Original Article

IFITM3 upregulates c-myc expression to promote hepatocellular carcinoma proliferation *via* the ERK1/2 signalling pathway

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Summary   **Interferon-induced transmembrane protein 3 (IFITM3) is associated with cancer development. Proto-oncogene c-myc can promote tumor proliferation. However, collections of IFITM3 and c-myc in hepatocellular carcinoma (HCC) and the potential role and mechanisms of IFITM3 in c-myc-mediated tumor proliferation remain unclear. In this study, we investigated a positive correlation between the expression of IFITM3 and c-myc in HCC. The down-regulation of IFITM3 significantly reduced c-myc expression and inhibited the proliferation of HCC *in vitro* and *in vivo*. In addition, upregulated c-myc expression restored the decrease in cell proliferation caused by the downregulation of IFITM3, while downregulation of c-myc reduced the proliferation of HCC enhanced by IFITM3. Mechanistically, IFITM3 regulates c-myc expression *via* the ERK1/2 signalling pathway. In conclusion, a novel path of IFITM3–ERK1/2–c-myc regulatory circuitry was identified, and its dysfunction may lead to HCC tumorigenesis.**

Keywords: IFITM3, c-myc, hepatocellular carcinoma, ERK1/2 signalling pathway, proliferation

1. Introduction

Hepatocellular carcinoma (HCC) is one of the most common primary malignancies of the liver and a leading cause of cancer-related mortality worldwide (*1,2*). Surgical resection is regarded as the primary treatment for HCC, but less than 20% of patients undergo timely radical surgical resection mainly due to high degree of malignancy and the rapid infiltrative growth of tumor cells (*3,4*). Thus, elucidating the mechanisms underlying HCC proliferation is critical to its treatment.

Interferon-induced transmembrane protein 3 (IFITM3) is a protein that is encoded by the IFITM3 gene in humans and that belongs to the interferon-inducible transmembrane protein family (*5*). Studies have shown that IFITM3 is involved in the processes of cellular differentiation, apoptosis, cell adhesion, and immune cell regulation (*6-9*). Recently, an increasing number of studies have focused on the role of IFITM3 in tumorigenesis and development. IFITM3 is reported to be significantly overexpressed in many tumors including colon cancer, astrocytoma, human glioma, myeloid leukemia, and prostate cancer, as well as HCC (*10-14*). In addition, the IFITM family seems to play an important role in the regulation of tumor cell proliferation (*15,16*). Moreover, the current authors previously reported that IFITM3 can promote HCC invasion and metastasis and that miR-29a can directly bind to IFITM3 to promote the growth of HCC cells (*17,18*). However, the downstream regulatory role played by IFITM3 in the proliferation of HCC is still unclear.

Proto-oncogene c-myc is one of the important members of the MYC gene family, and it participates in cellular metabolism, proliferation, and differentiation (*19,20*). c-myc is reported to play an important role in

the oncogenic transformation of normal cells and in tumor proliferation. For example, c-myc overexpression promotes oral cancer cell proliferation (*21*). Down-regulated c-myc can inhibit bladder cancer, prostate cancer, and gastric cancer cell proliferation (*22-24*). Many studies of HCC have also verified that down-regulated c-myc significantly inhibits tumor cell growth (*25,26*). However, the upstream regulatory role of c-myc in HCC has yet to be fully elucidated.

The current study confirmed that expression of the IFITM3 and c-myc genes is aberrantly overexpressed and positively correlated in HCC tissues. In addition, knockdown of IFITM3 inhibits HCC proliferation by downregulating c-myc expression *in vitro* and *in vivo*. Further investigations indicate that IFITM3 regulates c-myc *via* the ERK1/2 signalling pathway. Together, these findings have indicated a novel role for IFITM3 in activating the ERK1/2 signalling to promote the cell proliferation and growth of HCC.

2. Materials and Methods

2.1. *Human tissue specimens*

Subjects were 160 patients who underwent resection of HCC at the Second Affiliated Hospital of Nanchang University from January 2010 to December 2018. Tumor and adjacent tissue specimens were collected according to the protocol approved by the Ethics Committee of the Second Affiliated Hospital of Nanchang University. All patients provided informed consent.

