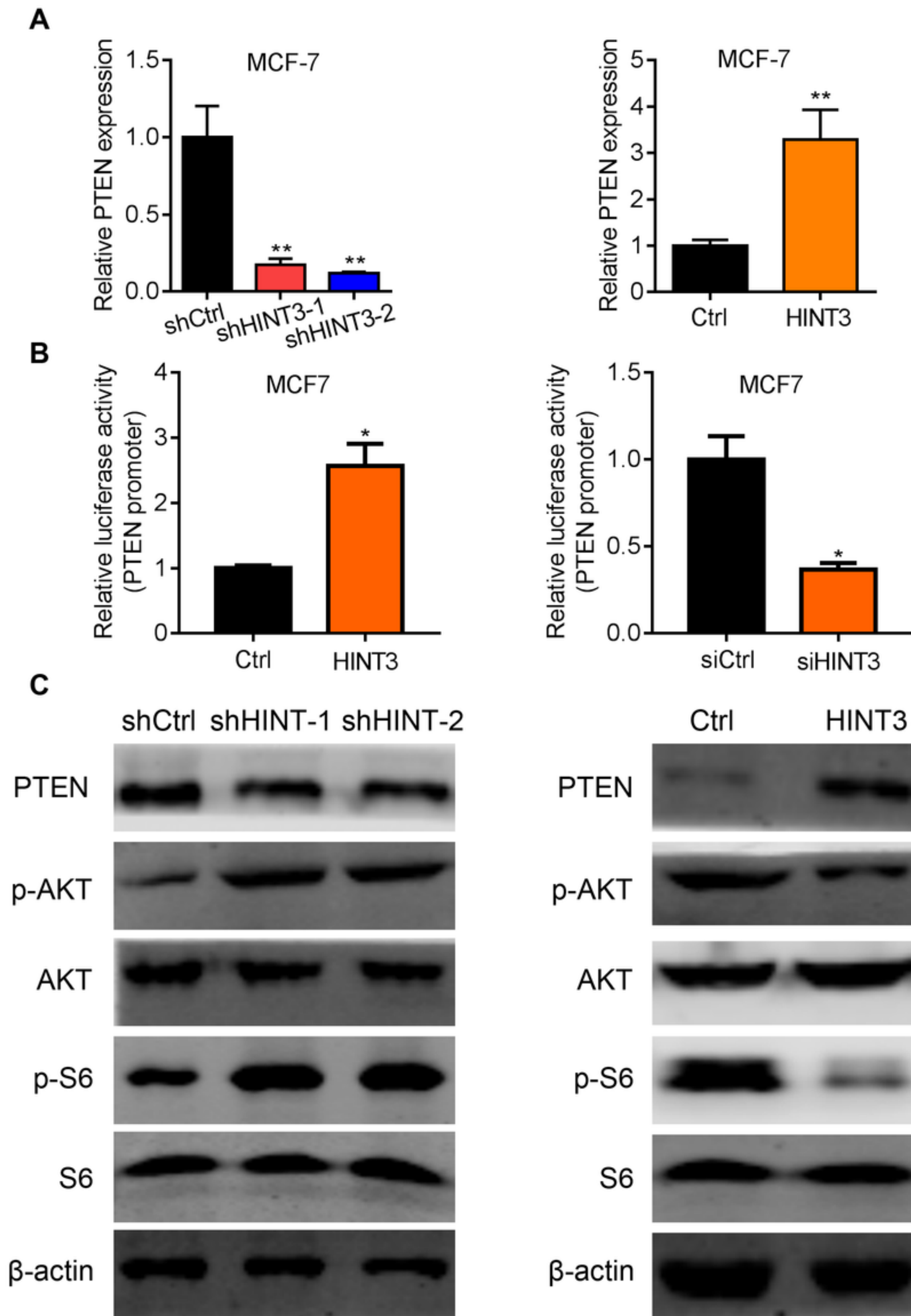

Figure 5

HINT3 inhibits PTEN/AKT signaling in BRCA cells. (A) qRT-PCR analysis of PTEN in shCtrl, shHINT3-1 and shHINT3-2 MCF7 and MDA-MB-231 cells. (B) qRT-PCR analysis of PTEN in Ctrl and HINT3 overexpressed

MCF7 and MDA-MB-231 cells. (C) shCtrl, shHINT3-1 and shHINT3-2, Ctrl and HINT3 overexpressed MCF7 and MDA-MB-231 cells were subjected to Western blot analysis of PTEN, p-AKT and AKT.

