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ORIGINAL ARTICLE

Upregulation of RUNX1 Suppresses Proliferation and Migration through Repressing VEGFA Expression in Hepatocellular Carcinoma

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Abstract

Hepatocellular carcinoma (HCC) is the most common type of primary liver cancer, and occurs in people with chronic liver diseases. Current treatment methods include surgery, transplant, and chemotherapy. Our study demonstrates runt-related transcription factor 1 (RUNX1) as a novel molecule in the initiation and development of HCC, and the role of its interaction with vascular endothelial growth factor A (VEGFA) in HCC. We showed the suppressive role of RUNX1 in the proliferation and migration of hepatocytes. In addition, the repressor RUNX1 functioned as a transcription factor on the promoter of VEGFA to inhibit the expression of VEGFA. Study in the HCC cells demonstrated that the suppression of HCC proliferation and migration was masked in the presence of overexpressed VEGFA. Introduction of RUNX1 into HCC mice model significantly limited the tumor growth. In summary, our study demonstrated that RUNX1 functions as a repressor in the HCC and this suppressive function was dependent on its effect on VEGFA.

Keywords Hepatocelluar carcinoma · RUNX1 · VEGFA · Tumor suppression

Introduction

Hepatocelluar carcinoma (HCC) is becoming one of the most severe and prevalent cancers in the world [1]. HCC, derived from hepatocytes, accounts for approximately 90% of primary cancers. The diagnosis of HCC reaches peak from the population between 55 and 65 years old [1]. The interaction between environmental and genetic factors has been long recognized as the initiator of cancer development, including HCC. Hepatic insults, such as liver cirrhosis hepatitis B and C viruses infection, persistent alcohol consumption, are defined as high risk factors for HCC [2]. Early diagnosis of HCC significantly favors the life expectancy (i.e. five-year survival rate) of patients, due to its effective treatment at early stage [3, 4].

RUNX1, runt-related transcription factor 1, was initially demonstrated in the acute myeloid leukemia [5]. Nowadays RUNX1 has been implicated as a tumor suppressor in a variety of solid cancers, including breast cancer, esophageal adenocarcinoma, colon cancer and prostate cancer [6, 7]. Low to medium levels of RUNX1 has been found in the human liver cancers [8], indicating its suppressive role in hepatocellular carcinoma.. However, RUNX1 may also play a role in carcinogenesis as an oncogene, depending on its regulated gene and its co-regulatory proteins that bind to targeted genes [6, 7, 9]. Therefore, our current study was designed to evaluate the role of RUNX1 in HCC.

Vascular endothelial growth factor (VEGFA) generally functions as angiogenesis and vascular permeability [10]. It has become evident that the function of VEGFA is involved in the regulation of immune cells in the tumor microenvironment, which impacts the immunological responses to the tumors [11]. VEGFA receptors-related downstream signaling pathways contributes to critical steps of carcinogenesis, regardless of angiogenesis [12]. VEGFA is produced by tumor and stromal cells. The VEGFA derived from stromal cells (i.e. macrophages, endothelial cells and fibroblasts) plays physiological roles in angiogenesis and cell permeability to facilitate the proliferation and migration of endothelial cells [13, 14]. In contrast, VEGFA secreted by tumor cells promotes a transition phenotype from epithelial to mesenchymal cells, which induces tumor invasion as a consequence [12]. In addition, the

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involvement of VEGFA in the liver HCC has been revealed [15, 16]. Our current study was to investigate the role of VEGFA in the hepatic carcinogenesis.

To determine how the interaction of RUNX1 and VEGFA impact hepatic carcinogenesis, we observed significant changes in expression level of RUNX1 and VEGFA in the human HCC. To determine the consequent functional changes, several cell lines were established with modification of the expression of RUNX1 and VEGFA. A RUNX1 binding site was identified in the VEGFA promoter region, which in turn suppressed the expression of VEGFA. Combined expression of RUNX1 and VEGFA in human liver cancer cell lines further demonstrated that RUNX1 suppresses liver cell proliferation and invasion dependent on VEGFA. At last, in vivo mouse experiment with liver carcinoma showed that expression of RUNX1 in the mice significantly inhibited the tumor growth.

Methods and Material

Human Tissue

Human samples were collected at The Second Affiliation Hospital of KunMing Medical University with approved consent form from each participant subject. The protocol for the research project has been approved by Ethic Committee of The Second Affiliation Hospital of KunMing Medical University and that it conforms to the provisions of the Declaration of Helsinki (as revised in Fortaleza, Brazil, October 2013).

Cell Line and Reagents

All the human fetal hepatocyte line L02, human hepatoma cell line Hep3b, and human hepatoblastoma cell line Huh-6 were purchased from Zhenhu biotechnology (Shanghai, China), Xuanya biotechnology (Shanghai, China) and Suer biotechnology (Shanghai, China), respectively. L02 cells, Hep3B and Huh-6 were cultured in DMEM or RPMI 1640 supplemented with 10% FBS, 100 IU/mL of penicillin, 100 μg/mL of streptomycin, 2 mM L-glutamine (Miaotong Biological technology, Shanghai, China) at 37 °C in 5% CO2.

Overexpression assay was performed using Lipofectamine 3000 (Thermo Fisher Scientific, Waltham, MA)-mediated transfection. Overexpression plasmids were constructed by inserting with full length human RUNX1 or VEGFA into expressing vector pCDNA3. Empty pCDNA3 vector was used as its negative control.

Real Time PCR

Total RNA was isolated from the cultured cells using Trizol (Thermo Fisher Scientific, Shanghai, China) following anufacturer's instruction. cDNA was synthesized with 1 mg of total RNA as template using cDNA synthesis kit (Generay, Shanghai, China). Primers used for specific amplification of target genes are described previously: Runx1 forward:5' CTGCTCCGTGCTGCCTAC 3′; reverese 5' AGCCATCA CAGTGACCAGAGT 3′ [17], Vegfa forward 5' AAGGAGGAGGGCAG AATCAT 3′; reverse: 5' CCAGGCCCTCGTCATTG 3′ [18]; GAPDH forward: 5'GCGAGATCGCACTCATCATCT-3′; Gapdh reverse: 5'-TCAGTGGTGGACCTGACC-3′. The PCR is initiated with 95 °C for 5 min, and consists of subsequent 40 cycles of 95 °C 20 s and 62 °C 30 s, followed by additional 72 °C 3 min as last cycle.

