

FHL1 inhibits the growth of tongue squamous cell carcinoma cells via G1/S cell cycle arrest

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Received August 11, 2014; Accepted April 30, 2015

DOI: 10.3892/mmr.2015.3844

Abstract. Four and a half LIM protein 1 (FHL1) has been characterized as a tumor suppressor in various types of tumor. However, the biological function and underlying mechanism of FHL1 in tongue squamous cell carcinoma (TSCC) remain to be elucidated. The present study demonstrated that FHL1 inhibits anchorage-dependent and -independent growth of TSCC cells *in vitro* and tumor growth in nude mice, as determined by cell proliferation and soft agar assays. Knockdown of FHL1 with FHL1 small interfering RNA (siRNA) promoted tumor growth in nude mice. Mechanistically, flow cytometric analysis showed that knockdown of FHL1 promoted G1/S cell cycle progression. Furthermore, expression of cell cycle-associated regulators, cyclin D and cyclin E, were detected by western blotting and reverse transcription-quantitative polymerase chain reaction. Cyclin D and cyclin E were markedly elevated at both the protein and mRNA level in the FHL1 siRNA-transfected cells. These results suggested that FHL1 has a tumor suppressive role in TSCC and that FHL1 may be a useful target for TSCC gene therapy.

Introduction

Squamous cell carcinoma (SCC) of the oral cavity is the sixth most frequent solid cancer worldwide (1). Tongue squamous cell carcinoma (TSCC) is the most common type of oral cancer and is well known for its high rate of proliferation and lymph node metastasis. The majority of TSCC patients are associated with smoking, heavy alcohol use and HPV infection (2-4). According to the American Cancer Society (5), while overall new cancer cases increased ~8%, new cases of TSCC increased by >37% in the same period. This indicates a major health problem associated with TSCC and suggests the immediate requirement for an improved understanding of this disease. To prevent and improve the outcomes of TSCC, it is necessary to further understand the molecular mechanism underlying the development and progression of TSCC and to develop new target therapies.

Four and a half LIM protein 1 (FHL1) is a member of the FHL protein family, which contains four complete LIM domains and an N-terminal half LIM domain (6). It has been reported that LIM domains function in protein-protein interactions with transcription factors, cell-signaling molecules and cytoskeleton-associated proteins (6,7). Previously, FHL1 has been demonstrated to be important in carcinogenesis. FHL1 expression is downregulated in various types of malignancy, including breast cancer, liver cancer, kidney cancer, prostate cancer, gastric cancer, lung cancer and oral squamous cell carcinoma (OSCC) (8-13). FHL1 exerts its tumor suppressive role via multiple mechanisms. FHL1 activates the tumor suppressor gene p21 (WAF1/CIP1) and represses the oncogene c-myc through interaction with Smad2, Smad3 and Smad4 in liver cancer cells (10). In breast cancer cells, FHL1 interacted with estrogen receptors and thus decreases the expression of pS2 and cathepsin D, two estrogen-responsive genes (9). In addition, FHL1 induces G1 and G2/M cell cycle arrest in lung cancer cells by inhibiting the expression of cyclin A, cyclin B and cyclin D as well as the induction of the cyclin-dependent

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Key words: four and a half LIM protein 1, tongue squamous cell carcinoma, cell growth, cell cycle, cyclin

kinase (CDK) inhibitors p21 and p27 (Kip1) (12). Although it has been reported that FHL1 expression is downregulated in OSCC, including TSCC (13), the biological function and the underlying molecular mechanisms of FHL1 in TSCC remain to be elucidated.

The present study aimed to investigate the function and mechanism of FHL1 in TSCC. Cell proliferation and soft agar assays were performed to detect whether FHL1 regulated anchorage-dependent and -independent growth of TSCC cells. The effects of FHL1 on cell migration and invasion were also examined by wound healing and Transwell assays. In addition, cell cycle assay, western blotting and reverse transcription-quantitative polymerase chain reaction (RT-qPCR) analysis were performed to detect the regulatory effects of FHL1 on the cell cycle, and the expression of cell cycle-associated regulators. Finally, a tumor formation assay was performed to detect the regulation of FHL1 on TSCC cell proliferation *in vivo*.

