

DLK1 as a Potential Target against Cancer Stem/Progenitor Cells of Hepatocellular Carcinoma

Xiao Xu^{1,2}, Rui-Fang Liu^{1,2}, Xin Zhang², Li-Yu Huang^{1,2}, Fei Chen², Qian-Lan Fei², and Ze-Guang Han^{1,2}

Abstract

Delta-like 1 homolog (DLK1; *Drosophila*) is a hepatic stem/progenitor cell marker in fetal livers that plays a vital role in oncogenesis of hepatocellular carcinoma (HCC). The aim of this study is to investigate whether DLK1 could serve as a potential therapeutic target against cancer stem/progenitor cells of HCC. DLK1⁺ and DLK1⁻ cells were sorted by fluorescence-activated cell sorting and magnetic-activated cell sorting, respectively, and then were evaluated by flow cytometry. The biological behaviors of these isolated cells and those with *DLK1* knockdown were assessed by growth curve, colony formation assay, spheroid colony formation, chemoresistance, and *in vivo* tumorigenicity. Adenovirus-mediated RNA interference was used to knockdown the endogenous *DLK1*. We found that DLK1⁺ population was less than 10% in almost all 17 HCC cell lines examined. DLK1⁺ HCC cells showed stronger ability of chemoresistance, colony formation, spheroid colony formation, and *in vivo* tumorigenicity compared with DLK1⁻ cells. The DLK1⁺ HCC cells could generate the progeny without DLK1 expression. Furthermore, *DLK1* knockdown could suppress the ability of proliferation, colony formation, spheroid colony formation, and *in vivo* tumorigenicity of Hep3B and Huh-7 HCC cells. Our data suggested that DLK1⁺ HCC cells have characteristics similar to those of cancer stem/progenitor cells. RNA interference against *DLK1* can suppress the malignant behaviors of HCC cells, possibly through directly disrupting cancer stem/progenitor cells, which suggested that DLK1 could be a potential therapeutic target against the HCC stem/progenitor cells. *Mol Cancer Ther*; 11(3); 629–38. ©2012 AACR.

Introduction

Hepatocellular carcinoma (HCC) is one of the most common cancers in Asia and the second leading cause of cancer-related mortality worldwide (1). It is urgent to identify new diagnostic markers and therapeutic strategies associated with the initiation and progression of HCC (2). During the last few years, increasing evidences have been shown to support the hypothesis that, among a hierarchy of heterogeneous cell populations within a tumor, a small subpopulation called cancer stem cells (CSC) is responsible for the tumor initiation, growth, metastasis, and recurrence (3–6). This cell subpopulation also exhibits the stem/progenitor cell-like characteristics such as self-renewal and differentiation. Previous studies suggested that CSCs existed in leukemia (7, 8) and some solid tumors, including breast cancer (9, 10), glioblastoma (11, 12), prostate cancer (13–16), gastric carcinoma (17, 18),

lung cancer (19), colon cancer (20, 21), as well as head and neck cancer (22). Similarly, it has been reported that potential CSCs with CD133, CD90, EpCAM, and OV6 membrane biomarkers could reside in HCC (23–29).

Others and we recently identified that delta-like 1 homolog (DLK1), a candidate hepatic stem/progenitor cell biomarker (30–32), was upregulated in HCC, indicating its role in tumorigenesis (33). As known, DLK1 was restrictedly expressed in a subpopulation of oval cells, hepatic progenitor cells, and in rat adult liver (34). DLK1 was also strongly expressed in mouse fetal liver of E10.5–E16.5, and obviously downregulated thereafter, where DLK1⁺ cells with highly proliferative ability can differentiate into both hepatocyte and biliary epithelial cell lineages (30). Moreover, the purified DLK1⁺ cells from mouse fetal liver were fully capable of repopulating the normal adult liver *in vivo* after transplantation (31), implying that DLK1⁺ cells are hepatic stem/progenitor cells. However, it is largely unknown whether DLK1 could serve as a biomarker of the stem/progenitor cells of HCC and even as a therapeutic target.

In this study, we investigated biological behaviors of the DLK1⁺ subpopulation of HCC cells, such as tumorigenicity, self-renewal, and chemoresistance, as well as the potential to be targeted by therapeutics.

Materials and Methods

Cell lines

Human HCC cell lines PLC/PRF/5, QGY7701, QGY-7703, Bel-7402, Sk-hep1, Bel-7404, YY-8103, SMMC7721,

Authors' Affiliations: ¹National Human Genome Center of Rui-Jin Hospital, Shanghai Jiaotong University School of Medicine; and ²Shanghai-MOST Key Laboratory for Disease and Health Genomics, Chinese National Human Genome Center at Shanghai, Shanghai, China

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Corresponding Author: Ze-Guang Han, Chinese National Human Genome Center at Shanghai, 351 Guo Shou-Jing Road, Shanghai 201203, China. Phone: 86-21-50801325; Fax: 86-21-50800402; E-mail: hanzg@chgc.sh.cn

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Focus, HepG2, Hep3B, Huh-7, SNU398, and hepatic cell line WRL68 were kindly provided and licensed by Cell Bank of Institute of Biochemistry and Cell Biology, Shanghai Institutes for Biological Sciences 5 years ago. MHCC-97L, MHCC-LM3, and MHCC-LM6 were kindly provided and licensed by Liver Cancer Institute & Zhongshan Hospital, Fudan University, 4 years ago. No authentication was done by the authors.

These cell lines were grown in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with heat-inactivated 10% fetal bovine serum (Gibco BRL) at 37°C in a 5% CO₂ humidified incubator.

