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FAM83D activates the MEK/ERK signaling pathway and promotes cell proliferation in hepatocellular carcinoma

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ABSTRACT

Publicly available microarray data suggests that the expression of FAM83D (Family with sequence similarity 83, member D) is elevated in a wide variety of tumor types, including hepatocellular carcinoma (HCC). However, its role in the pathogenesis of HCC has not been elucidated. Here, we showed that FAM83D was frequently up-regulated in HCC samples. Forced FAM83D expression in HCC cell lines significantly promoted their proliferation and colony formation while FAM83D knockdown resulted in the opposite effects. Mechanistic analyses indicated that FAM83D was able to activate the MEK/ERK signaling pathway and promote the entry into S phase of cell cycle progression. Taken together, these results demonstrate that FAM83D is a novel oncogene in HCC development and may constitute a potential therapeutic target in HCC.

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1. Introduction

Hepatocellular carcinoma (HCC) represents the major histological subtype of primary liver cancer, accounting for 70%–85% of the total liver cancer worldwide. It is the fifth most common cancer worldwide and the second major cause of cancer-related deaths worldwide [1,2]. As with other cancers, the development of HCC is a complex multistep process. Numerous signaling pathways that are related to the development of HCC have been identified, yet the deep mechanisms underlying the oncogenesis and cancer progression of HCC remain poorly understood [3–7].

FAM83 (Family with sequence similarity 83) family is classified as a protein family which shares a highly conserved DUF1669 domain in the N terminus. In humans, there are eight members in this family. While all FAM83 members share conservation in the N-terminal DUF1669, they vary greatly in size and lack any significant homology beyond this domain. Analysis of publically available microarray data revealed that many of the FAM83 members were overexpressed in various types of cancers [8]. Most interestingly, it is recently demonstrated that several FAM83 family members exhibit oncogenic properties and act as putative oncogenes in multiple human tumors. For example, FAM83A has been identified as a potential biomarker for lung cancer, which is expressed in over half of human lung cancer tissues and is especially higher in approximately 70% of lung adenocarcinomas [9]; FAM83A is up-regulated in breast cancer specimens and is associated with poor prognosis. In addition, FAM83A activates MAPK and PI3K-AKT signaling and confers resistance to clinical EGFR-TKIs (EGFR–tyrosine kinase inhibitors) in breast cancer [10]; FAM83B, another member of the FAM83 family, is significantly elevated in many cancers and involved in EGFR-mediated signaling and RAS-mediated transformation. Cells expressing elevated FAM83B levels has a decreased sensitivity to EGFR-TKIs [11]. It has also been reported that inhibition of FAM83B expression can suppress the activity of both CRAF and PI3K signaling, thus providing a foundation for future therapies aimed at targeting FAM83B to inhibit the growth of PI3K/AKT- and MAPK-driven cancers [12]. A recent study suggests that FAM83D is a novel oncogene in breast cancer development that at least partially acts through mTOR activation by downregulating tumor suppressor gene FBXW7 and has prognostic value for breast cancer patients [13].

Abbreviations: HCC, hepatocellular carcinoma; MEK, extracellular protein kinase; ERK, extracellular signal-regulated kinase; FAM83D, family with sequence similarity 83, member D; siRNA, small interference RNA; shRNA, short hairpin RNA; RNAi, RNA interference.

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However, little is known about the role of FAM83 members, especially FAM83D, in the development of HCC. We are particularly interested in FAM83D since our previous genome-wide approach suggested that FAM83D was markedly up-regulated in HCC clinical specimens compared to adjacent non-cancerous livers (unpublished data). Then we determined whether the highly expressed FAM83D could contribute to the pathogenesis of HCC. In the present work, we evaluate the expression of four common members of the FAM83 family in HCC and find that only FAM83D is frequently overexpressed in HCC tissues and cell lines. Overexpression of FAM83D significantly promotes cellular proliferation of HCC cells and accelerates the G1-S cell cycle transition, while FAM83D knockdown leads to the opposite results. Mechanism study indicates that FAM83D is involved in the activation of the MEK/ERK signaling pathway. Collectively, our findings provide new insight into the molecular pathogenesis of HCC and FAM83D may constitute a promising novel therapeutic target for HCC.

2. Materials and methods

2.1. Tissue specimens

All HCC specimens were obtained from HCC patients who underwent surgical resection with informed consent. Both the tumor and adjacent normal tissue were collected from each patient and were frozen at −80 °C until processed. The diagnosis of HCC was validated by pathological examination. The use of human tissues for research was approved by the institutional ethics committee of Nanjing Medical University.

2.2. Cell lines and cell transfection

The human HCC cell lines used in this study were obtained from the Chinese National Human Genome Center at Shanghai. All of them were maintained in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) (Gibco) and antibiotics (50 U/ml penicillin and 50 lg/ml streptomycin) (Gibco) at 37 °C in a humidified incubator with 5% CO_2. Cell transfection was performed using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions.