Materials and methods

Plasmids and small-interfering RNAs (siRNAs). The expression vector for FLAG-tagged FHL1 has been described previously (10). The cDNA target sequences of siRNA for FHL1 were siRNA-1: 5-AAGGAGGTGCACTATAAGAAC-3 and siRNA-2: 5-AATCTGGCCAACAAGCGCTTT-3 and were cloned into the vector pSilencer2.1-U6 (Ambion, Austin, TX, USA), respectively. All plasmids were verified by DNA sequencing.

Cell culture and transfection. The human TSCC Tca8113 cell line (Cell Institute, Chinese Academy of Sciences, Shanghai, China) and SCC6 cell line (provided by Dr Jing Sun, Department of Oral and Maxillofacial Surgery, Hospital of Stomatology, Tongji University, Shanghai, China) at passage 20 were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum at 37°C in a humidified atmosphere of 5% CO₂ in air. For the transfection assay, cells were seeded in 24-well or 6-well plates and transfected with the indicated plasmids using Lipofectamine 2000 according to the manufacturer's instructions (Invitrogen Life Technologies, Carlsbad, CA, USA).

Anchorage-dependent and -independent assays. Anchorage-dependent cell proliferation was analyzed by crystal violet assay as described previously (14). For the anchorage-independent growth assay, cells (1 × 10³) were seeded in a 12-well plate, with a bottom layer of 0.7% low-melting temperature agar in DMEM and a top layer of 0.35% agar in DMEM. Colonies were scored after 5 weeks of growth.

Western blotting. Cells were lysed in radioimmunoprecipitation assay buffer (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 1% NP40, 0.1% SDS, 0.5% DOC, 1 mM PMSF, and supplemented with a protease inhibitor cocktail) for 30 min on ice. The protein concentration was determined using a bicinchoninic acid assay (Pierce Biotechnology, Inc., Rockford, IL, USA). Equivalent quantities of protein were separated by 10% SDS-PAGE and blotted onto a nitrocellulose membrane (GE Healthcare, Amersham, UK). The membranes were blocked

in Tris-buffered saline containing Tween (TBST) supplemented with 10% nonfat milk for 1 h at room temperature. The membranes were then incubated with primary antibodies, diluted in TBST containing 10% nonfat milk. Following extensive washing with TBST, the membranes were incubated with a horseradish peroxidase-conjugated secondary antibody, followed by chemiluminescent detection with ECL detection reagent, according to the manufacturer's instructions (Pierce Biotechnology, Inc.). Images were captured and analysed using the Tanon-5200 Chemiluminescent imaging system (Tanon, Shanghai, China). The antibodies used in the present study were as follows: Rabbit polyclonal anti-human FHL1 (1:200; cat. no. sc-28691; Santa Cruz Biotechnology, Inc. Dallas, TX, USA), rabbit polyclonal anti-human GAPDH (1:5,000; cat. no. sc-25778; Santa Cruz Biotechnology, Inc.), rabbit monoclonal anti-human cyclin D (1:1,000; cat. no. ab134175; Abcam, Cambridge, UK), rabbit polyclonal anti-human cyclin E (1:500; cat. no. ab101324; Abcam), and horseradish peroxidase-conjugated goat anti-rabbit immunoglobulin G secondary antibody (1:10,000; cat. no. sc-2004; Santa Cruz Biotechnology, Inc.).

Cell migration and invasion assays. Wound healing assays were used to determine cell migration. Briefly, cells grown in 6-well plates as confluent monolayers were mechanically scratched using a 1-ml pipette tip to create the wound. Cells were washed with phosphate-buffered saline (PBS) and the debris was removed. Cells were cultured for 24 h in DMEM without serum to allow wound healing. A cell invasion assay was performed using a Transwell chamber according to the manufacturer's instructions (Corning Inc., Corning, NY, USA). Cells invaded through the Matrigel membrane were fixed with 4% paraformaldehyde and stained with crystal violet 24 h after seeding.