2.2. *Cell culture, plasmids, and reagents*

The human HCC cell lines MHCC97H, HCCLM3, Hep3B, Huh-7, and SMCC7721 were purchased from the Shanghai Institute of Cell Biology, China. They were cultured in DMEM (Thermo Fisher Scientific, Shanghai, China) with 10% FBS (Thermo Fisher Scientific) and 1% penicillin-streptomycin at 37°C and in a 5% CO_2 atmosphere. Four short hairpin RNAs (shRNAs) were designed with siRNA Target Finder (InvivoGen, Hong Kong, China). The target sites of shRNA are shown in Table S1 (*http://www.biosciencetrends.com/ action/getSupplementalData.php?ID=49*). Real-time quantitative PCR (qRT-PCR) and Western blotting (Figure S1, *http://www.biosciencetrends.com/action/ getSupplementalData.php?ID=49*) confirmed the effects of interference. The most effective interference was used in experiments. Expression plasmids for pcDNA3.1(+)-IFITM3 and pcDNA3.1(+)-c-myc are been described in refs. *17*, *18*, *27*, and *28*.

The following antibodies and reagents were used: IFITM3, c-myc, c-Jun, c-Fos, ERK1/2, p-ERK1/2 (T202/Y204), tubulin antibody (Abcam, Cambridge, MA, USA); Lipofectamine3000 (Invitrogen); Total Protein Extraction Kit (Applygen, Beijing, China); and U0126 (Sigma Chemical Co., St. Louis, MO, USA).

2.3. *qRT-PCR, western blotting, and immunohistochemical staining (IHC)*

qRT-PCR, Western blotting, and IHC were performed as described previously (*17,29,30*). The following primer pairs were used for qRT-PCR: IFITM3 (forward, 5'-ACTGTCCAAACCTTCTTCTCTCC-3', reverse, 5'-TCGCCAACCATCTTCCTGTC-3'), c-myc (forward, 5'- AATGAAAAGGCCCCCAAGGTAGTT ATCC-3', reverse, 5'- GTCGTTTCCGCAACAAGTCC TCTTC-3'); GAPDH (forward, 5'-CAGGGCTGCTTTT AACTCTGGT-3', reverse, 5'-GATTTTGGAGGGATC TCGCT-3') was used as an internal control.

2.4. *Cell proliferation and colony formation assays*

Cell proliferation and viability were assessed using an MTT assay. The differently transfected HCC cells were seeded on 96-well plates (1×10^3 cells/well). After culturing for 24, 48, 72, and 96 h, 10 µl of an MTT stock solution (5 mg/ml; Sigma) was added to each well with 100 µl of medium for 4 h at 37°C. The medium was replaced with 100 µl of dimethyl sulfoxide and the mixture was incubated at room temperature for 5 minutes. The absorbance was then measured at a wavelength of 570 nm.

To perform colony formation assay, 800 cells were seeded on 6-well plates. After 14 d, 4% paraformaldehyde was used to fix the cells, and cells were stained with 0.1% crystal violet. The number of colonies and their areas were counted.

2.5. *Animal experiments*

Animal protocols in accordance with the guidelines of the U.K. Animals (Scientific Procedures) Act, 1986, and EU Directive 2010/63/EU were approved by the Ethics Committee for Animal Experiments of the Second Affiliated Hospital of Nanchang University. The flanks of 6-week-old male BALB/c-nu/nu mice (SLAC Laboratory Animal Co., Ltd., Shanghai, China) were subcutaneously injected with 1×10^7 cells in 100 µl of PBS. After 14 d, the tumor tissues were resected, weighed, and imaged. The following formula was used to calculate tumor volume: (short diameter) × (longest diameter) × high × π /3.

2.6. *Statistical analysis*

All statistical analysis was performed with SPSS 17.0 (SPSS, Inc.). Results are expressed as the mean ± SD. The Student's *t*-test was used to analyze the differences between two groups, and one-way ANOVA was used to analyze differences when comparing more than two

groups. A *P* value of less than 0.05 was considered statistically significant.