Western Blot

Total proteins were extracted using RIPA buffer with addition of proteinase inhibitors. Bradford assay kit (Sigma, St. Louis, MO) was used for protein quantification. 15 μg of total protein was loaded into each well and transferred to PVDF membrane, which was subsequently incubated with 5% fat-free milk, and then primary antibody (1:1000) at 4 °C overnight. Secondary antibody (1:5000) was incubated with the membrane for 1 h at room temperature. The antibodies used in this study include: RUNX1 (ab91002, Abcam, Shanghai, China); GAPDH (sc-47,724, Santa Cruz, Dallas, TX); CDK4 (ab199728, Abcam, Shanghai, China); CDK6 (ab131469, Abcam, Shanghai, China); CyclinD1(ab134175, Abcam, Shanghai, China); E-cadherin (sc-21,791, Santa Cruz, Dallas, TX); Vimentin (sc-66,002, Santa Cruz, Dallas, TX); MMP2 (sc-53,630, Santa Cruz, Dallas, TX); VEGFA (sc-7269, Santa Cruz, Dallas, TX).

MTT Assay

When cells seeded in 24 wells plate reached 80% confluence, the medium was replaced with 10 μl sterile MTT dye (5 mg/mL). After 37 °C for 4 h incubation, medium was replaced with MTT containing solution with 150 μl dimethyl sulfoxide. The absorbance at 450 nm was obtained on an enzyme immunoassay analyzer (Damking, Shanghai, China).

Wound Healing Assay

1 mL pipette tip was used to scratch the monolayer of cells with around 80% confluence in culture wells. Detached cells were washed off and fresh medium was added. The pictures were taken 24 h after scratch to assess the healing process.

Transwell Assay

Cell invasion assay was performed using the 24-well transwell chambers (8 μm pore size, BD Biosciences). 3 × 105 cells in 100 μl serum-free medium was added into the top chambers

while DMEM containing 10% FBS was filled into the bottom chambers. Cells in the top chambers after 24 h of incubation at 37 °C were removed by a cotton-tipped applicator, and cells that migrated to the lower surface of the membrane were subjected to crystal violet staining for cell counting.

Flow Cytometry

Cells were fixed with cold 75% ethanol for 30 min, and then incubated with ribonuclease A at 37 °C for 20 min (Dingguo Changsheng Biotechnology, Beijing, China). Cells were stained with propidium iodide (50 μg/mL) at 4 °C overnight (Sigma, St. Louis, MO) and then analyzed on flow cytometry machine (BD, Franklin Lakes, NJ).

Luciferase Assay

The promoter region of VEGFA spanning from −2.2 kb to +50 bp relative to the transcription start site (TSS) was amplified and cloned into the pGL3-basic vector (Promega, Madison, WI). In parallel, the RUNX1 binding site around −790 bp from TSS was mutated from AACCACA to AAGGACA [19], which has been indicated previously for a critical RUNX1 site for VEGFA regulation [18]. Equal amount of Renilla expression vector pRL-TK (Promega, Madison, WI) was co-transfected for normalization of luciferase assay. Luciferase activity was measured 48-h post-transfection using the Dual-Glo Luciferase Assay System (Promega, Madison, WI).

In Vivo Inhibition of Diethylnitrosamine (DEN) -Induced Liver Tumors

Mice purchased from Yunnan animal care and experiment center, and housed at KunMing Medical University. The experiment has been approved by The Second Affiliation Hospital of KunMing Medical University. The generation of liver tumor model was described previously [20]. Briefly, infant male Balb/c mice were injected I.P. with DEN (N0258, Sigma-Aldrich, St. Louis, MO) at the dose of 50 μg/g on day 15 and maintained on sodium phenobarbital water (500 mg/mL, Sigma-Aldrich, St. Louis, MO) from weaning (day 28) for another 24 weeks. Tissues were collected after euthanasia of mice.

Statistical Analyses

Graphpad Prism 6 was used to perform statistical analyses. Unpaired t-test, one-way ANOVA and two-way ANOVA were used and indicated in the figure caption.

Results

Reduced Expression of RUNX1 in HCC

The mRNA expression level of RUNX1 in the liver tumor tissue and paratumor tissue were evaluated. Significant reduced expression of RUNX1 mRNA level was found in the tumor tissue, compared to paratumor control tissue (Fig. 1a, b). Immunohistochemistry staining and western blot were performed to test the protein level of RUNX1 in liver tumor tissue. We observed remarkable decrease of RUNX1 in the liver tumor tissue, compared to paratumor control tissue (Fig. 1c). Consistent with this, relative RUNX1 protein level was also found decreased in the liver tumor tissue by western blot (Fig. 1d). Furthermore, we tested several human liver cancer cell lines.and observed reduced expression of RUNX1 in all the liver tumor cell lines tested, at both mRNA (Fig. 1e) and protein levels (Fig. 1f).

Overexpression of RUNX1 Negatively Impacts Cell Proliferation

To investigate the effect of RUNX1 in the liver carcinogenesis, we transfected RUNX1-expressing vector into the Hep3B and Huh-6 cells to overexpress RUNX1. Colonies of Hep3B and Huh-6 overexpressing RUNX1 were selected. qRT-PCR assay confirmed significant increase of RUNX1 mRNA expression in those colonies (Fig. 2a). These transcriptional increases were additionally confirmed by western blot and quantification of the band intensity (Fig. 2b). MTT assay performed demonstrated significant suppression of cell proliferation rate in the cell colonies with overexpression of RUNX1, compared with the vector controls (Fig. 2c). Next, we analyzed the cell cycle by using flow cytometry. Decreased proportion of S and G0/G1 phases and increased proportion of G2/M phases were observed in the RUNX1-overexpressing cells (Fig. 2d). In addition, we also found significantly decreases in CDK4, CDK6 and Cyclin D1 protein expression in the RUNX1-overexpressing cells, compared with vector controls (Fig. 2e).