RT-qPCR. Total RNA was isolated using TRIzol reagent (Invitrogen Life Technologies) and reverse transcribed using SuperScript II Reverse Transcriptase (Invitrogen Life Technologies). qPCR was performed using the following primers: Cyclin D, sense GCTTCCTCTCCAGAGTGATC and antisense GTCCATGTTCTGCTGGGCCT; cyclin E, sense GAAGATTCCTATGGAAGACAGAC and antisense GCA CACTGGTGACAACCTGTC; FHL1, sense GAAGTGTGC TGGATGCAAGA and antisense GGGGGCTTCCTAGCT TTAGA; β-actin, sense ATCACCATTGGCAATGAGCG and antisense TTGAAGGTAGTTTCGTGGAT.

Animal experiments. A total of 10 BALB/c nude mice were purchased from Vital River Laboratories (Beijing, China). The mice were housed under specific pathogen-free conditions and fed a normal diet. Tca8113 (1 × 10⁷) cells were subcutaneously inoculated into the right flank of the 5-week old nude mice. Tumor size was monitored every week by measuring length and width with a caliper. Tumor volume was calculated using the following formula: Width² × length / 2. The mice were sacrificed by cervical dislocation 4 weeks later, the tumors were dissected out and immunohistochemistry was performed to detect the expression of FHL1. The present study was approved by the ethics committee of Anqing Municipal Hospital of Anhui Medical University (Anqing, China).