3. Results

3.1. *IFITM3 and c-myc expression are both upregulated and positively correlated in HCC tissues*

To explore the correlation between the levels of IFITM3 and c-myc expression in HCC, IFITM3 and c-myc expression was first detected in 160 HCC specimens and adjacent tissue specimens using qRT-PCR, IHC, and Western blotting. The results of qRT-PCR indicated that average levels of expression of IFITM3 and c-myc mRNA in cancer tissues were markedly higher than those in adjacent non-tumor tissues (Figure 1A). IHC confirmed that the IFITM3 protein was highly expressed in 61.25% (98 of 160) of the HCC specimens and that the c-myc protein was highly expressed in 54.38% (87 of 160) of the HCC specimens (Figure 1B). Western blotting further indicated that levels of IFITM3 and c-myc protein expression were markedly upregulated in HCC specimens ($p < 0.001$ for both, Figure 1C and D). Moreover, scatter plots confirmed that levels of IFITM3 and c-myc mRNA and protein were positively correlated in HCC specimens ($p < 0.001$ for both, Figure 1E).

3.2. *Downregulation of IFITM3 suppresses c-myc expression and inhibits HCC proliferation in vitro and in vivo*

In order to determine whether IFITM3 regulates the expression of c-myc in HCC cells, the levels of IFITM3 and c-myc expression were first detected in various HCC cells using qRT-PCR and Western blotting. Results indicated that the level of IFITM3 expression was positively correlated with the level of c-myc expression (Figure 2A). Subsequently, IFITM3-specific short hairpin RNA (shIFITM3-c and shIFITM3-d) was
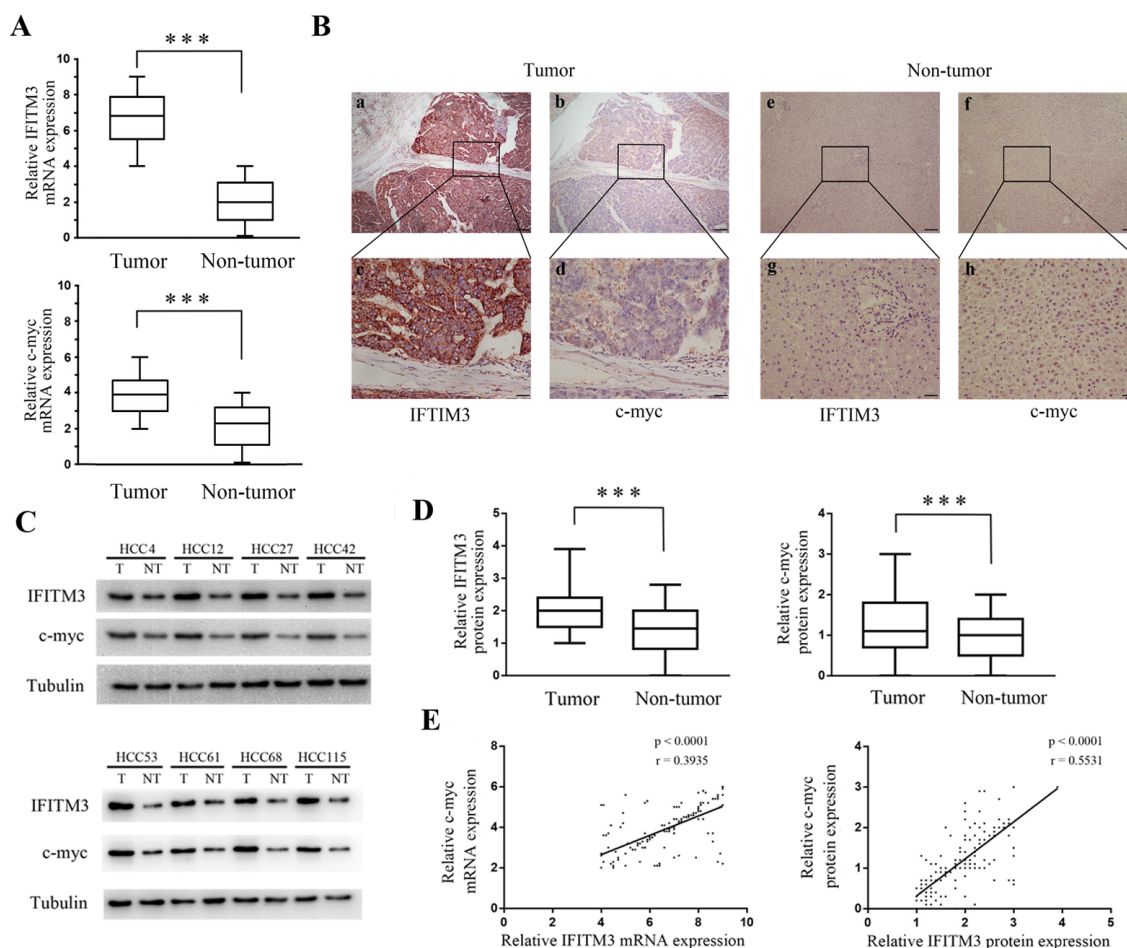