2.3. RNA extraction, semi-quantitative and real-time PCR

Total RNA was extracted from tissue or cell culture samples using TRIZOL reagent (Invitrogen) and was reverse transcribed into cDNA with a M-MLV reverse transcriptase kit (Promega). Semi-quantitative RT-PCR was performed and products were separated on 1% agarose gel-containing ethidium bromide. Quantitative real-time PCR was performed by the Thermal Cycler Dice Detection System with the SYBR Premix Ex Taq™ (Takara). The following primers were used to specially amplify the FAM83D gene and β-actin.

For FAM83D, forward: 5′-CTCTTCGGGCACCTACTTCC-3′, and reverse, 5′-ACCACTGCAATCACCTCTCG-3′;.

For β-actin: forward: 5′-AGAGCCTCGCCTTTGCCGATCC-3′, and reverse, 5′-CTGGGCCTCGTCGCCCACATA-3′.

2.4. Plasmid construction and RNA interference

The full-length cDNA of FAM83D (2445 bp, GenBank accession number NM_030919) was amplified from human liver cDNA library and cloned into the expression vector pcDNA3.1/myc-His(-)B-3 × FLAG-IRES-hrGFP, derived from pcDNA™3.1/myc-His(-)B (Invitrogen). FAM83D-specific small interference RNAs (siRNAs) were chemically synthesized (GenePharma) to suppress FAM83D expression in HCC cells: si-1, forward, 5-AUGGACGGAUGGCAAAUUAdTdT-3, and reverse, 5-UAAUUUGCCAUCCGUCCAUdTdT-3; si-2, forward, 5-CCUCUACUGUUAGUGAGGAdTdT-3, and reverse, 5-UCCUCACUAACAGUAGAGGdTdT-3;.

In addition, si-NC was also synthesized for use as a negative control: si-NC, forward, 5-UUCUCCGAACGUGUCACGUdTdT-3, and reverse, 5-ACGUGACACGUUCGGAGAAdTdT-3.

The oligonucleotides encoding short hairpin RNAs (shRNAs) for the continuous knockdown of endogenous FAM83D were synthezised and inserted into pSUPER (Oligoengine): sh-1, forward,5-GATCCCCATGGACGGATGGCAAATTATTCAAGAGATAATTTGCCATCCGTCCATTTTTTGGAAA-3, and reverse, 5-AGCTTTTCCAAAAAATGGACGGATGGCAAATTATCTCTTGAATAATTTGCCATCCGTCCATGGG-3; sh-2, forward,5-GATCCCCCCTCTACTGTTAGTGAGGATTCAAGAGATCCTCACTAACAGTAGAGGTTTTTGGAAA-3, and reverse, 5-AGCTTTTCCAAAAACCTCTACTGTTAGTGAGGATCTCTTGAATCCTCACTAACAGTAGAGGGGG-3; sh-NC containing irrelevant nucleotides was used as a negative control: sh-NC, forward, 5-GATCCCCTTCTCCGAACGTGTCACGTTTCAAGAGAACGTGACACGTTCGGAGAATTTTTGGAAA-3, and reverse, 5-AGCTTTTCCAAAAATTCTCGAACGTGTCACGTTCTCTTGAAACGTGACACGTTCGGAGAAGGG-3.

2.5. Cell proliferation

HCC cells were seeded at a density of 2000–5000 cells per well of 96-well plates and cultured for a week. Cell viability was measured using Cell Counting Kit-8 (Dojindo Laboratories) according to the manufacturer's instructions. The absorbance value at a wavelength of 450 nm was used as an indicator of cell viability.

2.6. Colony formation

HCC cells were plated at a density of 10,000–50,000 cells per 10 cm plate and cultured in the medium with addition of 0.6–1 mg/ml G418 (Life Technologies). After 3 weeks, the colonies stained with crystal violet were photographed and counted.

For the soft agar colony formation assay, 2000–5000 transfected cells were plated and grown in the medium containing 1% base agar and 0.5% top agar. After 2–3 weeks, colonies were counted and photographed under a dissecting microscope.

2.7. Cell cycle analysis

Serum starvation was used to induce cell cycle synchronization before cells were transfected. After 48 h, transfected cells were harvested as single cell suspensions. For DNA content detection, cells were fixed in 70% ethanol, washed with PBS and incubated with propidium iodide (10 μg/ml) and RNase A (10 mg/ml) for 30 min at 4 °C, followed by flow cytometric analysis using the FACSCalibur flow cytometer, CellQuest (BD Biosciences).

2.8. Bromodeoxyuridine (BrdU) incorporation assay

The transfected cells were treated with 20 μM BrdU (Sigma–Aldrich) for 2 h and incubated with an anti-BrdU antibody (1:100), followed by incubation with Alexa Flour®Dyes (1:200, Invitrogen). Confocal microscopy (Carl Zeiss) was performed to analyze the cellular incorporation of BrdU.