Overexpression of RUNX1 Negatively Impacts Cell Migration

The ability of migration of cancer cells beyond where the tumor was originated and formed is a critical characteristic for metastatic tumor. Therefore, we evaluated the cell migration and invasion in the aforementioned two cell colonies overexpressing RUNX1. The comparison of wound width between the vector controls and the RUNX1-overexpressing Hep3B or Huh-6 demonstrated a slower migration rate in the liver cancer cells that overexpressed RUNX1 (Fig. 3a). Transwell invasion assay showed a significant reduced migrated cell number in the RUNX1-ovreexpressing Hep3B

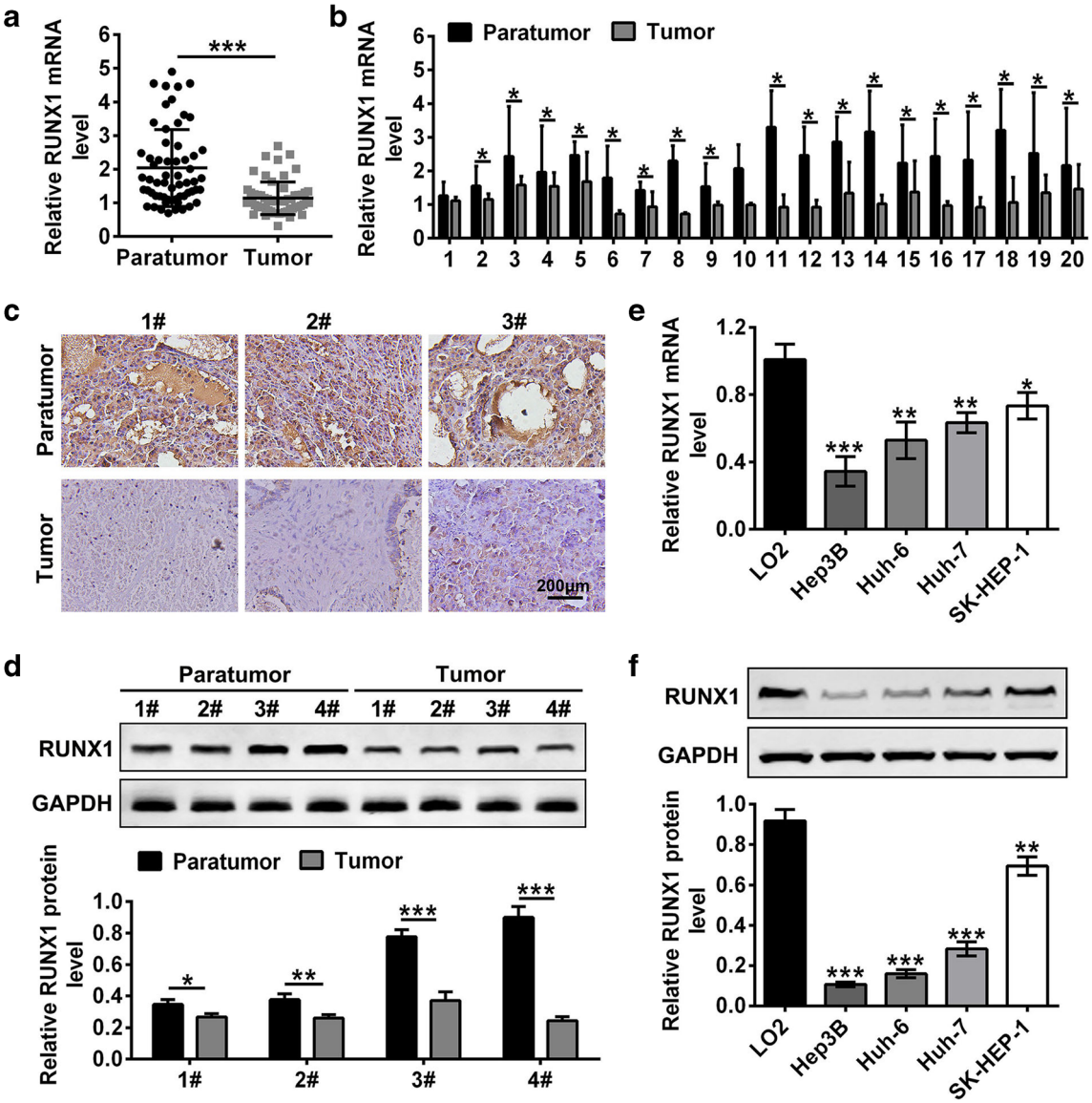

Fig. 1 Reduced expression of RUNX1 in liver cancer cells. RUNX1 mRNA level was tested in the human liver tumor and paratumor samples, presented in dots (**a**) and bar graph (**b**). Protein expression level of RUNX1 in the liver tumor was determined by immunohistochemical staining (**c**) and western blot (**d**). Comparison of RUNX1 expression in human hepatocellular carcinoma cell lines at transcription (**e**) and protein levels (**f**). In panel A, B and D, * indicates $P < 0.05$, *** $P < 0.001$ in unpaired t-test. In panel E and F, * indicates $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ in one-way ANOVE followed by multiple comparison with the control

and Huh-6 cells (Fig. 3b). In addition, proteins relevant to cell invasion and migration were tested, and RUNX1-overexpressing cells exhibited increased E-cadherin, decreased vimentin and matrix metalloproteinases-2 (MMP2) expression (Fig. 3c).