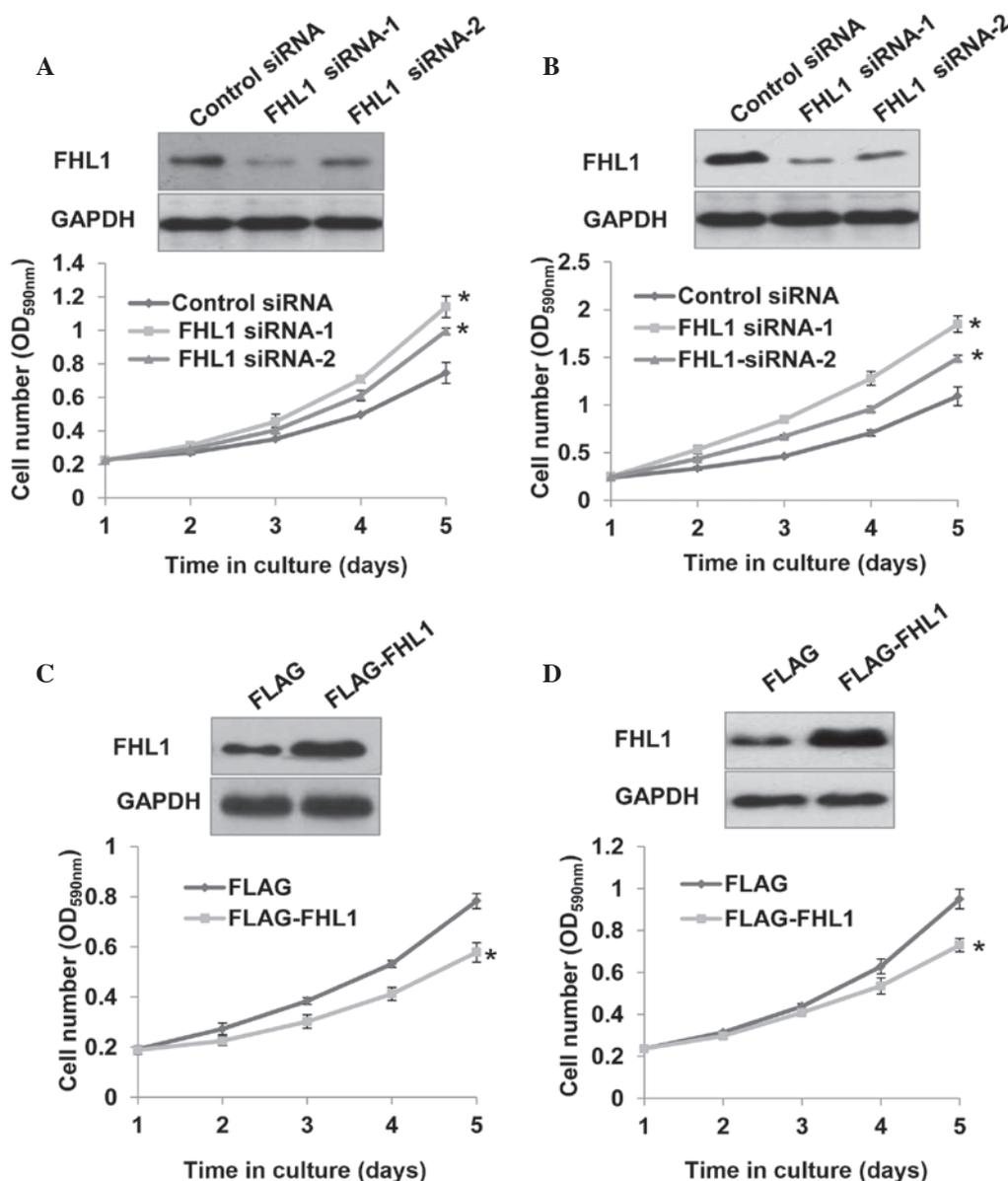


Figure 1. FHL1 inhibits anchorage-dependent growth of tongue cancer cells. (A and B) Anchorage-dependent growth assays in (A) Tca8113 and (B) SCC6 cells stably transfected with control siRNA or FHL1 siRNAs. Cell viability was determined at the indicated time points. Western blot analysis demonstrated the siRNA knockdown effects of anti-FHL1. GAPDH was used as a loading control. * $P < 0.05$ vs. control siRNA on day 5. (C and D) Anchorage-dependent growth assays in (C) Tca8113 and (D) SCC6 cells stably transfected with FLAG-tagged FHL1 or an empty vector (FLAG). The transfected cells were analyzed as in (A and B). * $P < 0.05$ vs. empty vector on day 5. FHL1, four and a half LIM protein 1; siRNA, small-interfering RNA.

Statistical analysis. Statistical significance in the cell growth assays between the control group and FHL1 overexpression or the knockdown group was examined by two-tailed Student's *t*-test. Statistical calculations were performed using SPSS 13.0 (SPSS, Inc., Chicago, IL, USA). $P < 0.05$ was considered to indicate a statistically significant difference.

Results

FHL1 inhibits TSCC cell proliferation in vitro. To investigate the effect of FHL1 on the proliferation of the TSCC cell lines Tca8113 and SCC6, stable cell lines expressing control siRNA, FHL1 siRNA-1 and FHL1 siRNA-2 were constructed. FHL1 protein expression was downregulated in cells transfected

with FHL1 siRNA-1 and FHL1 siRNA-2, particularly with FHL1 siRNA-1, compared with cells transfected with control siRNA (Fig. 1A and B). Notably, FHL1 knockdown with the two siRNAs, particularly FHL1 siRNA-1, markedly promoted the proliferation of Tca8113 and SCC6 cells (Fig. 1A and B). By contrast, overexpression of FHL1 inhibited Tca8113 and SCC6 cell proliferation (Fig. 1C and D). FHL1 siRNA-1 was used in the following experiments due to its more efficient knockdown of endogenous FHL1 and more notable effect on TSCC cell proliferation.