2.9. Western blot analysis

Cell extracts were collected using a 2 × loading lysis buffer (25 mmol/L Tris, pH 6.8, 1% SDS, 5 mmol/L EDTA and protease inhibitor cocktail, Sigma). Total protein extracts (20 μg) were subject to electrophoresis by SDS–PAGE on a 10% gel and then transferred

to a polyvinylidene difluoride (PVDF) membrane. After blocking with PBS containing 5% nonfat milk, the membrane was incubated with the appropriate primary antibody at room temperature for 2 h, followed by incubation with an IRDye 800DX-conjugated, affinity-purified secondary antibody. The labeled protein bands were detected using the Odyssey Infrared Imaging System (Li-COR Biosciences). β-actin was used as a loading control.

2.10. Antibodies and reagents

Antibodies against FAM83D, cyclin D1 and β-actin were obtained from Santa Cruz Biotechnology. Antibodies against MEK1/2, ERK1/2, phospho-MEK1/2, phospho-ERK1/2 as well as the MEK1 inhibitor PD98059 were obtained from Cell Signaling Technology.

2.11. Statistical analysis

Data between experimental groups were statistically analyzed by Student's t test using GraphPad Prism 5 software. All data are presented as means ± standard deviation (SD) and a two-tailed $p < 0.05$ was considered to be statistically significant.

3. Results

3.1. FAM83D is frequently up-regulated in HCC tissues and cell lines

To explore the potential effect of FAM83 members on HCC, we first used real-time PCR to measure the mRNA levels of four FAM83 members (FAM83A, FAM83B, FAM83C and FAM83D) in 30 paired HCC specimens and found that only the FAM83D mRNA level was significantly up-regulated in HCC tissues compared to the corresponding non-tumorous liver tissues (Fig. 1A). To verify this result, FAM83D expression was further accessed in additional 50 pairs of HCC samples by real-time PCR, where FAM83D was shown to be elevated in 21/50 (42%) of the HCC specimens at more than 2-fold higher levels (Fig. 1B). Eight pairs of typical cases were illustrated by semi-quantitative PCR assay (Fig. 1C). Moreover, the expression of FAM83D was elevated in most of the examined human HCC cell