Negative Regulation of VEGFA by RUNX1

VEGFA is a major regulator of normal and pathological angiogenesis. We observed significant increase of *Vegfa* expression in the human tumor samples, compared with their paratumor controls (Fig. 4a). Consistent increase of VEGFA expression was also demonstrated in the tumor samples at protein level, by immunohistochemical staining (Fig. 4b) and western blot (Fig. 4c). Correlation analysis revealed negative correlation between the expression of RUNX1 and VEGFA levels in the sampled tumors (Fig. 4d). Moreover, we tested the VEGFA expression in the established Hep3B and Huh-6 cell lines with overexpression of RUNX1. We observed significant reduction in the expression of VEGFA in both cell lines (Fig. 4e). To illustrate whether the suppression of VEGFA is directly regulated by RUNX1, the promoter

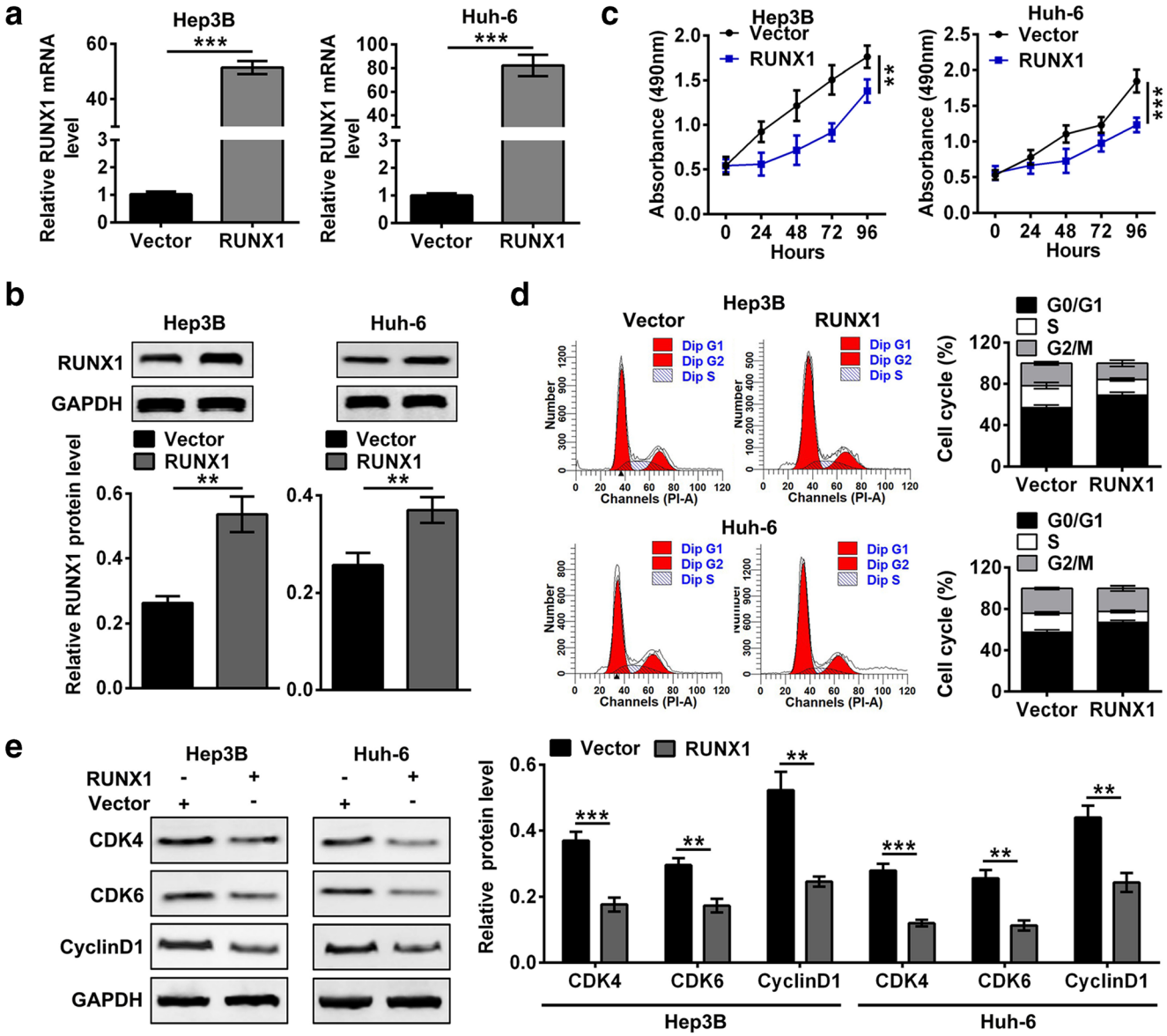

Fig. 2 Overexpression of RUNX1 negatively impacts cell proliferation. Overexpression of RUNX1 in the Hep3B and Huh-6 cells was confirmed by measuring the RNA (**a**) and protein levels (**b**) in the RUNX1-overexpressing cells. (**c**) Cell proliferation rate was determined by MTT in the RUNX1-overexpressing cells and its control. (**d**) Cell cycle was evaluated by flow cytometry in the RUNX1-overexpressing cells and its control. (**e**) Overexpression of RUNX1 suppresses the expression of cell cycle proteins. In panel A, B and E, ** $P < 0.01$, *** $P < 0.001$ in unpaired t-test. In panel C, ** $P < 0.01$, ***$P < 0.001$ for two-way ANOVE analysis

region of RUNX1 was cloned into the pGL3 vector. We observed significant repression of pGL3 luciferase with the wild-type VEGFA promoter, suggesting direct repressive regulation of RUNX1 on VEGFA expression. (Figure 4f).

Regulation of Cell Proliferation and Migration by RUNX1 Depends on VEGFA

Next, in the RUNX1-overexpressing Hep3B and Huh-6 cells, VEGFA-expressing vector was additionally transfected to generate Hep3B and Huh-6 cell lines that overexpress both RUNX1 and VEGFA. In the selected cell colonies, we confirmed significant increases of VEGFA expression, compared with the vector controls (Fig. 5a). These transcriptional increases of VEGFA also applied to the upregulation of VEGFA at protein level (Fig. 5b). Subsequently, we examined the cell proliferation in these genetically modified cell lines: Hep3b and Huh-6 cells with control vectors for RUNX1 and VEGFA; overexpression of RUNX1; overexpression of RUNX1 and VEGFA. Consistently, RUNX1 expression reduced the proliferation in both Hep3B and Huh-6 cells (Fig. 5c). Importantly, the reduced capability of proliferation was restored by the overexpression of VEGFA in both cells (Fig. 5c). Cell