Since anchorage-independent growth is one of the hallmarks of cancer cells, the effect of FHL1 on this phenotype was investigated using a soft agar assay. Knockdown of FHL1 increased anchorage-independent Tca8113 cell growth based

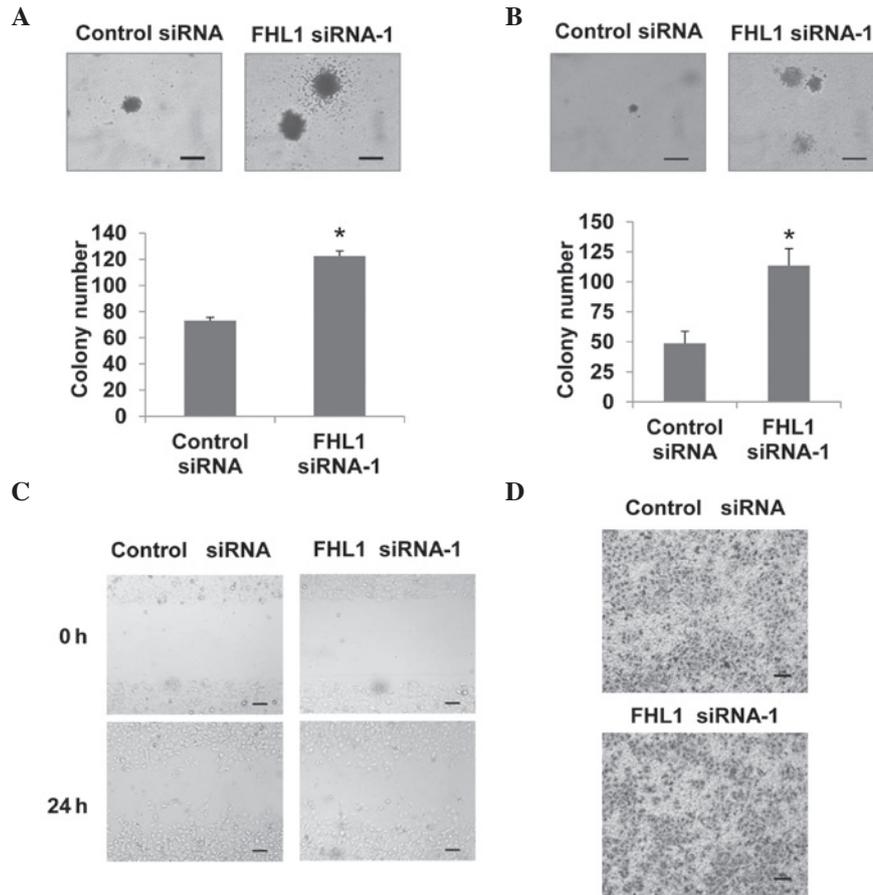


Figure 2. FHL1 inhibits anchorage-independent growth of tongue cancer cells. (A) Tca8113 and (B) SCC6 cells stably transfected with FHL1 siRNA-1 were plated in soft agar and analyzed. Scale bar=50 mm. *P<0.05 vs. control siRNA. (C and D) FHL1 does not affect Tca8113 cell migration and invasion. Wound healing assays were performed in Tca8113 cells stably transfected with control siRNA or FHL1 siRNA-1. (C) Images were captured at the indicated time points. Cell invasion ability was evaluated using a Matrigel invasion chamber. (D) Invasive cells were fixed and stained with crystal violet. Scale bar=100 mm. FHL1, four and a half LIM protein 1; siRNA, small-interfering RNA.