Figure 1. Levels of IFITM3 and c-myc expression are positively correlated in HCC. (A) qRT-PCR analysis of IFITM3 and c-myc mRNA expression in 160 HCC tumor specimens and adjacent normal tissue specimens (***$p < 0.001$, paired Student's *t*-test); **(B)** Representative IHC staining of IFITM3 and c-myc in HCC tissues. Magnification: a, b, e, and f: 100×; c, d, g, and h: 400×; **(C)** Representative Western blot analysis of IFITM3 and c-myc protein expression (T: tumor, NT: non-tumor tissues); **(D)** Quantification of IFITM3 and c-myc protein expression using Western blot analyses in 160 paired HCC specimens and adjacent normal tissue specimens (***$p < 0.001$, paired Student's *t*-test); **(E)** Scatter plots show a positive correlation between IFITM3 and c-myc in terms of levels of mRNA and protein (left, $r = 0.3935$, $p < 0.0001$; right, $r = 0.5531$, $p < 0.0001$).
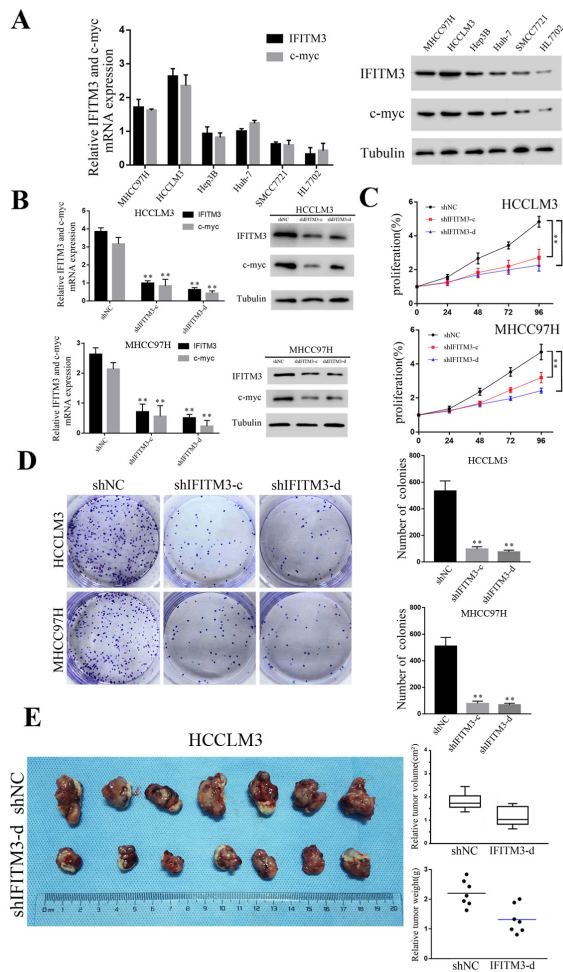
Figure 2. Downregulation of IFITM3 reduces c-myc expression and represses HCC proliferation. (A) qRT-PCR and Western blot analyses of IFITM3 and c-myc mRNA and protein expression in HL-7702 cells and HCC cell lines; (B) qRT-PCR and Western blot analyses were used to analyze IFITM3 and c-myc mRNA and protein expression in HCCLM3 and MHCC97H cells transfected with shIFITM3 (**$p < 0.01$); (C) An MTT proliferation assay indicated that IFITM3 knockdown significantly decreased cell proliferation in the HCCLM3 and MHCC97H cell lines (**$p < 0.01$); (D) Downregulation of IFITM3 suppressed colony formation in the HCCLM3 and MHCC97H cell lines (**$p < 0.01$); (E) A tumorigenicity assay indicated that knockdown of IFITM3 significantly suppressed tumor growth ($n = 7$ per group, $p < 0.05$, The formula for calculating the tumor volume was: (short diameter) × (longest diameter) × high × π /3).