Animal preparation

Male nonobese diabetic/severe combined immune deficiency (NOD/SCID) and BALB/c nude mice aged between 4 and 6 weeks were used to test the

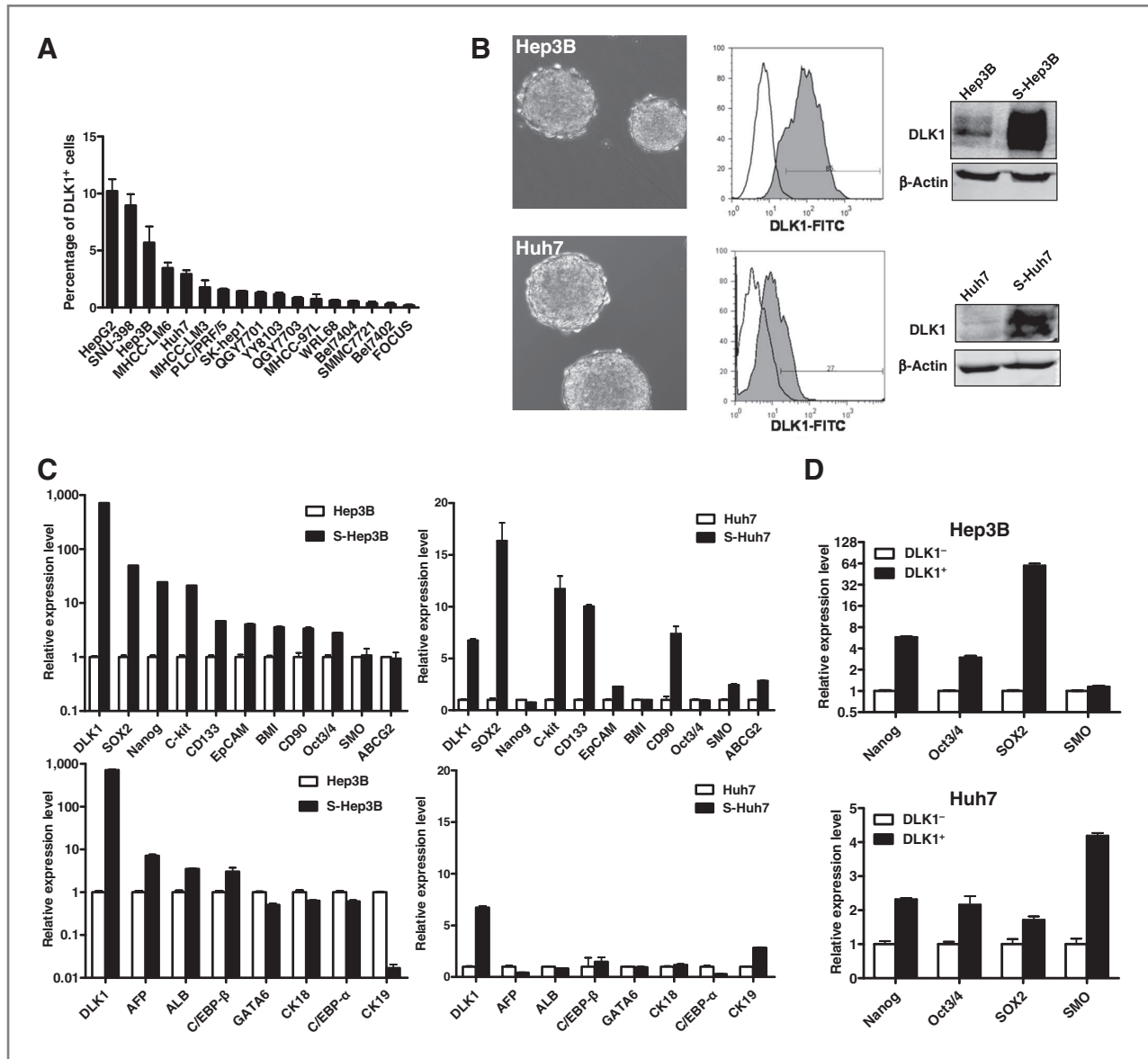
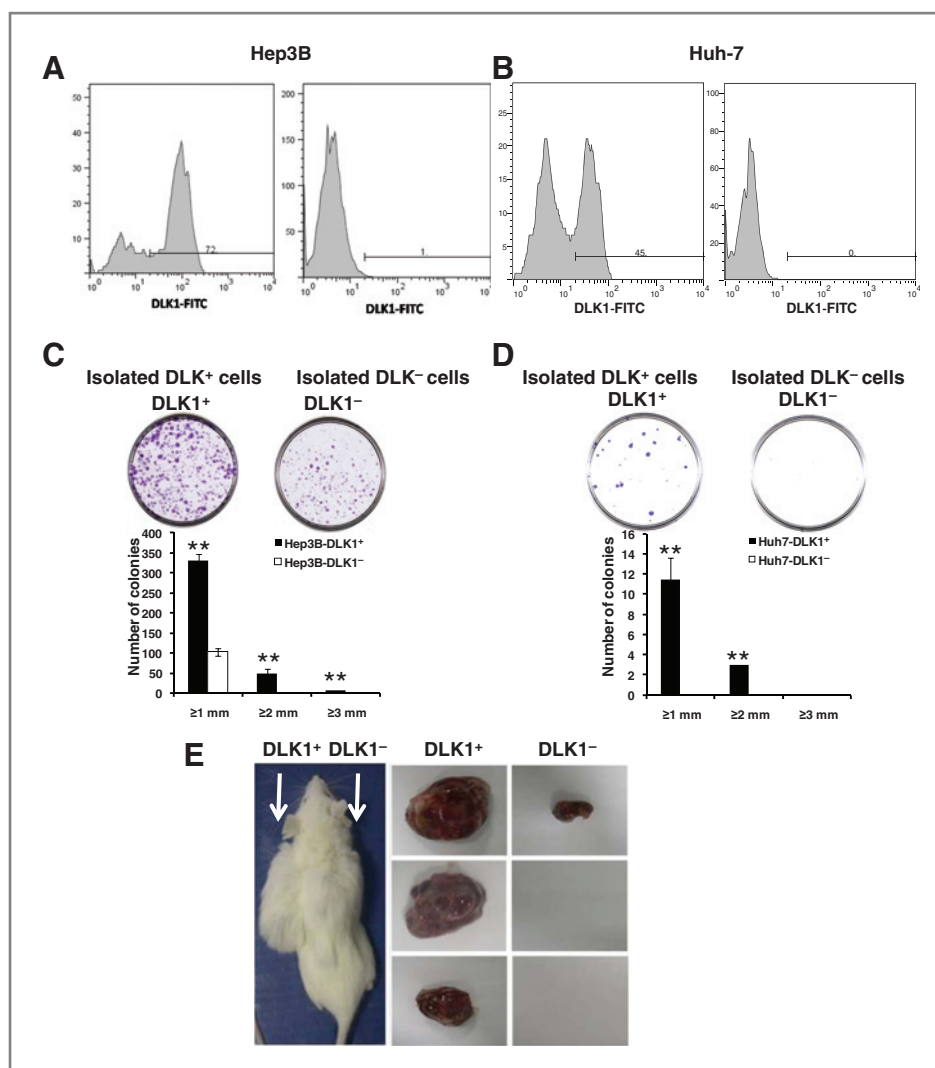