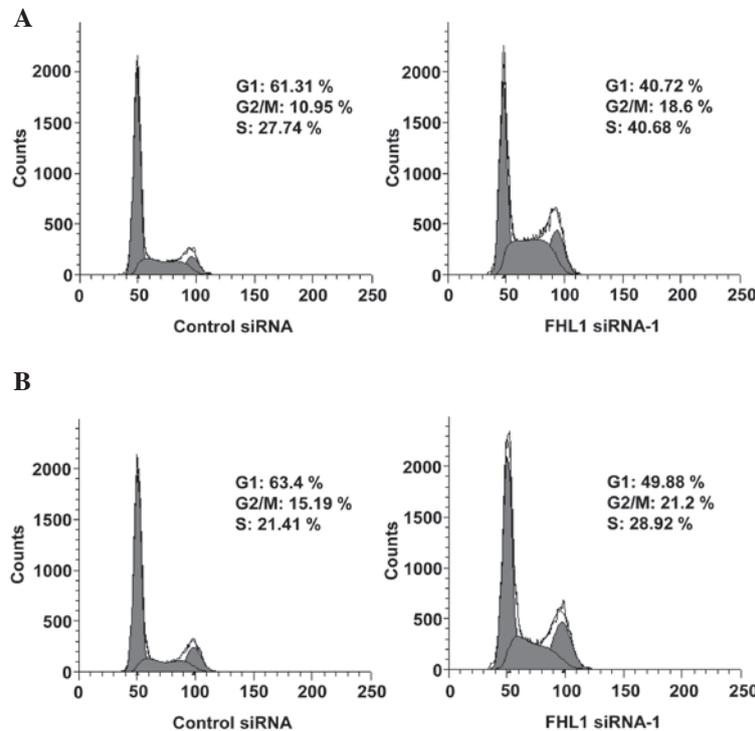


Figure 3. FHL1 induces cell cycle arrest at the G1 phase. (A) Tca8113 and (B) SCC6 cells stably transfected with control siRNA and FHL1 siRNA-1 were analyzed by flow cytometry. The experiments were repeated three times with similar results. FHL1, four and a half LIM protein 1; siRNA, small-interfering RNA.

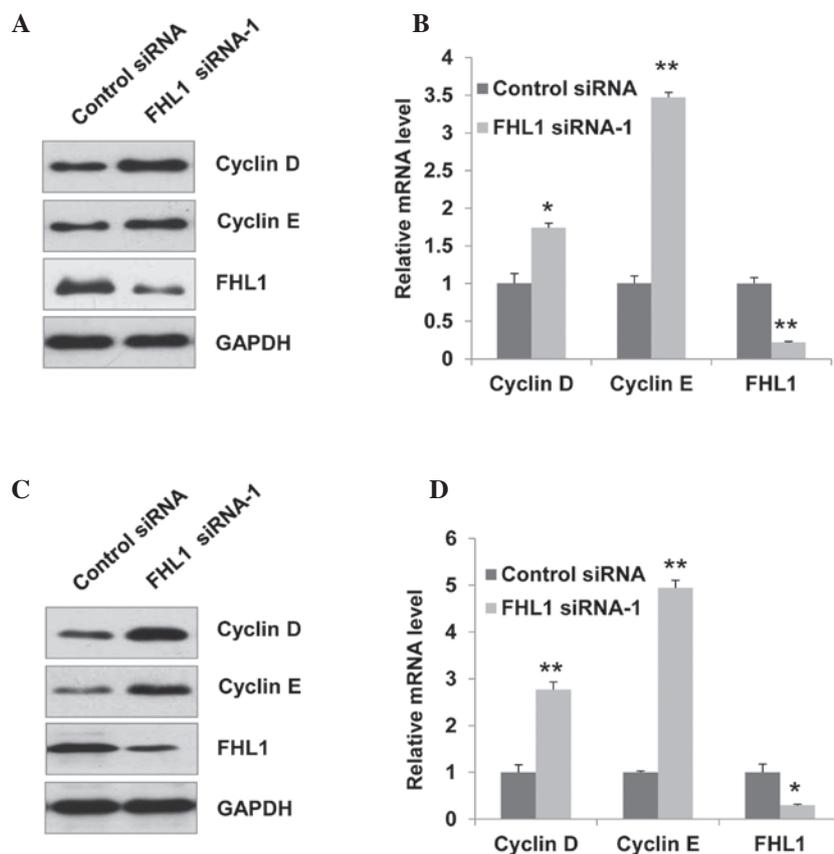


Figure 4. FHL1 regulates the expression of cell cycle-associated genes. Extracts from (A) Tca8113 and (C) SCC6 cells transfected with control siRNA and FHL1 siRNA-1 were used for western blot analysis with the indicated antibodies. (B and D) Reverse transcription quantitative polymerase chain reaction was performed to detect the mRNA levels of the indicated genes in (B) Tca8113 and (D) SCC6 cells. * $P < 0.05$ and ** $P < 0.01$ vs. the corresponding control siRNA. FHL1, four and a half LIM protein 1; siRNA, small-interfering RNA.