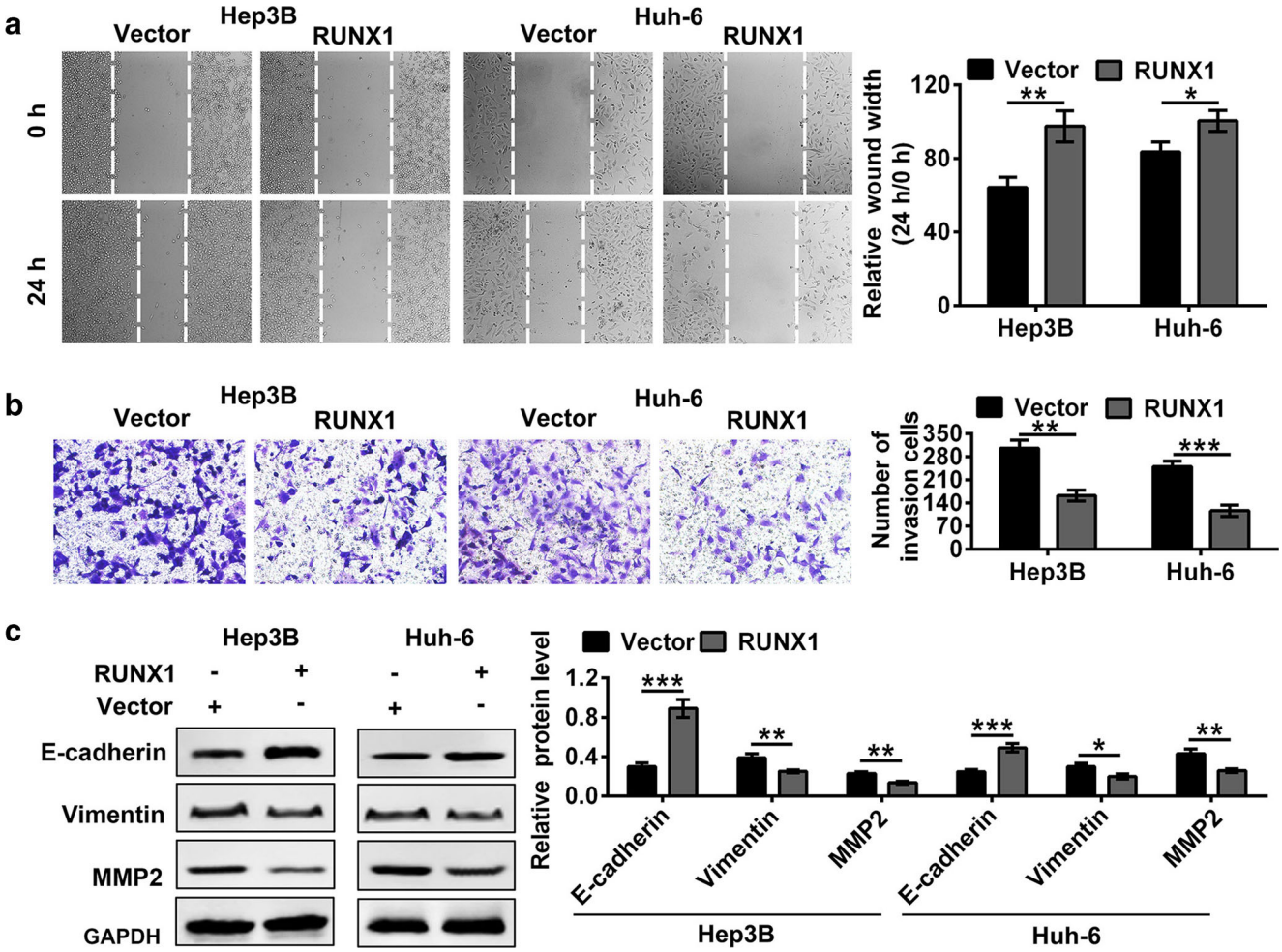

Fig. 3 Overexpression of RUNX1 negatively impacts cell migration. Cell migration (**a**) and cell invasion (**b**) were tested in the Hep3B and Huh-6 cells with overexpression of RUNX1, by wound healing and transwell assays, respectively. Cell migration-related proteins were measured by western blot in the cells overexpressing RUNX1 and its control (**c**). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ for unpaired t-test

cycle analysis illustrated that overexpression of VEGFA and RUNX1 in Hep3B and Huh-6 cells increased the proportion of S phase and reduced G2/M phases, compared with the cells overexpressing RUNX1 only (Fig. 5d). This altered distribution of cell cycle phases in RUNX1 + VEGFA overexpressing cells resemble that in the cells with control vectors (Fig. 5d). Consistently, restoration of VEGFA expression in the RUNX1-overexpressing Hep3B and Huh-6 cells significantly increased the capabilities of cell migration (decreased wound width) and cell invasion (increased translocated cell number) (Fig. 5e, f). Finally, cell cycle-related proteins (i.e. CDK4, CDK6, Cyclin D1) and cell migration-related proteins (i.e. E-cadherin, Vimentin, MMP2) are evaluated by western blot. In the Hep3B cells, overexpression of RUNX1 decreased the levels of CDK4, CDK6 and Cyclin D1, while additional overexpression of VEGFA normalized the expression of three proteins to the vector control level (Fig. 5g). For the cell migration-related proteins, E-cadherin was induced by RUNX1, while Vimentin and MMP2 were repressed by RUNX1 (Fig. 5g). Additional introduction of VEGFA normalized three proteins to the vector control level (Fig. 5g). Consistently, these change patterns were also observed in the Huh-6 cells (Fig. 5g).

Overexpression of RUNX1 Represses Tumor Growth In Vivo

Next, we evaluated the role of RUNX1 in the liver tumor growth in vivo. BALB/c mice treated with DEN and injected with RUNX1 expression vector were found with significantly smaller tumor volume, compared with the mice with control vector (Fig. 6a). In addition, the weight of tumor was smaller in the RUNX1 expressing mice (Fig. 6a). Immunohistochemical staining revealed less Ki67+ cells and VEGFA expression but momre RUNX1 expression and more VEGFA expression in the vector control mice, compared with the mice with RUNX1 expression (Fig. 6b).