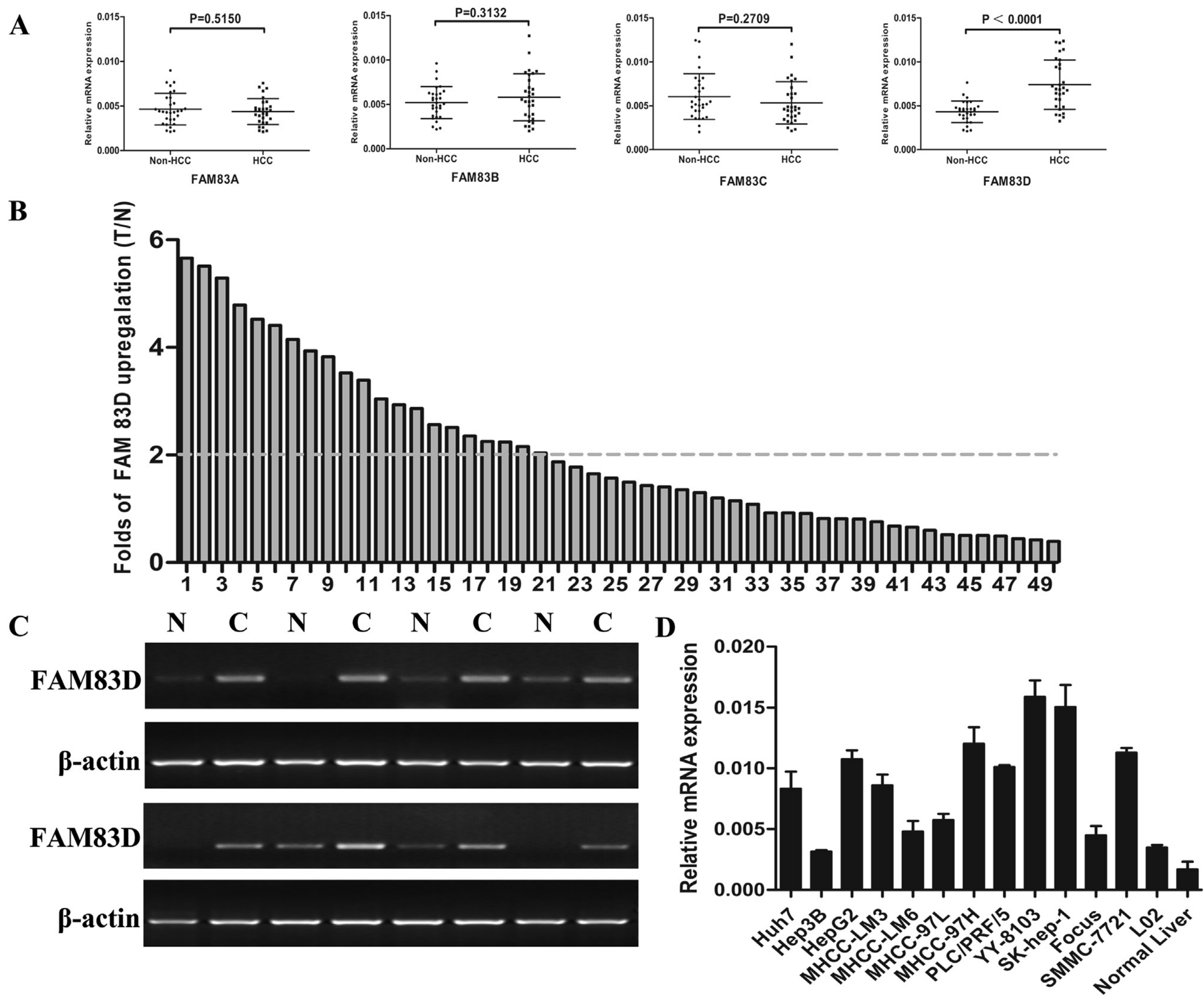

Fig. 1. FAM83D is overexpressed in HCC tissues and cell lines. (A) Real-time PCR was performed to measure the expression level of FAM8A-FAM83D in 30 pairs of HCC and the corresponding adjacent non-cancerous liver (non-HCC), where β-actin was used as an endogenous control. P value was calculated by student's t test. (B) Real-time PCR was carried out on additional 50 paired HCC samples and adjacent non-cancerous tissues. The columns show the up-regulated fold change of FAM83D mRNA level in HCCs, as compared to that in the corresponding adjacent non-cancerous liver, where β-actin was used as an internal control. (C) Semi-quantitative PCR assay results of 8 pairs of HCC (C) and the adjacent non-HCC liver tissue (N). (D) The expression levels of FAM83D was determined by real-time PCR in 12 HCC cell lines as well as the normal human liver cell line L02 and normal adult liver tissue, with β-actin as an internal control.

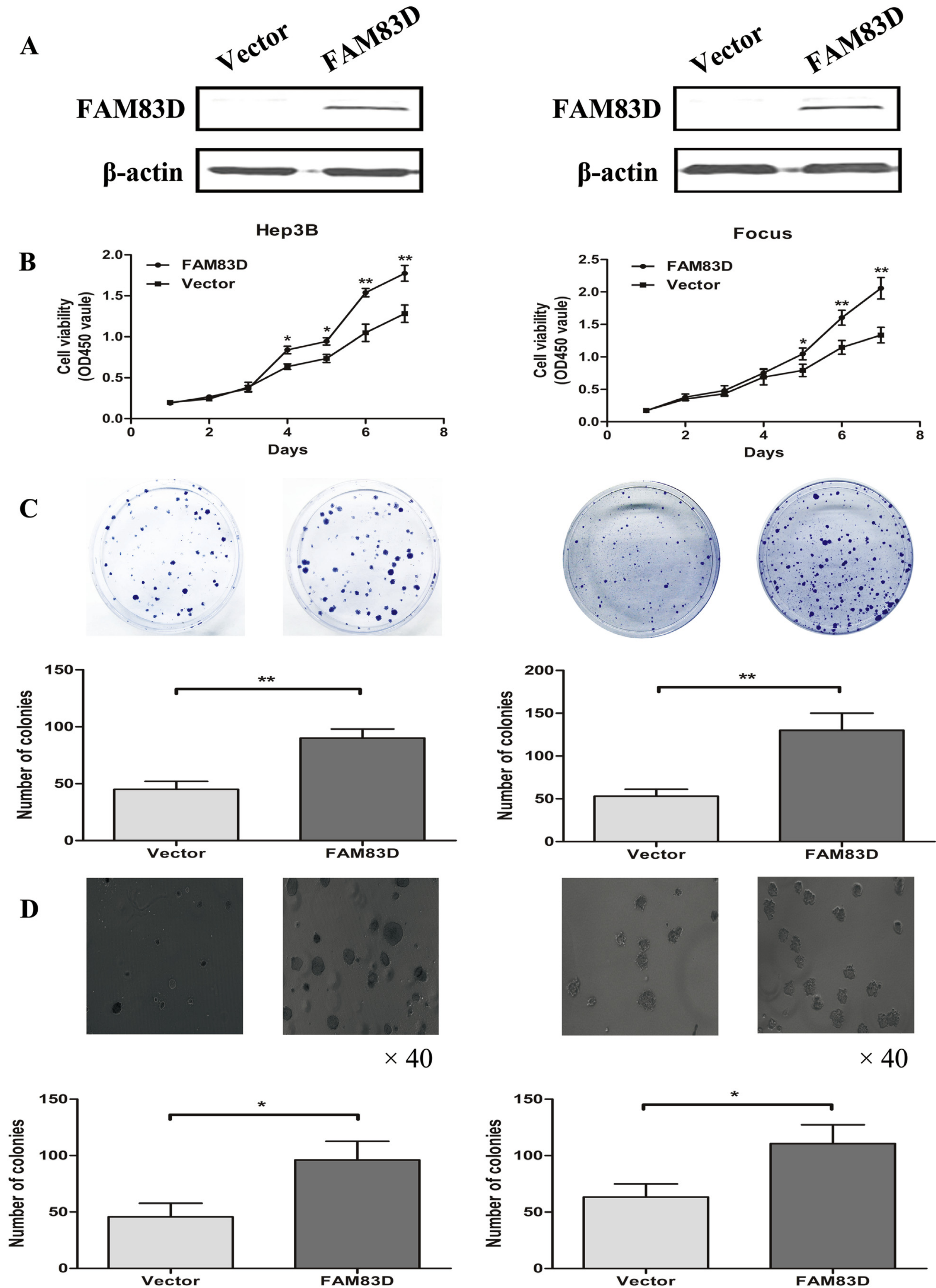

Fig. 2. Overexpression of FAM83D promotes HCC cellular proliferation and colony formation in vitro. (A and B) Ectopic FAM83D promoted proliferation of Hep3B and Focus cells. The expression of ectopic FAM83D was detected by western blot assay, with β-actin as a loading control. (C) Overexpression of FAM83D enhanced the anchorage-dependent colony