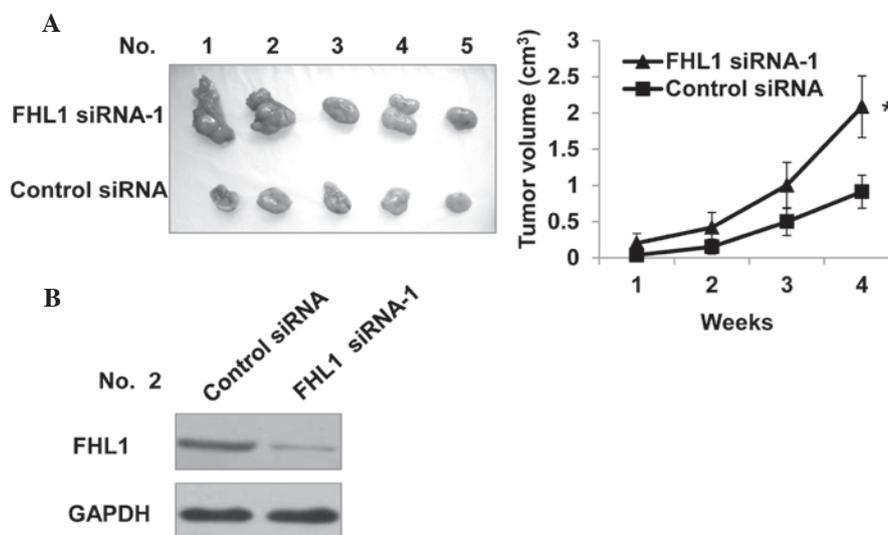


Figure 5. Knockdown of FHL1 promotes Tca8113 cell growth in nude mice. (A) Tca8113 cells stably expressing FHL1 siRNA-1 were injected into nude mice. At the indicated time points, tumors were measured with Vernier calipers (mean \pm standard deviation; $n = 5$). * $P < 0.01$ vs. control siRNA at week 4. (B) Western blot analysis of representative excised tumor from (A). FHL1, four and a half LIM protein 1; siRNA, small-interfering RNA.

on the size and the number of colonies (Fig. 2A). Similar results were observed in SCC6 cells (Fig. 2B). Taken together, these results demonstrated that FHL1 inhibits anchorage-dependent and -independent growth of TSCC cells.

FHL1 does not affect Tca8113 cell migration and invasion. Since TSCC is characterized by a high metastatic rate (15,16), the present study aimed to determine whether FHL1 modulates TSCC migration and invasion in Tca8113 cells. Wound

healing assays were performed to evaluate cell migration ability. Knockdown of FHL1 did not affect Tca8113 cell migration (Fig. 2C). In addition, FHL1 did not affect cell invasion according to the transwell assay (Fig. 2D).

FHL1 induces G1/S cell cycle arrest in TSCC cells. To elucidate the mechanism underlying the growth inhibition of TSCC cells by FHL1, the effect of FHL1 on the cell cycle of Tca8113 and SCC6 cells was examined. In comparison with control siRNA, the reduction of endogenous FHL1 cells using FHL1 siRNA caused a clear decrease in the proportion of cells in the G1 phase (from 61.31 to 40.72% for Tca8113 cells; from 63.4 to 49.88% for SCC6 cells) and an increase in the proportion of cells in S phase (from 27.74 to 40.68% for Tca8113 cells; from 21.41 to 28.92% for SCC6 cells; Fig. 3A and B). Taken together, these data suggest that FHL1 induces G1/S cell cycle arrest in TSCC cells.

FHL1 regulates the expression of cyclin D and cyclin E in TSCC cells. To further elucidate the molecular mechanism by which FHL1 expression induces G1/S cell cycle arrest, the expression of cyclin D and cyclin E, which promote G1/S transition, was determined by western blot analysis. Knockdown of endogenous FHL1 in Tca8113 cells increased the expression of cyclin D and cyclin E (Fig. 4A). Consistent with the results of FHL1 modulation of protein expression, FHL1 knockdown increased the expression of cyclin D and cyclin E at the mRNA level (Fig. 4B). Similar results were observed in SCC6 cells (Fig. 4C and D).