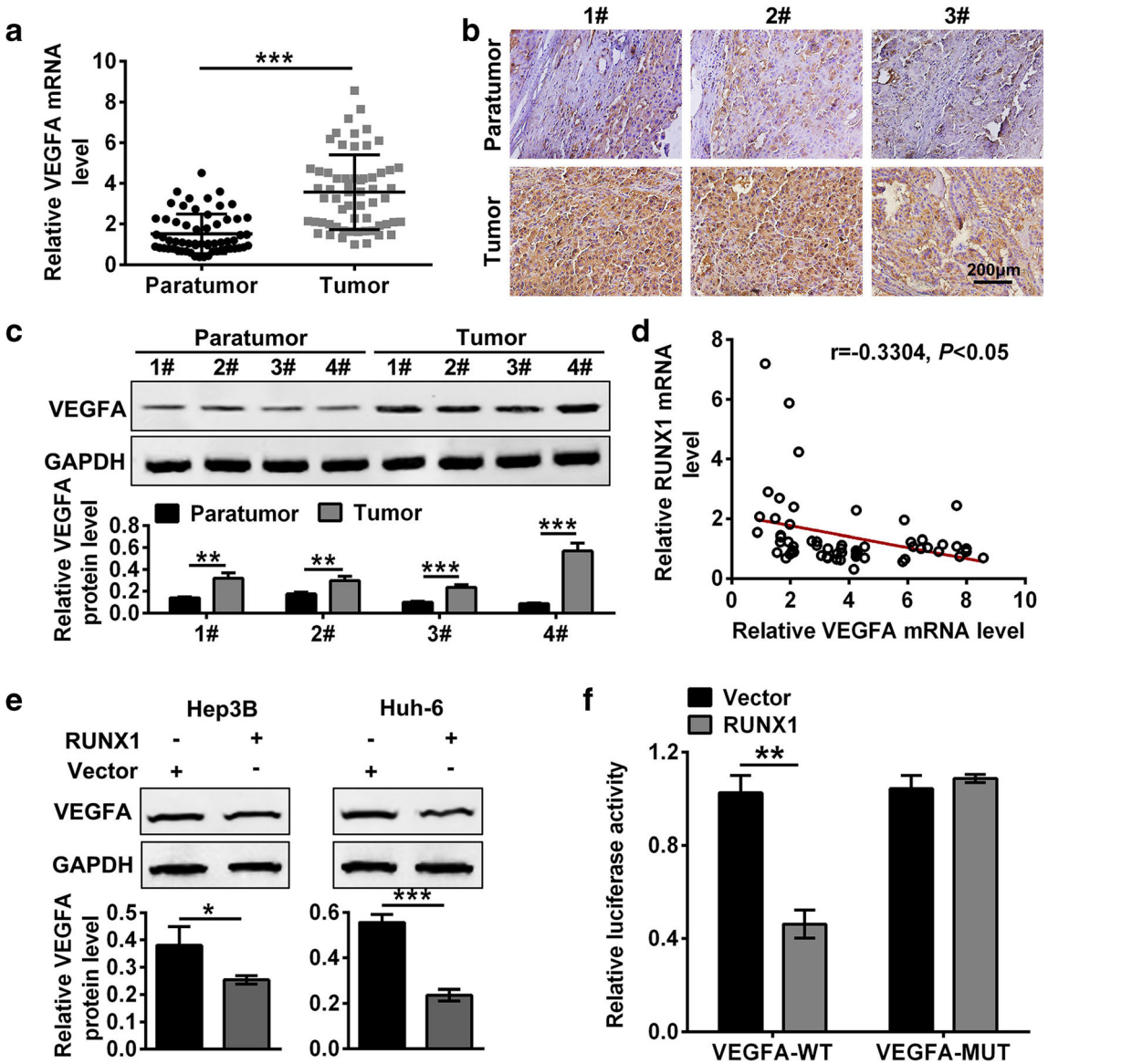

Fig. 4 Negative regulation of VEGFA by RUNX1. VEGFA mRNA level was tested in the human liver tumor and paratumor samples (**a**). Protein expression level of RUNX1 in the liver tumor was determined by immunohistochemical staining (**b**) and western blot (**c**). Negative correlation between RUNX1 and VEGFA in human hepatocellular carcinoma samples (**d**). Overexpression of RUNX1 in the human liver cancer cells suppresses the expression of VEGFA, measured by western blot (**e**). Luciferase assay showed the regulatory role of RUNX1 on the VEGFA promoter (**f**). In panel A, C and E, * indicates $P < 0.05$, *** $P < 0.001$ in unpaired t-test. In panel F, ** $P < 0.01$ in two-way ANOVE followed by multiple comparison between RUNX1-overexpressing cells and vector control

Discussion

The mortality rates of HCC has been steadily increasing for the past decade [4]. However, the limited therapeutic options greatly hinder the prevention and treatment of this severe cancer [16]. In our current study, we demonstrated the role of RUNX1 and its regulation on VEGFA in the carcinogenesis of hepatocytes. This may provide essential insights to the role of RUNX1 in HCC and consideration of RUNX1 as a target for therapy or diagnostic marker for HCC.

RUNX1 is a member of the core-binding transcription factors and is indispensable for hematopoiesis in vertebrates [5]. Somatic mutations in RUNX1 results in myeloid malignancies [5]. In addition, RUNX1 is also strongly expressed in breast epithelia [21], and knockout of RUNX1 caused a phenotype of hyperproliferation in breast epithelia [22]. However, its role in HCC has never been explored. Our study was intrigued by an observation of the decreased expression of RUNX1 in the human HCC samples (Fig. 1). Subsequently, we tested a number of human liver cancer cell lines and found consistent decreases of RUNX1 (Fig. 1). These observations indicated a potential