Figure 1. DLK1⁺ cells in spheroid colonies derived from HCC cell lines. **A**, the percentage of DLK1⁺ cells in 17 HCC cell lines was detected by flow cytometry. **B**, spheroid colony formation of HCC cell lines cultured in serum-free media. The spheroid colonies (left) were derived from both Hep3B and Huh-7 HCC cell lines, and then DLK1⁺ cells of spheroids were analyzed by flow cytometry (middle), and DLK1 expression was detected by Western blotting assay (right). **C**, real-time RT-PCR analysis on some genes relevant to stemness and differentiation of hepatic stem/progenitor cells was carried out in spheroids, where parent cells were used as controls. **D**, comparison of *Nanog*, *SOX2*, *SMO*, and *Oct3/4* mRNA levels in DLK1⁺ and DLK1⁻ Huh-7 and Hep3B cells sorted by MACS, where the mRNA levels of these genes in DLK1⁻ HCC cells were normalized as 1. Each experiment was carried out 3 times independently.

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Figure 2. DLK1⁺ cells exhibited stronger colony formation and tumorigenicity. A and B, the purity of DLK1⁺ and DLK1⁻ cells isolated from Hep3B (A) and Huh-7 cells (B) was analyzed by FACS. C and D, the colony formation of DLK1⁺ HCC cells isolated from Hep3B and Huh-7 cells was significantly enhanced, as compared with that of DLK1⁻ HCC cells. **, $P < 0.01$. The experiment was carried out 3 times independently. E, *in vivo* tumorigenicity was evaluated by subcutaneous injection of 1×10^4 DLK1⁺ and DLK1⁻ Huh7 cells in the same NOD/SCID mice, as shown by arrows. The representative xenograft tumors were surgically removed (right).



tumorigenicity of sorted cells from HCC cell lines or HCC cells with *DLK1* knockdown.

Total RNA isolation and quantitative reverse transcription PCR

Total RNA isolated with TRIzol Reagent (Invitrogen) was reverse transcribed with M-MLV Reverse Transcriptase (Promega) according to manufacturer's instructions. Quantitative real-time reverse transcription (RT)-PCR was conducted with the primers listed in Supplementary Table S1. Each sample was analyzed in triplicate. Data were normalized to β -actin before comparative analysis.

Cell proliferation and colony formation assay

HCC cells were plated at a density of 3,000 to 4,000 cells per well in 96-well plates and allowed to grow for 5 to 7 days. Cell proliferation was assessed every day using Cell Counting Kit-8 (Dojindo Laboratories). HCC cells were plated at a density of 2,000 to 5,000 cells per plate in 60 or 100 mm plates and allowed to grow for 18 to 21 days, and colonies were stained with crystal violet.

Flow cytometry analysis

Cells were harvested and incubated with primary antibody biotinylated antihuman Pref-1/DLK1/FA1 antibody (R&D) followed by anti-biotin-fluorescein isothiocyanate (FITC). Isotype-matched mouse immunoglobulins served as controls. Samples were analyzed with FACSCalibur flow cytometer, CellQuest, and Flowjo5.7.2 software (BD Biosciences).

Isolation of DLK1⁺ HCC cells by fluorescence-activated cell sorting and magnetic-activated cell sorting

For fluorescence-activated cell sorting (FACS), cells were stained with the antibody mentioned above and then sorted on a BD FACS Aria flow cytometer (BD Biosciences). Only the top 2% most brightly stained cells and the bottom 20% most dimly stained cells were selected as the positive and negative population, respectively. For magnetic-activated cell sorting (MACS), cells were labeled with the biotinylated antihuman pref-1/dlk1/FA1 antibody and followed with anti-biotin-microbeads and separated on MACS LS

Table 1. *In vivo* tumor development experiments of DLK1⁺ and DLK1⁻ cells sorted from HCC cell lines Huh7 in NOD/SCID mice

Cell type ^a	Cell numbers injected	Tumor incidence ^b	P ^c
Huh7-DLK1 ⁺	10,000	5/5	0.0476
Huh7-DLK1 ⁻	10,000	1/5	

^aDLK1⁺ and DLK1⁻ cells freshly isolated from HCC cell lines Huh7 were injected subcutaneously in NOD/SCID mice.