Knockdown of FHL1 promotes TSCC cell growth in nude mice. Subsequently, the effect of FHL1 on TSCC cell growth in nude mice was determined. A total of 10 mice were injected with Tca8113 cells stably transfected with control siRNA or FHL1 siRNA-1. As shown in Fig. 5A, all mice inoculated with Tca8113 cells developed tumors after 4 weeks. Notably, knockdown of FHL1 increased Tca8113 tumor growth, which is consistent with the stimulatory role of FHL1 knockdown in cell growth *in vitro*. As expected, the tumors in mice inoculated with Tca8113 cells expressing FHL1 siRNA had reduced protein levels of FHL1 compared with control siRNA (Fig. 5B).

Discussion

The present study provided for the first time, to the best of our knowledge, several lines of evidence demonstrating a novel role for FHL1 in TSCC. Firstly, FHL1 inhibits anchorage-dependent and -independent growth of TSCC cells. Secondly, knockdown of FHL1 inhibits G1/S cell cycle arrest, accompanied by upregulation of important cell cycle regulators, including cyclin D and cyclin E. Finally, knockdown of endogenous FHL1 promotes tumor growth in nude mice. These results suggest that FHL1 functions as a tumor suppressor in TSCC and that FHL1 may be a useful target for TSCC gene therapy.

FHL1 does not affect TSCC cell migration and invasion, which is consistent with a previous study demonstrating that expression of FHL1 does not correlate with node metastasis (13). These findings indicate that FHL1 may

be important in the development of TSCC, but not in the progression of TSCC.

Knockdown of endogenous FHL1 upregulates the protein and mRNA levels of cyclin D and cyclin E. In normal human cells, cellular division is an ordered, tightly regulated process, involving multiple cell cycle checkpoints that ensure genomic integrity. Cyclins and their associated CDKs are the central machinery that govern cell cycle progression (17,18). During the G1 phase, cyclin D binds and activates CDK4 and CDK6, which leads to partial inactivation of RB, RBL1 and RBL2 proteins. In addition, CDK2-cyclin E complexes further phosphorylate these proteins and drive the G1/S transition. Altered regulation of the cell cycle is a hallmark of several types of human cancer (19). Overexpression of cyclin D is common in human cancers of epithelial cell origin (20). In tongue tumors, cyclin D gene amplification was detected in 88% of the tumors (21). Patients with head and neck squamous cell carcinoma (HNSCC) that were strongly positive for cyclin D had reduced overall and disease-free survival. Cyclin D may be used as a predictor of long-term outcomes for patients with HNSCC (22). Various types of cancer, including breast cancer, lung cancer, cervical cancer, endometrial cancer and gastrointestinal cancer, overexpress cyclin E protein or mRNA (23). In addition, cyclin E overexpression has been proposed as a marker of poor clinical outcome in breast cancer (24). The fact that FHL1 can regulate cyclin D and cyclin E suggests a critical role for FHL1 in cell cycle regulation and cellular division.

It has been reported that FHL1 is frequently downregulated in primary OSCC tissues compared with the corresponding normal oral tissues (13). One of the mechanisms of FHL1 downregulation in OSCC is hypermethylation of CpG islands within FHL1 gene promoter regions, which is also found in bladder cancer (13,25). Due to the importance of FHL1 in the regulation of TSCC cell growth, it would be interesting to investigate whether the FHL1 promoter is methylated in TSCC and to determine other mechanisms underlying FHL1 downregulation in TSCC.

Acknowledgements

This study was supported by the National Natural Science Foundation of China (grant nos. 31200565, 31071174 and 81330053), the Beijing Natural Science Foundation (grant no. 5132027) and the Beijing Nova Program (grant no. Z131102000413034). Anqing Municipal Hospital of Anhui Medical University and Beijing Institute of Biotechnology contributed equally to this work.

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