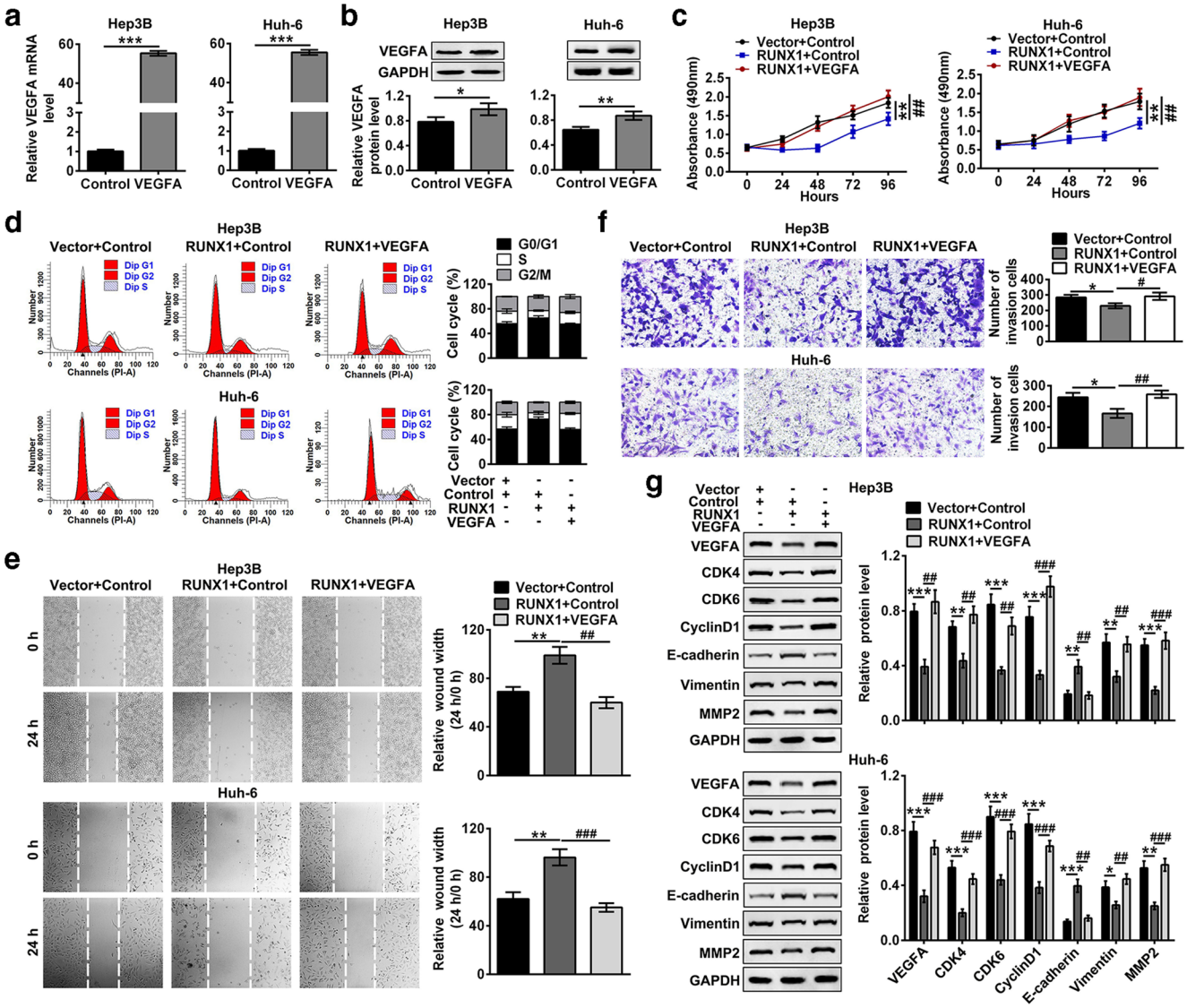

Fig. 5 Regulation of cell proliferation and migration by RUNX1 depends on VEGFA. VEGFA was overexpressed in the Hep3B and Huh-6 cells that overexpress RUNX1. The expression level of VEGFA was confirmed at mRNA (**a**) and protein (**b**) levels. (**c**) Cell proliferation rate was determined by MTT in the RUNX1 and VEGFA double-overexpressing cells and its control. (**d**) Cell cycle was evaluated by flow cytometry in the RUNX1 and VEGFA double-overexpressing cells and its control. Cell migration (**e**) and cell invasion (**f**) were tested in the Hep3B and Huh-6 cells with double overexpression of RUNX1 and VEGFA, by wound healing and transwell assays, respectively. Cell cycle-related and migration-related proteins were measured by western blot in the cells overexpressing RUNX1 and VEGFA as well as its control (G). In panel A and B, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ in unpaired t-test. In panel C, ** $P < 0.01$, ## $P < 0.001$ in two-way ANOVE analysis. In panel E, F and G, *, # $P < 0.05$, **, ## $P < 0.01$ and ***, ### $P < 0.001$ in one-way ANOVE analysis

repressive role of RUNX1 in the hepatic carcinoma as it was initially demonstrated in metastasis [18]. To illustrate this observed correlation between RUNX1 and tumor, we generated and screened cell lines that stably express RUNX1 (Fig. 2a, b). Overexpression of RUNX1 lead to a change in cell cycle distribution, with increased G0/G1 phases and decreased S and G2/M phases (Fig. 2d). The increased G0/G1 phases could be due to the arrest of cells at G1/S checkpoints, which lead to decreased cell in the G2/M phase [23]. This is consistent with the observation of decreased cell proliferation (Fig. 2c). CDK4, CDK6 and Cycline D1 are critical proteins responsible for the induction of cell entry into S phase [23], reduced expression of which contribute to the accumulation of cells in the G0/G1 phases, and subsequent reduction in cells in the S and G2/M phases. As a result of the cell cycle changes, cells with the overexpression of RUNX1 were trapped mainly in the G0/G1 phases, leading to reduced synthesis of cell division materials and inhibition of cell division and thereafter proliferation.