^bThe number of tumors detected/number of injections.

^cFisher exact probability P value.

column (Miltenyi Biotec) according to manufacturer's instructions. MACS was conducted twice or thrice to obtain a purity of more than 50% DLK1⁺ cell population, and the remaining DLK1⁻ HCC cells was greater than 95%.

Self-renewal assay

For self-renewal, DLK1⁺ and DLK1⁻ HCC cells sorted by FACS were cultured for 28 days *in vitro*, and then the proportion of DLK1⁺ cells was assessed by flow cytometry. Moreover, DLK1⁺ cells were subcutaneously injected to mice, and then DLK1⁺ cells of xenograft tumor were also detected by flow cytometry.

Spheroid colony formation assay

HCC cells were seeded at a density of 1,000 cells/mL in ultra low attachment plate (Corning, costar) in DMEM/ Nutrient Mixture F-12 Ham (Sigma) supplemented with recombinant human basic fibroblast growth factor (20 ng/mL), B-27 (1×), recombinant human epidermal growth factor (20 ng/mL), L-glutamine (200 mmol/L; Invitrogen). Cells were cultured at 37°C in 5% CO₂ humidified incubator.

***In vivo* tumorigenicity in mice**

The sorted Huh-7 and Hep3B DLK1⁺ cells were mixed with an equal volume of Matrigel (BD Bioscience), respectively, and then subcutaneously injected into the flank of male NOD/SCID mice (1 × 10⁴, 200 μL per site). To avoid individual difference, equal amount of DLK1⁻ cells as controls were subcutaneously injected into the opposite flank of the same mice. For assessing the effect of *DLK1* knockdown on HCC cells, Huh-7 and Hep3B cells infected by recombinant adenovirus vectors with short hairpin RNA (shRNA) 874 and 1011 targeting *DLK1* were harvested and then subcutaneously injected (Hep3B, 4 × 10⁶ cells; Huh-7, 2 × 10⁶ cells, 200 μL per site) into the backs of anterior limbs of male BALB/c nude mice. When the palpable tumor occurred, tumor size was recorded and monitored once or twice a week. Tumor size was measured as the longest surface length (mm; L) and width (mm; W). Tumor volume

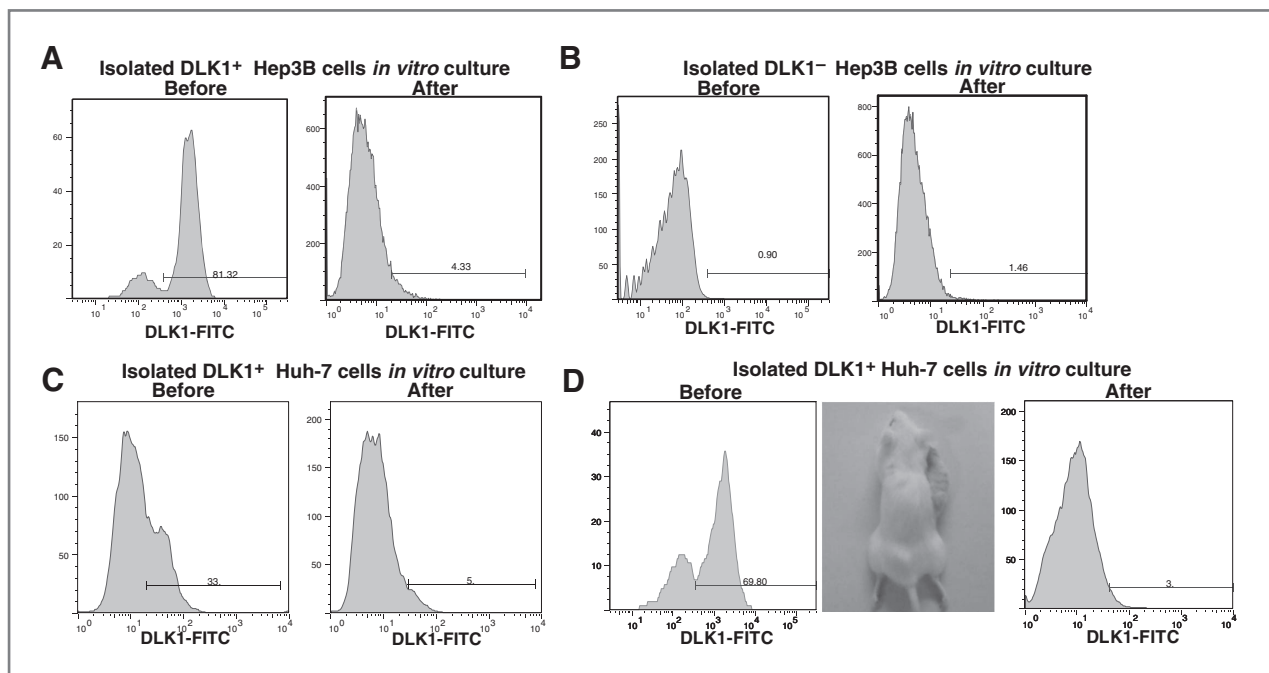


Figure 3. DLK1⁺ HCC cells possess the ability of self-renewal. The isolated DLK1⁺ (A, left) and DLK1⁻ (B, left) cells from Hep3B cell line were cultured for 4 weeks, respectively, and then the proportion of DLK1⁺ cells was analyzed by flow cytometry (A and B, right). C, the isolated DLK1⁺ cells sorted by MACS from Huh-7 cell line were also cultured and analyzed. D, the isolated DLK1⁺ cells from Huh-7 cell line were analyzed by flow cytometry (left) and then were subcutaneously injected into NOD/SCID mice for *in vivo* tumorigenicity (middle). The xenograft tumor derived from these DLK1⁺ cells were harvested and digested to cell suspension, which was subjected to flow cytometry for measuring percentage of DLK1⁺ cells (right). These experiments were carried out 3 times independently.