lines when compared to the normal human liver cell line L02 and normal adult liver tissue (Fig. 1D).

In addition, the correlation between clinicopathological characteristics of these 50 HCC patients and expression of FAM83D was evaluated (Supplementary Table 1). Elevated FAM83D expression in HCC patients was significantly associated with tumor size and tumor stage, suggesting that FAM83D might have a stimulatory role in the progression of HCC. Moreover, FAM83D expression was correlated with HBsAg, implying that FAM83D is perhaps connected with HBV infection of HCC patients.

3.2. *Overexpression of FAM83D promotes HCC cell proliferation and colony formation in vitro*

To investigate the role of FAM83D in HCC, we first examined the effect of FAM83D overexpression on HCC cells. So we transfected the recombinant plasmid pcDNA3.1-FAM83D containing the FAM83D gene into Hep3B and Focus cell lines which express FAM83D at a relatively low level. After evaluating the detectability of the recombinant FAM83D plasmid (Fig. 2A), we observed that cell growth and colony formation were significantly promoted by FAM83D overexpression when compared to that of the cells transfected with the empty vector (Fig. 2B and C). Moreover, an anchorage-independent colony formation assay in soft agar was performed to evaluate the effect of FAM83D on malignant behavior and the results revealed that FAM83D overexpression significantly enhanced the anchorage-independent growth of Hep3B and Focus cell lines in soft agar (Fig. 2D). These data suggested that FAM83D overexpression accelerates HCC cell proliferation and colony formation in vitro.

3.3. *Knockdown of FAM83D inhibits HCC cell proliferation and colony formation in vitro*

To determine whether FAM83D is necessary for the proliferation of HCC cells, we used chemically synthezised siRNAs (si-NC, si-1, si-2) and constructed the corresponding shRNA plasmids (sh-NC, sh-1, sh-2) to knockdown endogenous FAM83D in 2 HCC cell lines (SK-hep-1 and YY-8103) with relatively high FAM83D level. The efficient inhibition of FAM83D expression in siRNA-treated cells was verified by western blot (Fig. 3A). As expected, we observed significant growth suppression of HCC cell lines treated with siRNAs in comparison with the si-NC-transfected cells (Fig. 3B). Moreover, FAM83D depletion by shRNAs substantially inhibited the colony formation of FAM83D-overexpressing HCC cell lines compared to the control shRNA-NC-infected cells (Fig. 3C). Furthermore, down-regulation of FAM83D decreased the anchorage-independent growth of these HCC cell lines in soft agar compared to the cells transfected with the negative control shRNAs (Fig. 3D). These collective data indicated that endogenous expression of FAM83D is essential for maintaining cell proliferation and colony formation in HCC cells.

3.4. *FAM83D facilitates the G1-S cell cycle transition and enhances the MEK/ERK signaling pathway*

In search of the cellular events involved in the effects of FAM83D on HCC cell proliferation, we performed a flow cytometric analysis to determine whether FAM83D could affect cell cycle distribution. The results showed that the proportion in the S-phase was remarkably increased in Focus cells transfected with recombinant pcDNA3.1-FAM83D plasmid than in those treated with empty pcDNA3.1 vector. In contrast, the fraction of cancer cells in the S-phase was notably decreased upon FAM83D knockdown with si-1 or si-2 in SK-hep-1 cells than in those treated with the control si-NC (Fig. 4A). In addition, de novo DNA synthesis was evaluated by BrdU incorporation assay in the transfected cells. Expectedly, FAM83D overexpression led to the significant increase of BrdU-incorporating ratios in Focus cells as demonstrated by immunofluorescence assay with BrdU antibody, whereas siRNA reduced the BrdU-incorporated proportion of SK-hep-1 cells (Supplementary Fig. S1). These results inferred that FAM83D promotes the growth regulation of HCC cells by modulating the G1–S transition.

To further clarify the mechanism by which FAM83D affects the cell proliferation, several important signaling molecules for cell proliferation were investigated by western blot analysis after FAM83D overexpression and knockdown. Interestingly, our data showed that forced FAM83D expression led to a striking increase of p-MEK1/2, p-ERK1/2 and cyclin D1 protein levels in the Focus cells when compared with that of the cells transfected the empty vector, while the total protein levels of MEK and ERK were not changed. In contrast, the opposite results were obtained with the knockdown of FAM83D in SK-hep-1 cells (Fig. 4B). These results implied that FAM83D might play an important role in activating the MEK/ERK signaling pathway in HCC cells.