stably transfected into HCCLM3 and MHCC97H cells to inhibit the expression of IFITM3. qRT-PCR and Western blot analysis confirmed that the knockdown of IFITM3 significantly decreased the expression of c-myc mRNA and protein in HCCLM3 and MHCC97H cells (Figure 2B). In addition, the proliferation capacity of cells was significantly inhibited in shIFITM3-c and shIFITM3-d cells ($p < 0.01$, Figure 2C). The number of colonies was significantly reduced in IFITM3-suppressed cells ($p < 0.01$, Figure 2D). In addition, a tumorigenicity assay similarly indicated that the downregulation of IFITM3 significantly suppressed tumor growth ($p < 0.01$, Figure 2E).
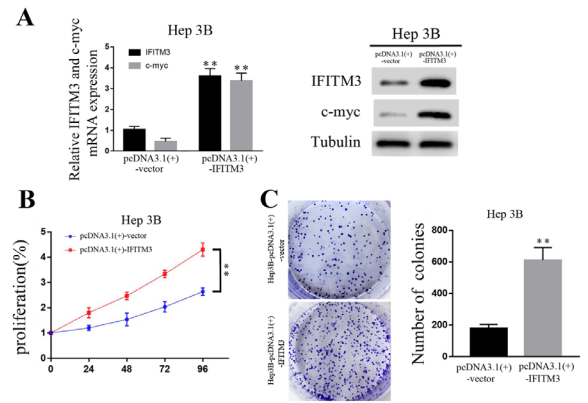

Figure 3. Overexpression of IFITM3 increases c-myc expression and promotes HCC proliferation. (A) qRT-PCR and Western blot analyses were used to analyze IFITM3 and c-myc mRNA and protein expression in Hep3B cells transfected with the pcDNA3.1(+)-IFITM3 plasmid (**$p < 0.01$); (B) An MTT proliferation assay indicated that IFITM3 overexpression significantly increased cell proliferation in the Hep3B cell line (**$p < 0.01$); (C) Upregulation of IFITM3 promoted colony formation in the Hep3B cell line (**$p < 0.01$).

To confirm the specificity of tumorigenicity, the plasmid pcDNA3.1(+)-IFITM3 was transfected into Hep3B cells to increase IFITM3 expression. Results indicated that overexpression of IFITM3 significantly increased the expression of c-myc (Figure 3A). The upregulation of IFITM3 markedly promoted the proliferation of HCC cells *in vitro* (Figure 3B and C). Taken together, these results confirmed that downregulation of IFITM3 reduced the expression of c-myc and inhibited the proliferation of HCC cells *in vitro* and *in vivo*.

3.3. *c-myc is the key for IFITM3-mediated HCC cell proliferation*

In order to further confirm that IFITM3 promotes the proliferation of HCC cells by regulating c-myc. We first increased the c-myc expression in IFITM3-knockdown HCC cells, and then observed the expression of IFITM3 and c-myc and cell-proliferation abilities. Western blotting indicated that the downregulation of IFITM3 reduced the expression of c-myc, while the upregulation of c-myc attenuated the loss of c-myc expression in IFITM3-knockdown HCCLM3 and MHCC97H cells (Figure 4A). The proliferation experiment also indicated that the down-regulation of IFITM3 significantly reduced the proliferation of HCCLM3 and MHCC97H cells, while the up-regulation of c-myc rescued the decreased proliferation ability caused by the knockdown of IFITM3 (Figure 4B).