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Table 2. Percentage of DLK1⁺ cell subpopulation after Huh-7 and Hep3B cells were treated with different dosages of doxorubicin

Doxorubicin (μg/mL)	Percentage of DLK1 ⁺ cells (%)	
	Hep3B	Huh7
0	5.7	4.08
2	6.42	13.71
4	7.45	14.07
6	9.95	19.95
8	22.34	23.8

NOTE: Huh-7 and Hep3B cells were treated with different dosages of doxorubicin, and then percentage of DLK1⁺ cell subpopulation was evaluated by flow cytometry.

($V; \text{mm}^3$) = $LW^2/2$. All study protocols were approved by the Animal Experimental Ethics Committee.

Statistical analysis

Statistical analysis was conducted by Student *t* test (GraphPad Prism 5). Some data presented in figures are derived from 3 independent replicates and was showed as mean ± SD. $P < 0.05$ was considered statistically significant.

Results

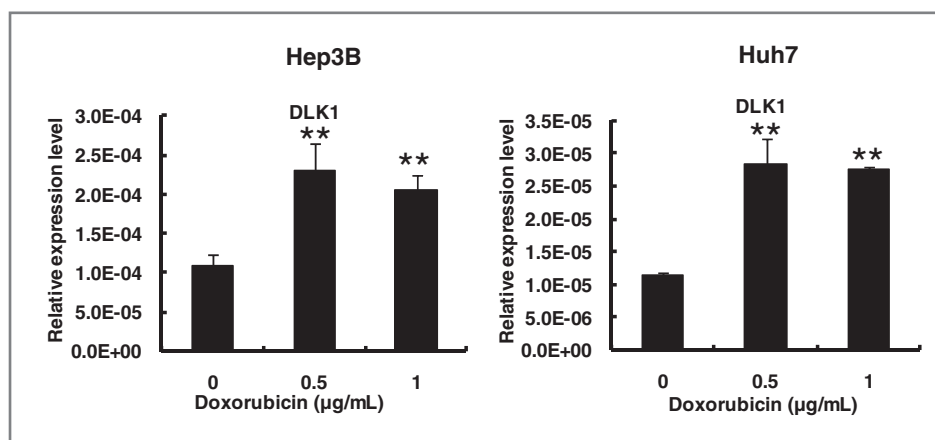
DLK1⁺ HCC cells were enriched in spheroid colonies

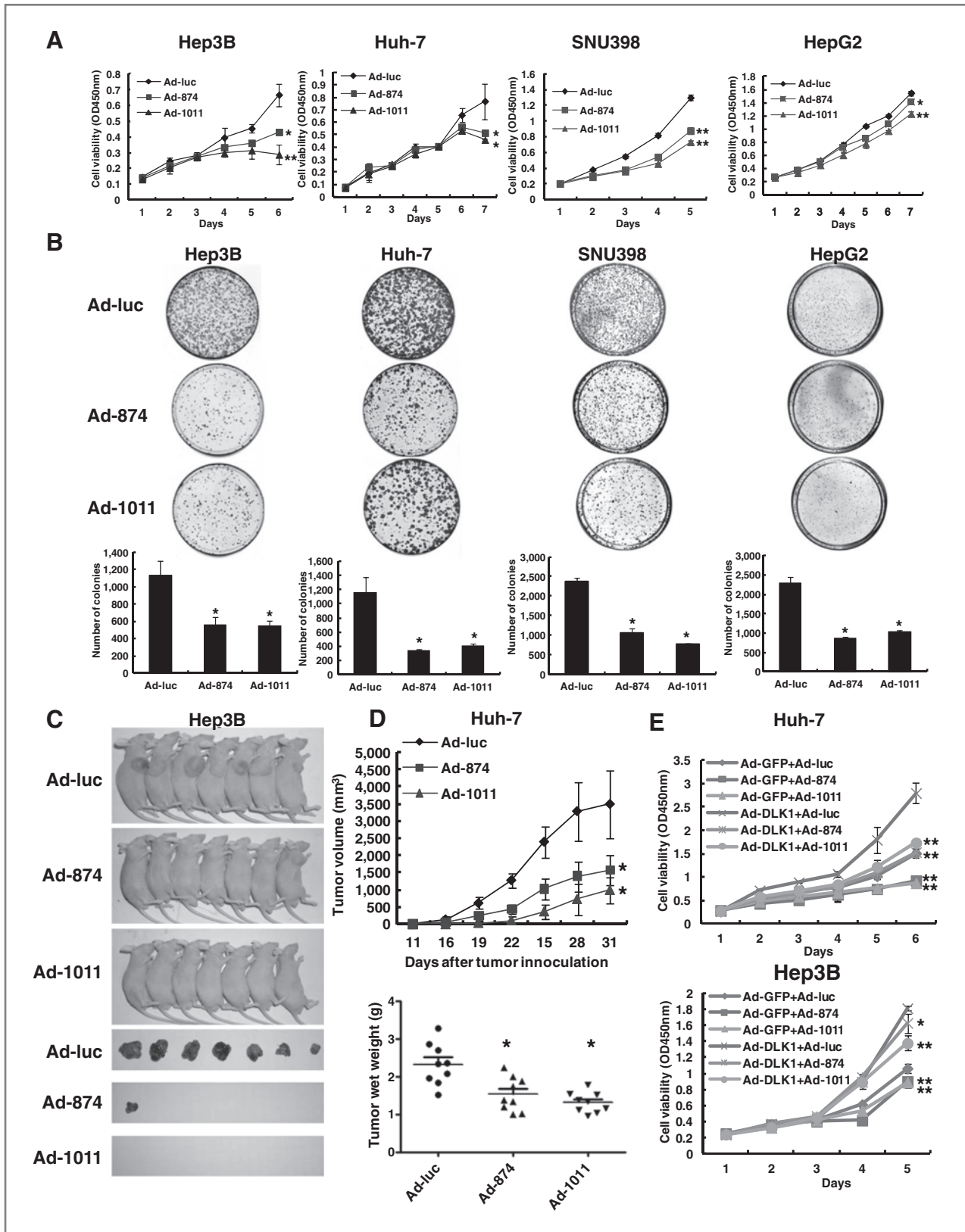
To determine whether DLK1⁺ cells are present in human HCC cell lines as a small subpopulation, we assessed DLK1 expression in 17 liver cancer cell lines with biotinylated antihuman pref-1/dlk1/FA1 antibody and followed by anti-biotin-FITC as a second antibody. The data showed that only a very small DLK1⁺ subpopulation (0.18%–10.22%) exists in these cell lines examined (Fig. 1A and Supplementary Fig. S1). To further determine whether DLK1⁺ cells could be a subpopulation of CSCs, spheroid colony formation assay was conducted in which