To further confirm whether the phenotypic changes of the proliferation promoted by FAM83D overexpression were through a MEK/ERK-dependent signaling pathway, Focus and Hep-3B cells were treated with the MEK1 inhibitor PD98059. Intriguingly, the results showed that the MEK1 inhibitor PD98059 almost rescued the proliferation-promoting role of FAM83D in Focus and Hep-3B cells (Fig. 4C and D), suggesting a critical role of MEK/ERK signaling in the proliferation of HCC cells. Therefore, we inferred that FAM83D promotes HCC cell proliferation partially through enhancing the MEK/ERK signaling pathway.

4. Discussion

HCC is a highly malignant cancer worldwide and its molecular etiology is heterogeneous. Despite great achievements have been attained in therapeutic modalities and strategies of HCC, the long-term survival rate for HCC is still poor. Therefore novel effective approaches against drug targets are urgently needed [14,15].

The FAM83 family is an 8-member family of proteins based solely on the presence of the highly conserved DUF1669. Various microarray studies suggest that FAM83 member mRNAs are significantly elevated in a wide variety of tumor types. For example, FAM83D expression is reported to be elevated in breast cancer [16], hepatocellular carcinoma [17,18], ovarian cancer [19] and metastatic lung adenocarcinomas [20]. A recent study demonstrates that FAM83D promotes oncogenic properties upon overexpression and physically interacts with the tumor suppressor FBXW7 in breast cancer cells. Moreover, elevated expression of FAM83D is associated with poor disease-free survival of breast cancer patients [13].

Our previous gene–expression profiles using the genome-wide method indicated that FAM83D was up-regulated in HCC tissues compared with the corresponding non-HCC tissues (unpublished data). Therefore, we speculate that FAM83D may act as a possible

formation of Hep3B and Focus cells, as shown by representative plates of cells transfected with the FAM83D expression vector and empty control vector. The histograms represent mean with standard deviation of colonies from triplicate tests. (D) Forced FAM83D expression promoted the anchorage-independent colony formation of Hep3B and Focus cells in soft agar. The histograms represent mean with standard deviation of colonies from triplicate tests. Here all experiments were performed independently 3 times. A t test was used to evaluate the statistical significance of these experiments, as compared to the control. $^{*}P < 0.05$; $^{**}P < 0.01$. Original magnification, ×40.