Secondly, we decreased the expression of c-myc in IFITM3-overexpressing Hep3B cells, and then measured the levels of IFITM3 and c-myc proteins as well as cell proliferation. Western blotting indicated that overexpression of IFITM3 significantly upregulated
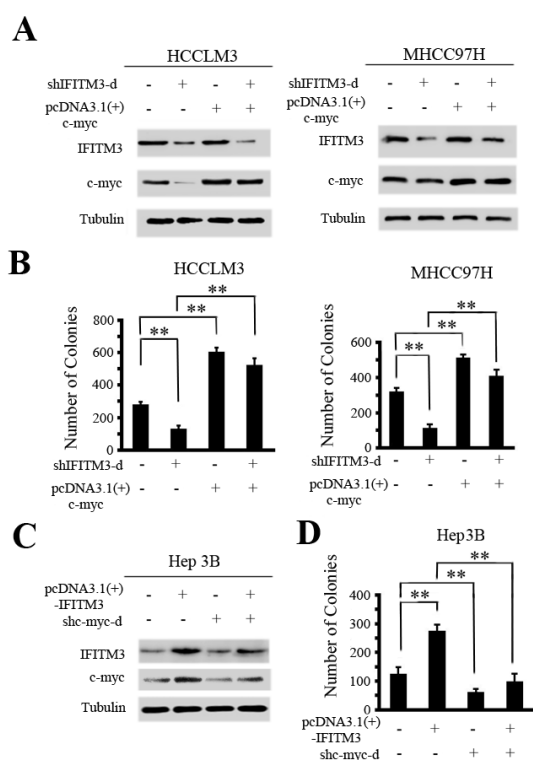
Figure 4. c-myc is essential for IFITM3-mediated HCC proliferation. (A) Levels of IFITM3 and c-myc protein were detected with Western blot analysis. The overexpression of c-myc attenuated the loss of c-myc expression in HCCLM3- and MHCC97H-shIFITM3-d cells; **(B)** Overexpression of c-myc rescued colony formation in HCCLM3- and MHCC97H-shIFITM3-d cells (**$p < 0.01$); **(C)** The downregulation of c-myc expression significantly inhibited the increase in c-myc expression in Hep3B-IFITM3 cells; **(D)** Inhibition of c-myc reduced colony formation in IFITM3-overexpressing Hep3B cells (**$p < 0.01$).

c-myc expression, while downregulation of c-myc dramatically inhibited the increase in c-myc expression induced by IFITM3 in Hep3B cells (Figure 4C). Moreover, the knockdown of c-myc reduced IFITM3-enhanced cell proliferation (Figure 4D). These results demonstrate that c-myc is necessary for IFITM3-mediated HCC cell proliferation.

3.4. IFITM3 regulates c-myc expression via ERK1/2 signalling in HCC cells

c-myc is reported to be a target of ERK1/2 signalling (*19,31,32*), Therefore, the hypothesis was that IFITM3 regulates c-myc *via* the ERK1/2 signalling pathway in HCC cells. To verify this hypothesis, changes in the expression of phosphorylated ERK1/2 (p-ERK1/2) and total ERK1/2 were first detected in IFITM3-knockdown HCCLM3 and MHCC97H cells. Western blotting indicated that the down-regulation of IFITM3 significantly decreased the level of p-ERK1/2 but did not have a significant effect on the expression of total ERK1/2 protein in HCCLM3 and MHCC97H cells (Figure 5A). In contrast, up-regulation of IFITM3
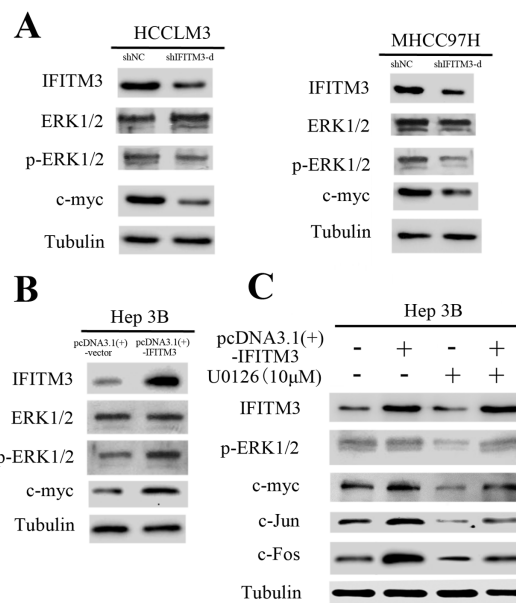

Figure 5. IFITM3 regulates c-myc expression via the ERK1/2 signalling pathway. (A and **B)** Levels of IFITM3, ERK1/2, p-ERK1/2, and c-myc protein expression were assessed with Western blotting in IFITM3-knockdown and IFITM3-overexpressing HCC cells; **(C)** Western blot analysis indicated levels of IFITM3 overexpression and ERK1/2 signalling inhibition (U0126, 10μM) and their effects on p-ERK1/2, c-myc, c-Jun, and c-Fos in Hep3B cells.