Hep3B, Huh-7, SK-Hep1, and HepG2 cells were cultured with serum-free media. DLK1⁺ cells were enriched in these spheroid colonies after culture, as detected by flow cytometry, in which DLK1⁺ cells accounted for 85% of Hep3B spheroids at passage 8 (Fig. 1B, left, middle), 27% of Huh-7 cells at passage 5 (Fig. 1B, left, middle), 21% of SK-hep1 and 28% of HepG2 cells at passage 2 (Supplementary Fig. S2). The enrichment of DLK1 protein in Hep3B (8.36-fold) and Huh-7 (8.41-fold) spheroid colonies compared with the parent cells was confirmed by Western blotting assay (Fig. 1B, right). The finding that DLK1⁺ cells serve as major body of spheroids suggested that DLK1 could be a potential biomarker of HCC stem/progenitor cells.

We evaluated the expression of some genes related to stemness, self-renewal, and differentiation of hepatic stem/progenitor cells in spheroids and their corresponding parent Hep3B or Huh7 cells. The results showed that, in addition to DLK1, the expression of some well-known biomarkers of stem cells or progenitor cells, such as *Nanog*, *SOX2*, *c-kit*, *CD133*, *EpCAM*, *BMI*, *CD90*, and *Oct3/4*, were upregulated in the spheroid colonies compared with parent Hep3B and/or Huh-7 cells (Fig. 1C, top). We also evaluated the expression of some genes related to liver differentiation. The expression of some hepatic markers, such as *AFP* and *ALB*, was increased in spheroids than that in parent Hep3B cells; whereas *CK19*, a biliary biomarker, was decreased in spheroids derived from Hep3B cells (Fig. 1C, left, bottom). Conversely, both *AFP* and *ALB* were downregulated whereas *CK19* was upregulated in spheroids derived from Huh-7 cells (Fig. 1C, right, bottom). That could reflect heterozygosity and differentiation potential of the spheroids derived from Hep3B and Huh-7 cells, respectively. Remarkably, we noticed that the expression level of *DLK1* mRNA was very high in spheroids, especially in spheroids derived from Hep3B cells. The DLK1⁺ Huh-7 and Hep3B cells were sorted and isolated, and the expression of stem cell biomarkers in these cells was evaluated. The expression of *Nanog*, *SOX2*, *Oct3/4*, and *SMO* were

Figure 4. DLK1⁺ HCC cells exhibited chemoresistance to doxorubicin. After doxorubicin treatment, mRNA level of *DLK1* was significantly upregulated as measured by real-time RT-PCR. **, $P < 0.01$, as compared with that of untreated cells. These experiments were carried out 3 times independently.





higher in the isolated DLK1⁺ cells than that in DLK1⁻ cells (Fig. 1D). These data suggested that DLK1⁺ subpopulation of HCC could serve as a part of cancer stem/progenitor cells.

DLK1⁺ HCC cells exhibit stronger proliferation and tumorigenicity

To explore whether DLK1⁺ cells could exhibit stronger tumorigenicity than DLK1⁻ cells, we chose the Huh-7 and Hep3B cell lines as cell model to address the issue, because the 2 cell lines with relatively higher DLK1 expression level have been widely used in laboratories. It should be pointed out that, although HepG2, SNU-398, and MHCC-LM6 also showed higher DLK1 expression, HepG2 cell line was derived from hepatoblastoma, not HCC cell lines, both SNU-398 and MHCC-LM6 cell lines just have been used in a few laboratories so far. We sorted DLK1⁺ and DLK1⁻ cells from Hep3B and Huh-7 cell lines with FACS, and then observed their proliferative and tumorigenic ability. The proportion of the sorted DLK1⁺ cells was ranged from 45% to 72%, whereas the purity of the remaining DLK1⁻ subpopulation was at least 95% (Fig. 2A and B). Significantly, the sorted DLK1⁺ subpopulation from Hep3B cells exhibited stronger colony formation ability than DLK1⁻ cells ($P < 0.01$), where the colonies derive from DLK1⁺ cells were obviously larger than that from DLK1⁻ population (Fig. 2C). Larger colonies maybe reflect stronger proliferative ability. The similar result was also found in DLK1⁺ cell subpopulation from Huh-7 cells (Fig. 2D).