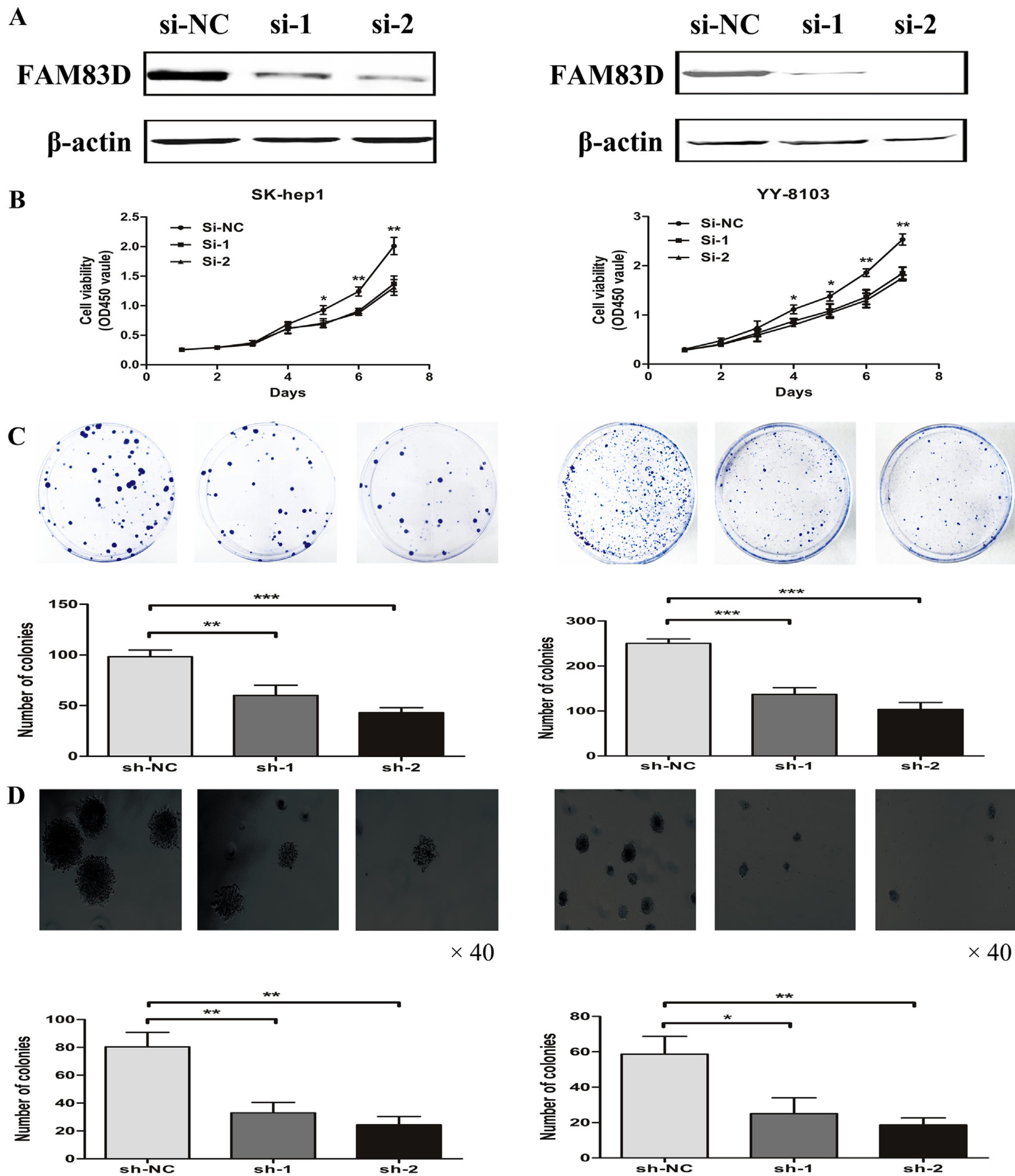

Fig. 3. Knockdown of FAM83D inhibits HCC cellular proliferation and colony formation in vitro. (A and B) Knockdown of endogenous FAM83D suppresses the proliferation of SK-hep-1 and YY-8103 cell lines. The efficiency of two siRNA (si-1, si-2) against endogenous FAM83D was evaluated by western blot, where si-NC was used as a negative control. (C) FAM83D RNAi suppressed colony formation in SK-hep-1 and YY-8103 cells, as shown by representative plates of cells transfected with the FAM83D shRNA constructs and shRNA-NC control. The histograms represent mean with standard deviation of colonies from triplicate tests. (D) FAM83D RNAi limited colony formation of SK-hep-1 and YY-8103 cells in soft agar. The histograms represent mean with standard deviation of colonies from triplicate tests. Here all experiments were performed independently 3 times. A t test was used to evaluate the statistical significance of these experiments, as compared to the control. *$P < 0.05$; **$P < 0.01$. Original magnification, ×40.

oncogene in HCC as it did in breast cancer. To confirm this hypothesis, we detected mRNA levels of four common members of the FAM83 family and found that only FAM83D was frequently elevated in HCC specimens. Besides, high expression of FAM83D was also found in HCC cell lines. These data suggested that FAM83D might be involved in HCC carcinogenesis. Further in vitro experiments revealed that FAM83D overexpression accelerated the proliferation and colony formation of HCC cells, while FAM83D knockdown led to a suppression of cell growth. Flow cytometric analysis and BrdU incorporation assay indicated that overexpression of FAM83D