The overexpression of RUNX1 in Hep3B and Huh-6 also weakened the capability of cell migration and invasion (Fig. 3a, b). To explore the potential mechanism, we demonstrated significantly increased expression of E-cardherin and reduced

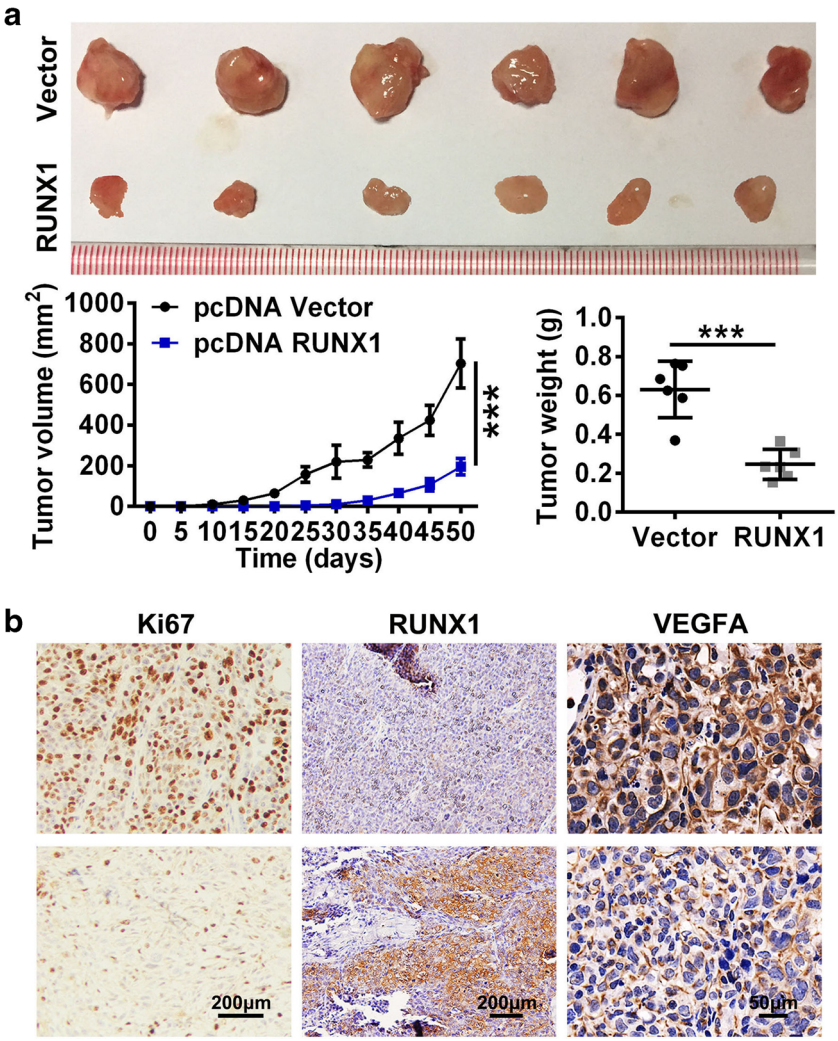

Fig. 6 Overexpression of RUNX1 represses tumor growth in vivo. **a** Liver tumor volume and weight were measured in the BALB/c mice injected with control vector and RUNX1-expressing vector. **b** Histochemical staining of Ki67, RUNX1 and VEGFA in the liver tumors from BALB/c mice injected with control vector and RUNX1-expressing vector. *** $P < 0.001$ in two-way ANOVA analysis in the test of tumor volume. *** $P < 0.001$ in unpaired t-test in tumor weight measurement

expression of Vimentin and MMP2 in the RUNX1-overexpressing cells (Fig. 3c). E-cadherin is a major hemophilic cell-cell adhesion molecule that inhibits motility of cells [24]. The elimination of its expression is a significant driver for the morphological changes associated with EMT. Therefore, our obtained data of increase in E-cardhein suggests the conversion of immobile cells into migratory and invasive cells [24]. In addition, Viementin are required for initiation and migration of cancer cells that have undergone EMT [25, 26]. Previous publication has reported that Vimentin deficient mice exhibit systemic defects related to development and wound repair [25]. Combined with the fact that MMP2 is an important determinant of cancer cell migration [27], we demonstrated that RUNX1 negatively regulate cell migration and invasion through its impacts, at least partially, on E-cadherin, Vimentin and MMP2.

VEGFA is a homodimeric glycoprotein and a key mediator of angiogenesis in cancer [10]. In cancer, VEGFA is upregulated by oncogene to facilitate the formation of new vasculature around the tumor, allowing it to grow exponentially [10]. RUNX1 has been shown to negatively regulate VEGFA expression in disease involving vascular endothelial cells [18]. Since RUNX1 significantly modulates the capability of proliferation, migration, and invasion in hepatic cancer cells, we questioned whether VEGFA is an effector downstream of RUNX1 in HCC. We observed a negative correlation of RUNX1 and VEGFA expression in the human HCC samples (Fig. 4d). Importantly, RUNX1 directly binds to VEGFA promoter as a transcription factor to repress the expression of VEGFA (Fig. 4f). This data is consistent with previous finding that RUNX1 acts as a repressor for VEGFA in the context of acute myeloid leukemia, in which aberration of RUNX1 results in upregulation of VEGFA [18]. Overexpression of RUNX1, a tumor repressor, significantly reduced the carcinogenic properties in the Hep3B and Huh-6 cells (Fig. 2). However, overexpression of VEGFA in these RUNX1-overexpressing cells was found to restore the carcinogenic properties (i.e. proliferation, migration, invasion)

(Fig. 5). Moreover, the overexpression of VEGFA-induced carcinogenic properties was characterized by enhanced phase of cell division and the capability of EMT (Fig. 5). These data showed VEGFA functions as a carcinogenic inducer in the HCC, consistent with its reported role in HCC mouse model [28] and head and neck carcinoma patients [16]. Most importantly, we observed that introduction of RUNX1 was able to suppress hepatocyte tumorigenesis and this suppression of tumor was associated with decreased expression of VEGFA in the genetically modified mouse model (Fig. 6), indicating the application potential by introduction or activation of RUNX1 or suppression of VEGFA in HCC therapy.

In summary, our study demonstrated that RUNX1 plays an important role in the HCC models we tested. Its partner VEGFA was also involved in the liver cancer. The demonstration of RUNX1 as a tumor repressor in the HCC was novel and this finding could be applied in further study to test the therapeutic role of RUNX1 in the liver cancer and the potential to use RUNX1 as a diagnostic marker for HCC.

Author's Contributions CL and JH conceived and designed the experiments, DWX and BX analyzed and interpreted the results of the experiments, JL and BLL performed the experiments.

Data Availability All data generated or analyzed during this study are included in this published article.

Compliance with Ethical Standards

Ethics Approval and Consent to Participate Not Applicable.

Patient Consent for Publication Not Applicable.

Competing Interests The authors state that there are no conflicts of interest to disclose.

Informed Consent Written informed consent was obtained from a legally authorized representative(s) for anonymized patient information to be published in this article.

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