To further investigate the *in vivo* tumorigenicity of DLK1⁺ HCC cells, the purified DLK1⁺ Huh-7 cells (1×10^4) were injected subcutaneously into 5 NOD/SCID mice, the equal amount of DLK1⁻ cells as control was injected into opposite flank of the same mice simultaneously. DLK1⁺ subpopulation exhibited stronger *in vivo* tumorigenicity than DLK1⁻ cells ($P = 0.0476$), where the xenograft tumors derived from DLK1⁺ cells were significantly larger than that from DLK1⁻ cells (Fig. 2E, Table 1). These data suggested that DLK1⁺ Huh-7 cells could be initiating cells of malignant tumor or cancer stem/progenitor cells of HCC. However, in this work, we also conducted the same experiment with 1×10^4 DLK1⁺ and DLK1⁻ Hep3B cells, no visible tumor was found under observation period (data not shown).

DLK1⁺ HCC cells possess potential of self-renewal

To evaluate the self-renewal capacity of DLK1⁺ HCC cells, the sorted DLK1⁺ (purity is 81%) Hep3B cells were cultured *in vitro* for 4 weeks and then detected by

flow cytometry. Remarkably, the proportion of DLK1⁺ cells was decreased to 4.33%, which was similar to the proportion (5%) of DLK1⁺ cells in parent Hep3B cells (Fig. 3A). In contrast, the proportion of DLK1⁺ cells in sorted DLK1⁻ cells was increased from 0.9% to 1.46% after *in vitro* culture (Fig. 3B). We carried out a similar experiment in which DLK1⁺ and DLK1⁻ Hep3B cells were sorted by MACS. The result showed that the proportion of DLK1⁺ cells of the 2 populations reconverted to 3% after *in vitro* culture (Supplementary Fig. S3A). Additional results from MACS positively sorted DLK1⁺ Huh-7 cells showed that the proportion of DLK1⁺ cells was decreased from 33% to 5% after *in vitro* culture (Fig. 3C). We studied xenograft tumor models (Fig. 3D, middle) established from the sorted DLK1⁺ Huh-7 cells (purity of 69.8%; Fig. 3D, left), using flow cytometry analysis and immunohistochemical staining (Supplementary Fig. S3B). The results showed that only a very small proportion of DLK1⁺ HCC cells (3%) existed in the xenograft tumor, whereas the majority of the tumor model was DLK1⁻ cells (Fig. 3D, right). The data suggested that DLK1⁺ HCC cells could generate DLK1⁻ subpopulation and maintain as a small population within in Hep3B and Huh-7 cell lines through the self-renewal.

Chemoresistance of DLK1⁺ HCC cells

It has been reported that cancer stem/progenitor cells in many solid tumors are resistant to routine chemotherapy. To address whether DLK1⁺ HCC cells could be involved in chemoresistance, we treated Hep3B and Huh-7 cells with doxorubicin, a routine chemotherapeutic drug. The percentage of DLK1⁺ cells in Hep3B and Huh-7 cell lines was obviously increased after treatment in a dose-dependent manner, from 5.7% and 4.08% to 22.34% and 23.8%, respectively (Table 2). The expression level of DLK1 mRNA elevated also (Fig. 4). In addition, other chemotherapeutic drugs, including epirubicin, cisplatin, and 5-FU, were used to treat Hep3B and Huh-7 and HepG2 cells. The elevated DLK1 expression was also detected by RT-PCR (Supplementary Fig. S4A–D). We also detected the elevated DLK1⁺ cells in HepG2 cell lines by flow cytometry (Supplementary Fig. S4E). These data suggested that DLK1⁺ HCC cells possess stronger chemoresistance to chemotherapeutic drugs than DLK1⁻ HCC cells.

DLK1 could serve as a therapeutic target

Our previous study showed that the upregulated DLK1 could contribute to oncogenesis of HCC (33). This

Figure 5. DLK1 knockdown could suppress proliferation and tumorigenicity of HCC cells. A, adenovirus-mediated shRNA target to endogenous DLK1 can inhibit cell proliferation of Hep3B, Huh-7 SNU398, and HepG2 HCC cells. B, the colony formation of Hep3B, Huh-7, SNU398, and HepG2 HCC cells was suppressed by the adenovirus-mediated shRNA (top). The number of colonies was counted and statistically analyzed (bottom). These experiments were carried out 3 times independently. Hep3B (C) and Huh-7 HCC cells (D) with adenovirus-mediated shRNA against DLK1 were subcutaneously injected into BALB/c nu mice ($n = 7-9$ per group). Tumor size was evaluated (D, top). After 31 days observation, these xenograft tumors were surgically removed (C, bottom) or weighted (D, bottom). E, overexpression of DLK1 overcame the effect of DLK1 knockdown and promoted growth of Hep3B and Huh-7 cell lines. Knockdown of DLK1 expression in Huh-7 and Hep3B cell lines that with DLK1 overexpression inhibited their proliferation too. One or 2 asterisks indicate that P value is less than 0.05 or 0.01, respectively, as compared with those with the adenovirus-mediated shRNA against luciferase used as control.