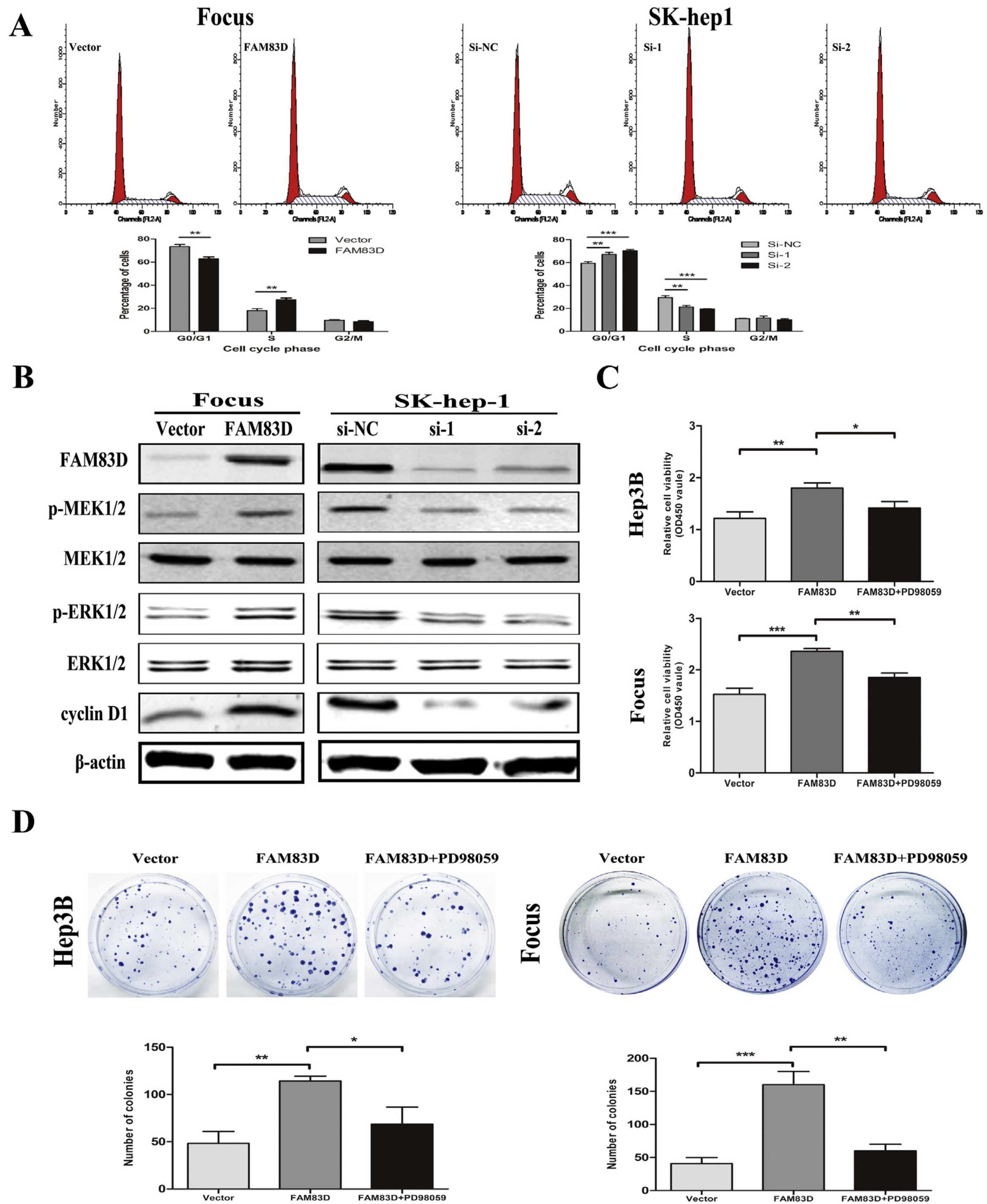

Fig. 4. FAM83D leads to accelerated G1-S transition of cell cycle progression and activates MEK/ERK signaling. (A) The cell cycle analysis was performed after HCC cells were infected with plasmids or siRNAs. Representative cell cycle distributions were shown and the histogram columns represent the average percentages of G0/G1, S and G2/M phases. (B) The expression levels of several components involved in the MEK/ERK pathway were detected by Western blotting of FAM83D overexpression in Focus or FAM83D knockdown in SK-hep-1 cells, where β-actin was used as a control. (C and D) The effects of ectopic FAM83D overexpression on cell viability (C) and colony formation (D) in Hep-3B and Focus cells were neutralized by the specific MEK1 inhibitor PD98059. All the above experiments were repeated 3 times. A t test was used to evauate the statistical significance of these experiments, as compared to the control. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

promoted the G1/S transition in Focus cells. Likewise, knockdown of FAM83D in SK-hep-1 cells led to an arrest in G0/G1 phase.

The MEK/ERK signaling pathway regulates many fundamental cellular functions such as cell proliferation, survival or cell motility. Up-regulation of this pathway is crucial in the promotion or development of tumor cell growth [21]. An increased expression and activation of the MEK1/2 and ERK1/2 kinases has been detected in human and mice primary liver tumors [22,23]. Moreover, several active forms of the MEK/ERK pathway components including p-MEK1/2 and p-ERK1/2 are correlated with poor prognosis in HCC patients [24,25]. In addition, MEK/ERK pathway can be activated by Hepatitis-B virus (HBV) or C virus (HCV) infection and thereby

might contribute to HCC carcinogenesis by facilitating the proliferation and survival of infected cells [26]. The critical role of the MEK/ERK pathway in HCC tumorigenesis strongly suggests that the kinases MEK1/2 or ERK1/2 could be promising therapeutic targets. Indeed, the chemical MEK1/2 kinase inhibitors blocks in vitro and in vivo proliferation of various tumor models, including HCC [27,28]. RNAi strategy against MEK1 prevents ERK activation and abolishes hepatocarcinoma growth [29]. Therefore, in our study, we performed western blot to explore whether the MEK/ERK signaling pathway was activated by FAM83D in HCC cells. Intriguingly, p-MEK1/2, p-ERK1/2 and cyclin D1 protein levels was apparently elevated by FAM83D overexpression in Focus cells and observably inhibited by FAM83D depletion in SK-hep-1 cells. Therefore, we speculated that FAM83D was capable of promoting HCC cell proliferation by enhancing the MEK/ERK signaling. To further confirm our assumption, we used a known MEK1 inhibitor PD98059 in the cell proliferation assay. As expected, the proliferation promotion effect of FAM83D overexpression in Focus and Hep3B cells were significantly rescued by the MEK1 inhibitor PD98059. Thus, our data indicated that FAM83D activates the MEK/ERK signaling pathway to promote HCC cell proliferation. However, whether FAM83D activates the MEK/ERK signaling pathway through HBV infection stills needs future investigations since we found that FAM83D expression was also correlated with HBsAg in HCC patients.

To sum up, our findings highlight a novel mechanism in which FAM83D functions as a tumor promoter in HCC cells. Therefore, FAM83D overexpression might be a prognostic marker and potential novel therapeutic target for the treatment of HCC.

Conflict of interest

The authors have no conflict of interest.

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Transparency document

The transparency document associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.bbrc.2015.01.108.

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.bbrc.2015.01.108.

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