expression significantly increased the level of p-ERK1/2 but had not effect on total ERK1/2 protein expression in Hep3B cells (Figure 5B). In addition, up-regulation of IFITM3 expression increase the expression of other downstream genes in the ERK1/2 signalling pathway, *i.e.* c-Jun and c-Fos, in Hep3B cells. However, blockade of ERK1/2 signalling dramatically inhibited the increase in c-myc, c-Jun, and c-Fos expression in Hep3B-pcDNA3.1(+)-IFITM3 cells (Figure 5C). These findings confirmed that IFITM3 regulates c-myc *via* the ERK1/2 signalling pathway.

4. Discussion

IFITM3 is a member of the IFITM family, which plays an important in regulating cellular differentiation, apoptosis, inflammation, and immune cell regulation (*6,7,9,14*). Recently, mounting evidence has indicated that IFITM3 plays a crucial role in oncogenesis and development. For example, Gan *et al.* found that knockdown of IFITM3 suppressed the growth of oral squamous cell carcinoma cells *via* the CCND1-CDK4/6-pRB axis (*33*). Another study found that knockdown of IFITM3 expression inhibited gastric cancer cell migration, invasion, and proliferation (*34*). A previous study by the current authors confirmed that IFITM3 is significantly overexpressed in HCC tissues and is associated with a poor prognosis (*17*). The current authors previously also reported that overexpression of IFITM3 enhances HCC invasion and

proliferation (*17,18*). These results indicate that IFITM3 can promote HCC tumorigenesis and development.

The current study investigated the downstream gene by which IFITM3 promotes HCC proliferation. Previous studies have confirmed that c-myc is overexpressed in many tumors and that it promotes tumor proliferation. For example, Tang *et al*. reported that LncRNA GLCC1 promotes colorectal carcinogenesis and tumor proliferation by stabilizing c-myc (*35*). Another study found that silencing of HMGA1 significantly inhibited gastric cancer cell proliferation by regulating c-myc expression (*36*). In addition, Yang *et al*. reported that the mistletoe extract fraxini inhibits the proliferation of HCC by down-regulating c-myc expression (*37*). These studies indicated that c-myc is a master regulator of tumor proliferation. The current study identified a novel regulatory mechanism in which IFITM3 promotes HCC cell proliferation by increasing c-myc expression. First, our results showed that IFITM3 and c-myc expression were significantly upregulated and were positively correlated in HCC tissues. In addition, downregulation of IFITM3 was found to reduce the expression of c-myc and to decrease HCC proliferation *in vitro* and *in vivo*. In addition, overexpression of c-myc rescued the decreased proliferation caused by the knockdown of IFITM3, while the inhibition of c-myc significantly reduced proliferation that increased by IFITM3 overexpression. These results suggest that one of the mechanisms by which IFITM3 promotes HCC proliferation is the upregulation of c-myc expression.

Next, the mechanism by which IFITM3 regulates c-myc was further investigated. c-myc is reported to be a target of ERK1/2 signalling, which is involved in the oncogenesis and development of many cancers (*19,31,32*). The relationship between IFITM3 and the ERK1/2 signalling pathway was first investigated. Interestingly, downregulation of IFITM3 was confirmed to decrease the level of p-ERK1/2, whereas overexpression of IFITM3 increased p-ERK1/2 expression. Moreover, upregulation of IFITM3 was found to also increase the other downstream genes in the ERK1/2 pathway, including c-Jun and c-Fos, whereas the blockade of ERK1/2 signalling dramatically inhibited the increase in c-myc, c-Jun, and c-Fos expression in Hep3B-pcDNA3.1(+)-IFITM3 cells. In conclusion, these findings have shown that IFITM3 regulates c-myc expression *via* the ERK1/2 signalling pathway.

In summary, the current study demonstrated that IFITM3 and c-myc expression are positively correlated in HCC tissues. Silencing IFITM3 decreases the expression of c-myc, inhibiting HCC cell proliferation *in vitro* and *in vivo*. In addition, we confirmed that IFITM3 regulates c-myc expression *via* the ERK1/2 pathway. The newly identified IFITM3-ERK1/2-c-myc axis facilitates the proliferation of HCC and represents a valuable target for treatment of HCC.

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