work suggested that DLK1⁺ HCC cells are potential cancer stem/progenitor cells playing a role in tumorigenicity, indicating its potential of being a therapeutic target. To confirm this hypothesis, we constructed adenovirus-mediated RNA interference to knock down endogenous *DLK1* in Hep3B, Huh-7, SNU398, and HepG2 cells, and then observed their effect on proliferation and tumorigenicity of HCC cells. In *DLK1* knockdown cells, the growth and colony formation of Hep3B, Huh-7, SNU398, and HepG2 cells were attenuated, as compared with controls that were infected with vector containing RNA interference against luciferase (Fig. 5A and B). Moreover, *in vivo* tumorigenicity of both Hep3B and Huh-7 cells with *DLK1* knockdown was also reduced and even disappeared in BALB/c nude mice after 1 month observation (Fig. 5C and D, Supplementary Fig. S5). However, overexpression of DLK1 can reverse the suppressed cell growth of Hep3B and Huh7 cells, which was induced by *DLK1* knockdown, and even promote their proliferation (Fig. 5E). These data suggested that DLK1, a regulator of cell growth, could serve as a potential therapeutic target for HCC.

Next, we evaluated whether the adenovirus-mediated RNA interference can affect spheroid colony formation in Hep3B and Huh-7 cells. The result showed that the adenovirus-mediated *DLK1* knockdown can significantly reduce the formation of spheroid colonies derived from Huh-7 and Hep3B cells, compared with control vectors (Supplementary Table S2). Then these obtained spheroid cells were treated with the adenovirus-mediated *DLK1* interference. We observed that the *DLK1* knockdown reduced the reformation of spheroid colonies (Supplementary Table S3). We concluded that adenovirus-mediated RNA interference against *DLK1* attenuated the *in vivo* tumorigenicity of HCC cells, probably through targeting the cancer stem/progenitor cells.

Discussion

More and more evidences have been reported that oncogenesis, development, and relapse of many cancers could be ascribed to CSCs. Some putative stem cell biomarkers were used to identify this small subpopulation of cells with high tumorigenicity. HCC, like other cancers, could be original from the malignant stem/progenitor cells. Although some membrane molecules such as CD90 were identified as the markers of liver CSCs (25), other membrane molecules relevant to hepatic development were also proposed as markers of HCC stem/progenitor cells.

Previous data showed that DLK1 played a vital role in liver development and HCC oncogenesis (30, 33, 35–37). DLK1⁺ cells from mouse fetal liver were able to differentiate into hepatocytes and biliary epithelial cell lineages and exhibited stronger proliferative ability than DLK1⁻ cells (30). In this work, DLK1⁺ cells, as a very small subpopulation, were found in all 17 HCC cell

lines examined, however, DLK1⁺ cells were enriched in spheroids after cultured in serum-free media, along with the upregulation of some known stem/progenitor cell-like markers, such as Nanog, SMO, SOX2, Oct3/4, CD133, CD90, and EpCAM, suggesting that DLK1⁺ cells could be a subset of HCC cancer stem/progenitor cells. In addition, the fact that spheroid colonies formed in serum-free culture indicated that DLK1⁺ cells could be resistant to poor environment, which are consistent with known characteristics of cancer stem/progenitor cells. Significantly, the sorted DLK1⁺ HCC cells exhibited stronger ability of *in vitro* clonogenicity and *in vivo* tumorigenicity in mice. Moreover, the purified DLK1⁺ HCC cells could produce DLK1⁻ cells after 4 weeks *in vitro* culture, and the proportion of DLK1⁺ cells in xenograft tumor eventually reconverted to the original level (<5%) found in parent cells. These data collectively suggested that DLK1 could be a potential biomarker for cancer stem/progenitor cells, and DLK1⁺ HCC cells can maintain in a small subset within the whole cell population through self-renewal. However, 1×10^4 DLK1⁺ Hep3B cells did not induce visible tumor under observation period, which implied that, unlike Huh-7 cells, tumor induced from Hep3B could need longer time or more Hep3B cells. Hep3B could possess different inheritable program for tumorigenicity *in vivo*. Thus, more cell lines should be used for evaluating tumorigenicity, and the minimum number of DLK1⁺ HCC cells necessary for induction of xenograft tumor should be investigated in future.

CSCs are highly resistant to chemotherapy in several solid tumors (38–40). We observed an increased proportion of DLK1⁺ cells in HCC cell lines treated with doxorubicin, cisplatin, epirubicin, and 5-FU, indicating that DLK1⁺ HCC cells are associated with HCC chemoresistance to certain anticancer drugs. Our previous studies identified that DLK1 could contribute to oncogenesis of HCC (33). Thus, we further examined whether DLK1 could serve as a therapeutic target against HCC stem/progenitor cells. The *DLK1* knockdown attenuated spheroid colony formation and inhibited proliferation and reduced *in vivo* tumorigenicity of HCC cells, confirming the potential of DLK1 being a therapeutic target.

Taken together, our study showed the cellular behaviors of DLK1⁺ HCC cells as potential HCC stem/progenitor cells and DLK1 as a possible therapeutic target against HCC stem/progenitor cells. Although these findings provided deeper insight into HCC stem cells, further studies need to be carried out in future. With regard to HCC treatment, in addition to RNA interference against *DLK1*, other strategies such as therapeutic antibodies and chemical inhibitors of DLK1 activity also could be developed for HCC administration in future.

Disclosure of Potential Conflicts of interest

No potential conflicts of interest were disclosed.

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