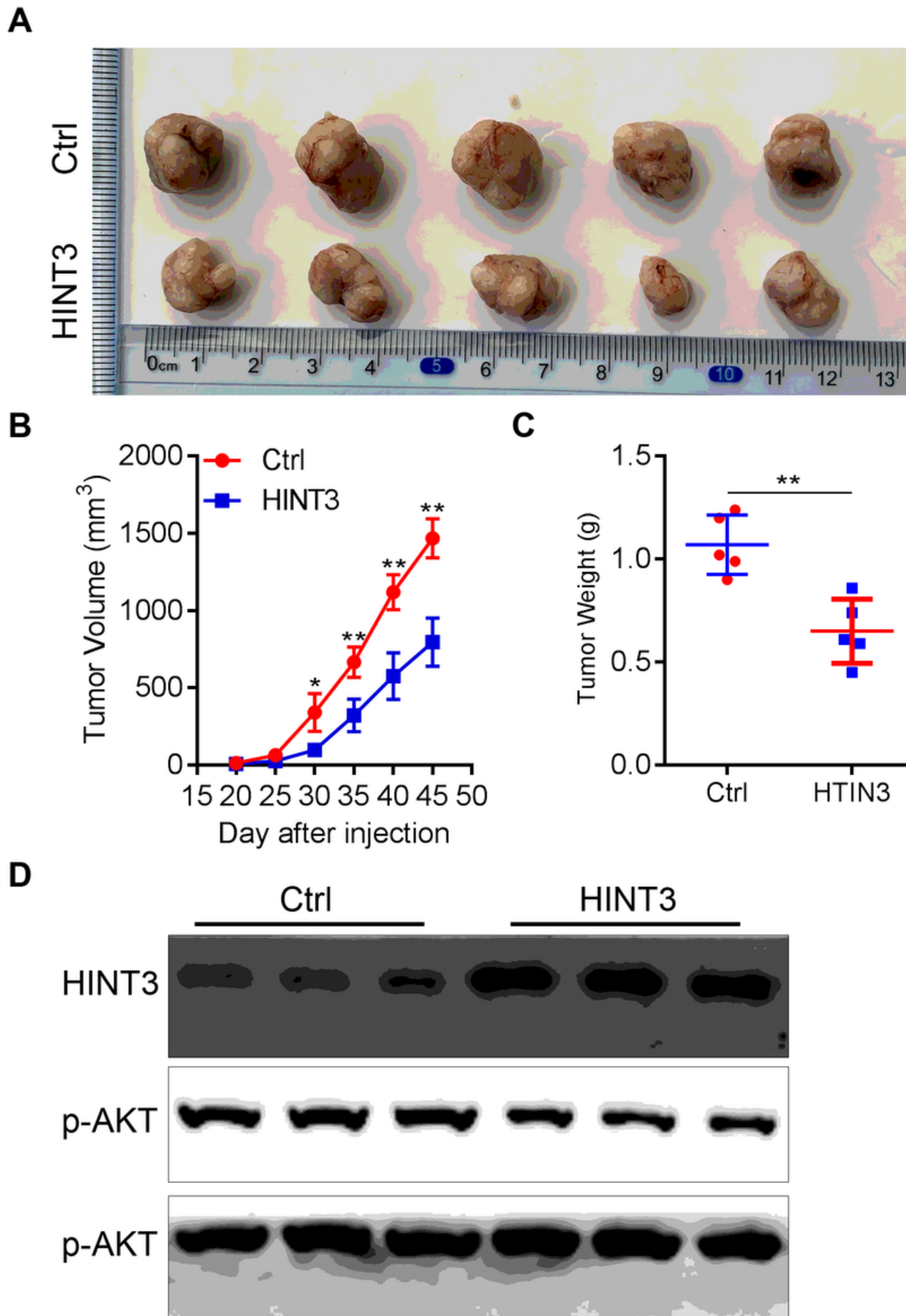

Figure 6

HINT3 overexpression represses AKT activity and tumorigenesis of MDA-MB-231 cells. (A-C) MDA-MB-231 cells expressing Ctrl or HINT3 overexpressed lentivirus were subcutaneously implanted into the right armpit of 4-week old female nude mice. The mice were sacrificed by the 45 days after implantation. The

images of tumors were shown in A. The tumor growth curve was shown in B. Tumor weight was in C. *$p<0.05$. **$p<0.01$. (D) Western blot analysis of HINT3, p-AKT and AKT in the tumor tissues. AKT acts as internal control.

Supplementary Files

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